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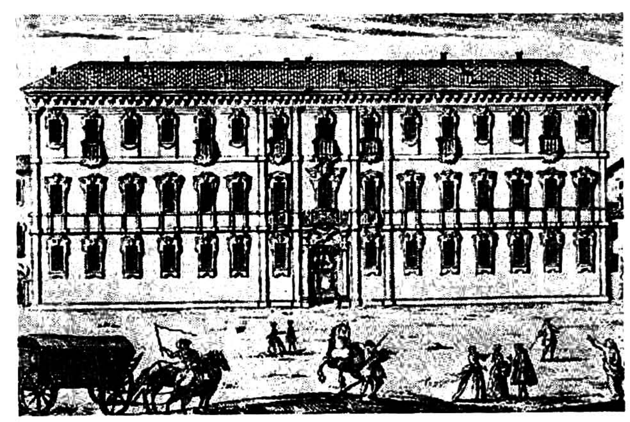
SPECIAL ISSUE



TSWETT
MEMORIAL SYMPOSIUM

Organized by the Fondazione Carlo Erba:

Second Russian – Italian Symposium in memory of M. S. Tswett,

Milan, October 13th and 14th, 1969

The picture shown on this page is an old etching of the Palazzo Visconti at Milan, where the Symposium was held.

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PREFACE

This issue contains a symposium organised by the Fondazione Carlo Erba (Milano) in memory of M. TSWETT the inventor of chromatography.

TSWETT was born in Italy in 1872 of a Russian father and an Italian mother and died in 1919. A series of commemorative symposia is being held with the participation of Russian and Italian chromatographers. The first of these was held in Moscow in 1968, the second (the present one) on October 13th and 14th, 1969 in Milan.

Papers on all aspects of chromatography were presented and discussed. This issue also includes two papers, one by KISELEV and the other by POSHKUS, which were read by title only at the symposium as the authors were unable to attend.

Several of the contributions are not being published here because they are summaries of work published previously (*e.g.* the papers by LEDERER and by BONIFORTI) and others because the manuscripts did not reach the editor in time (the papers by MARKELOV and by SHEMJAKIN); again others will be published elsewhere.

THE EDITOR

CHROM. 4592

M. S. TSWETT—HIS LIFE

K. SAKODYNSKY
Karpov's Institute of Physical Chemistry, Moscow (U.S.S.R.)

It is not infrequent in the history of science that, for some reason or other, discoveries remain in prolonged obscurity and only gain recognition and extensive application after they have appeared much later in papers by other scientists. Such was the fate of a discovery made in the early twentieth century by Mikhail Semenovich Tswett, a young Russian botanist and exceptionally modest and painstaking scientist. His discovery has transformed modern analytical chemistry and resulted in basically new concepts of control and automation in chemical processes.

Little is known about Mikhail Semenovich Tswett himself[1,2]. He was born on May 14, 1872* in the little town of Asti in Northern Italy. His father, Semen Nikolaye-

Fig. 1. Mikhail Semenovich Tswett.

* Sometimes his birth is erroneously given as May 19. This mistake is traced down to a form once filled in by Tswett himself.

Fig. 2. Hotel "Reale", where Tswett was born on May 14, 1872.

vich Tswett, held a high position in the civil service, and his mother was an Italian, Maria Dorozza by name. Tswett belonged to the Orthodox Church, and was educated in Switzerland, where he first went to Galliard's College in Lausanne and then to the St. Anthony Gymnasium in Geneva. In 1891 he entered the Faculty of Natural Sciences

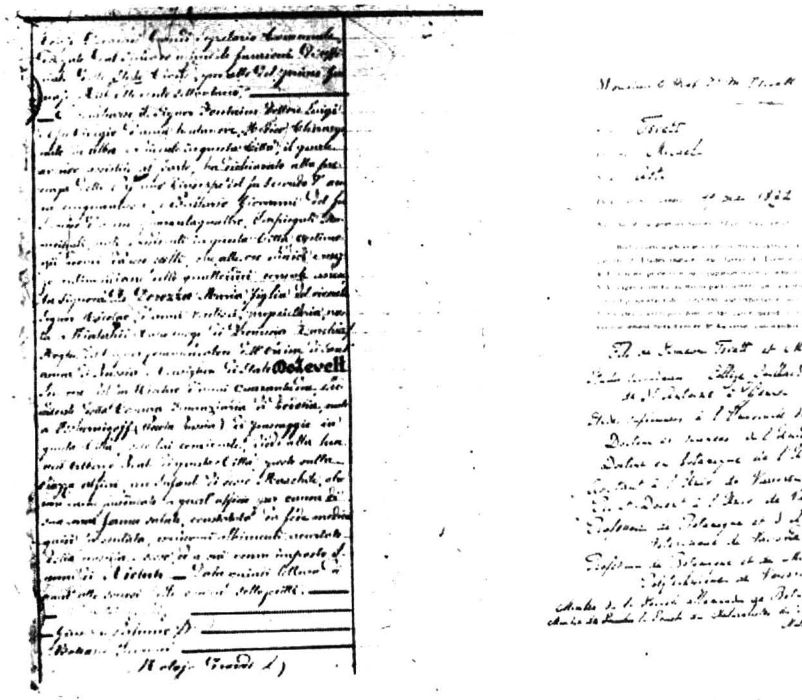


Fig. 3. The birth certificate of Tswett.

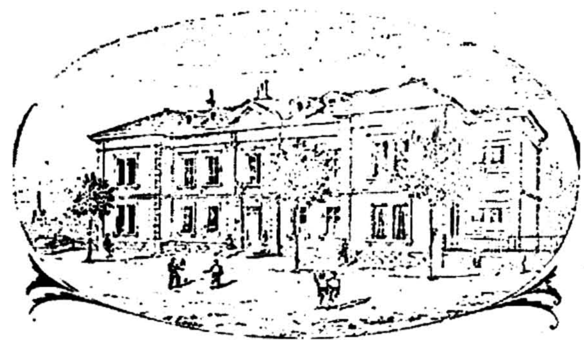
Fig. 4. A *curriculum vitae* form filled in by Tswett.

de Haller, Charles, ingénieur, Genève.
Wilson, Georges.
Moore, Lancelot.
Paris, Frédéric, négociant, Kreuzlingen.
Auberjonois, René, artiste-peintre, Paris.
Mitrofanof, Vladimir.
de Haller, Albert, pasteur, Montreux.
Welti, Oswald, Amérique.
Wood, Frank.
Browne, Beauchamp.
Mitrofanof, Paul.
Estivant, Léon.
Tsvett, Michel.
Thompson, William.
Rattray, Haldane.
Thébault, Alfred, employé de banque, Anvers.
Blattner, Eugène, aspirant de marine. † 1899.
Cart, Gustave, Algérie.
Luginbühl, Ernest, fabricant, Fillinges (Haute-Savoie).

Fig. 5. The list of students in Galliard's College in Lausanne.

at Geneva University; his interests at that time included botany, chemistry and physics. Much of his time there was taken up by his first research work at the general botanical laboratory, for which he was awarded a University prize. It was in this laboratory that Tswett worked for his Doctorate thesis in cell physiology, which was successfully presented in 1896. In 1896 he migrated to Russia, where he first stayed in Simferopol for half a year, and then moved to Petersburg to work in Professor Lesgaft's private institute for women.

Tswett's first years in Russia were not easy. His doctorate degree was not recognised and was of no use, so he had to work again for a degree. In his letters to Briquet, a friend in Geneva, he complained of a difficult life and was frank in his criticism of Russian society. He even planned to leave Russia. But by and by things straightened out and though some difficulties still remained, he made friends among his countrymen, and eventually settled down to work with enthusiasm*.



Le Collège Galliard sur la place de Chauderon à Lausanne.

Fig. 6. The college building, Galliard (now destroyed).

* It might be of interest to cite here CHARLES BAEHNI, who contributed considerably to the search of all available materials bearing on Tswett's life: "Like most Slavs, he possessed a certain charm which created a friendly atmosphere around him... Although he denied this fact in his letters, he was fundamentally a Russian and did not hesitate to return to his country".

ÉTUDES

DE

PHYSIOLOGIE CELLULAIRE

Contributions à la connaissance
des mouvements du protoplasme, des membranes plasmiques
et des chloroplastes.

DISSERTATION

PRÉSENTÉE A LA FACULTÉ DES SCIENCES DE L'UNIVERSITÉ
DE GENÈVE POUR L'OBTENTION
DU GRADE DE DOCTEUR ÈS SCIENCES

PAR

Michel TSWETT

GENÈVE
IMPRIMERIE REY & MALAVALLON
précédemment Aubert-Schuchardt.
1896

Fig. 7. The dissertation of Tswett presented at the University of Geneva.

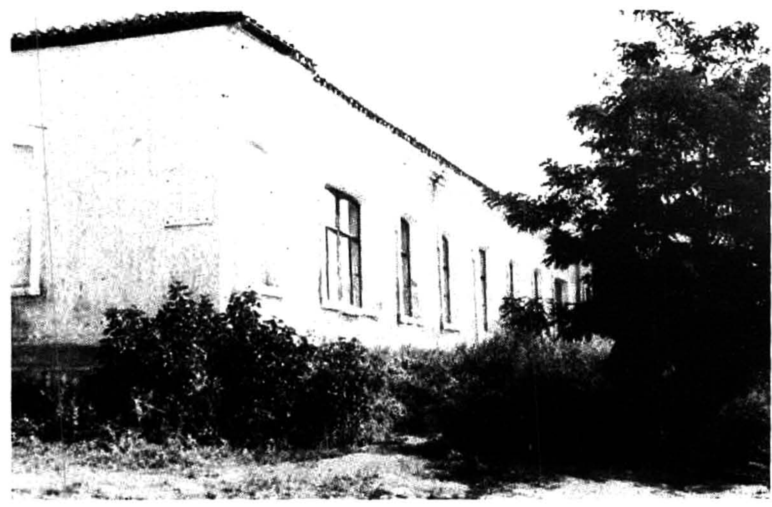


Fig. 8. The house of Steven in Simferopol.

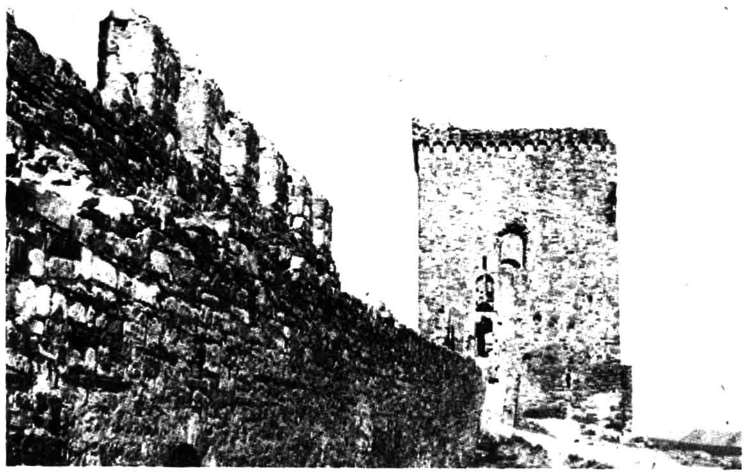
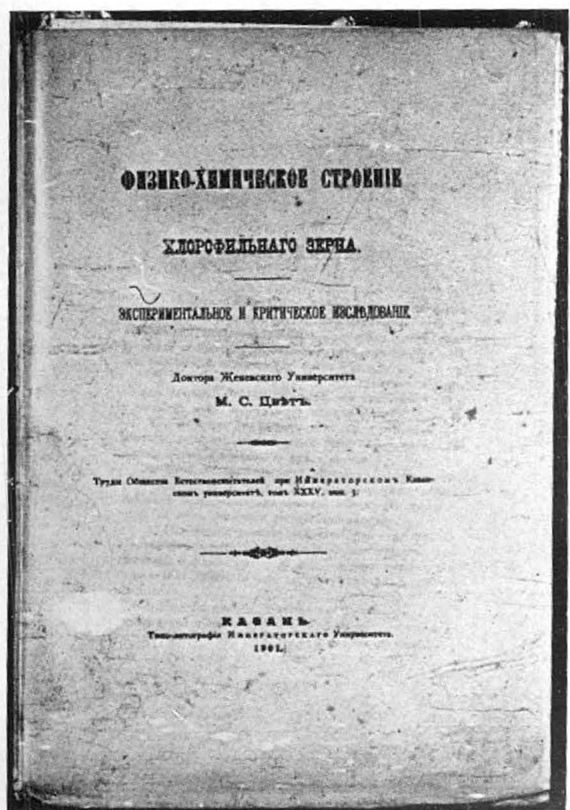


Fig. 9. The old castle in Sudak built by Genuese where Tswett was during his vacation in 1898.

Fig. 10. One of the letters written by Tswett to his friend Briquet.

Fig. 11. A letter written by M. Tswett to the dean of the University of Kazan.



ФИЗИКО-ХИМИЧЕСКОЕ СТРОЕНІЕ

ХЛОРОФИЛЬНАГО ЗЕРНА.

ЭКСПЕРИМЕНТАЛЬНОЕ И КРИТИЧЕСКОЕ ИЗСЛѢДОВАНІЕ

Доктора Женевскаго Университета

М. С. Цвѣтъ.

КАЗАНЬ.

1901.

Fig. 12. The title of the Russian Master's dissertation.

In Petersburg, Tswett made the acquaintance with and had the friendship of many a distinguished Russian botanist, particularly Academician A. S. Famintsin, in whose laboratory he worked without having a position on the staff. In 1900, on the recommendation of several prominent scientists, Tswett was given a membership to the Petersburg Society of Natural Scientists.

Fg. 13. The main building of Kazan University.

Curriculum vitae

Fig. 14 and Fig. 15. The *curriculum vitae* of Tswett.

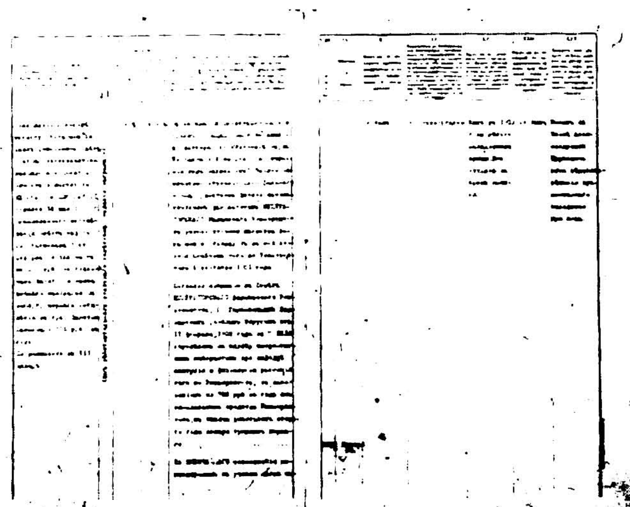


Fig. 16. The service form of Tswett as an assistant of Warsaw Polytechnical Institute

Fig. 17. The University building, Warsaw.

By the year 1901 Tswett had prepared his thesis for his Russian Master's degree entitled "Physical and chemical study of chlorophyll. Experiments and analysis". After the Master's qualifying examination on September 21, Tswett presented his dissertation at Kazan University. In addition to a successful presentation, Tswett proved to be a brilliant lecturer with a talent and alacrity for vivid and simple interpretation of the most complex phenomena.

In January 1902, Tswett moved to Warsaw where he first took the insignificant position of a supernumerary laboratory assistant, then that of an instructor at the Department of Plant Anatomy and Physiology, but before the end of 1902 he was promoted to an assistant professorship and was qualified to lecture. Tswett spent fourteen years in Warsaw. His discovery of chromatography belongs to this Warsaw period, though the fundamentals of the method had been outlined in his dissertation for his Russian Master's degree. In 1907, Tswett became a Reader in botany and agriculture at the Warsaw Veterinary Institute, and in 1908 was put on the staff of the Warsaw Polytechnical Institute as a Reader in botany and microbiology at the Chemistry and Mining Department. Whilst in Warsaw Tswett met Helena Alexandrovna Trusevich, and in late 1908 they married.

Fig. 18. The Polytechnical Institute building in Warsaw.

Fig. 19. The house on Mokotovskaya Street in Warsaw.

This initial period in Warsaw was a fruitful one for the young research worker As early as in Petersburg, Tswett had given particular attention to the central problem of his life, the mystery of green chlorophyll. He persisted in searching for a physical method to separate even the most complex mixtures, and he was convinced that chlorophyll was not a simple substance.

М. С. ЦВѢТЪ

ХРОМОФИЛЛЫ

ВЪ РАСТИТЕЛЬНОМЪ И ЖИВОТНОМЪ МІРѢ

ВАРШАВА

1910

M. TSVETT. Les Chromophylles dans les Mondes Végétal et Animal

М. С. ЦВѢТЪ

ХРОМОФИЛЛЫ

ВЪ РАСТИТЕЛЬНОМЪ И ЖИВОТНОМЪ МІРѢ.

Neue Tatsachen sind an sich nur eine geringfügige Bereicherung der Wissenschaft. Ihren Wert erhalten sie erst durch den Zusammenhang in welchem sie mit bereits bekannten und mit künftig bekannt werdenden stehen.

Ostwald.

ВАРШАВА

ТИПОГРАФІЯ ВАРШАВСКАГО УЧЕБНАГО ОКРУГА.

1910.

Fig. 20. Title of the book by Tswett presented as his Doctor's thesis in 1910

Fig. 21. The first page of this book with some words written by Tswett (see Fig. 20).

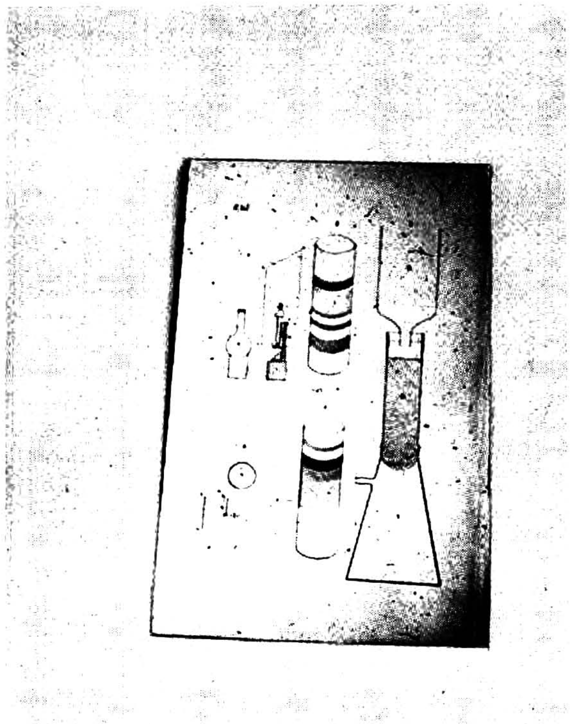


Fig. 22. The first chromatograph and the first chromatogram.

Numerous experiments, and days and nights of questing were spent until the method was found. It was quite simple. Its simplicity was its main advantage, which unfortunately was not appreciated by many of his contemporaries. The method is so simple that one cannot help being surprised that it had not been found before Tswett's time though many had almost discovered it.

Tswett's experiments whereby complex substances could be separated in a chalk-filled tube not only inaugurated an attack on the mystery of the green leaf, but also laid the foundation for a new method of separation—chromatography. Tswett wrote: "Thus the prospects open up for the construction of a new method of physical separation of the various substances present in organic liquids. The method is based on the ability of soluble substances to mix with various solid mineral and organic substances to form physical adsorption compounds". In addition, Tswett framed the idea of his discovery in the following poetic words: "Similarly to light beams in the spectrum" the different components of a complex pigment are regularly distributed one after another in the adsorption column and thus lend themselves to qualitative and quantitative analysis. I have called such a multi-coloured preparation a CHROMATOGRAM, and the respective method of analysis a CHROMATOGRAPHIC METHOD*.

* Gr. CHROMA—colour, GRAPHEIN—writing. In this connection H. PURNELL[4], an Oxford professor, remarked: "It is pleasing to think that Tswett, whose name in Russian means literally COLOUR or TINT, used this chance to demonstrate his sense of humour".

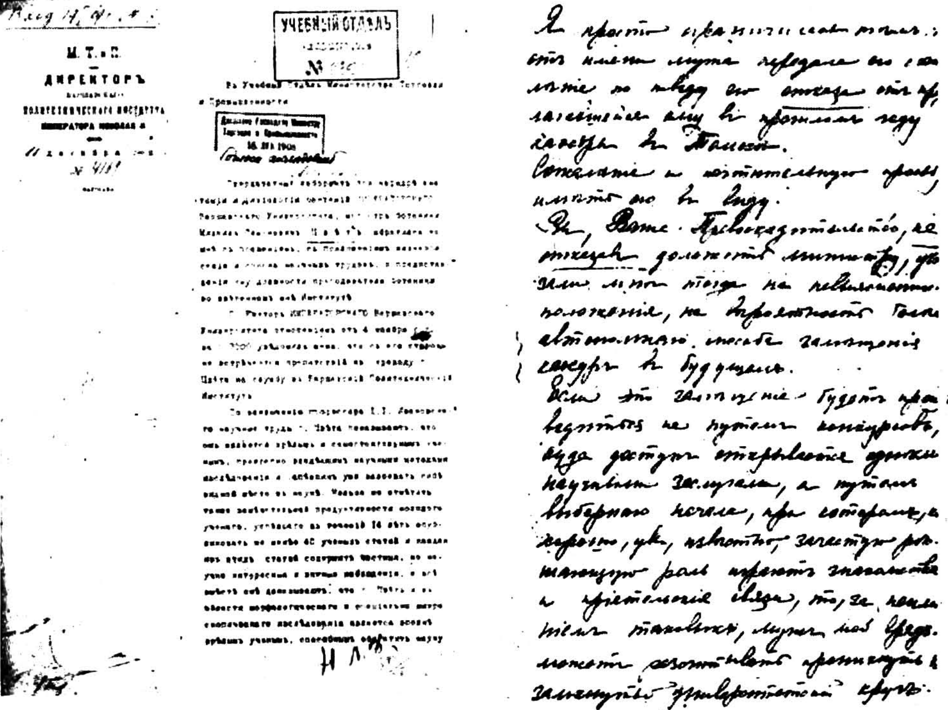


Fig. 23. A letter from the director of the Warsaw Polytechnical Institute about Tswett.

Fig. 24. Part of a letter by Elena Tswett.

It may seem strange that Tswett initially restricted his method to coloured substances only. However, in his very early work he made it absolutely clear that the adsorption analysis utilized primarily for pigments could also be used for colourless, in other words invisibly "coloured" substances.

Tswett's first work on adsorption chromatographic analysis was published in Russian in 1903 in the Proceedings of the Warsaw Society of Natural Scientists, unlike most of his papers which were written in German or French. On November 29, 1910 he presented his Doctorate dissertation in botany "Chromatophylls in plants and animals". This work was later awarded the M. A. Akhmatov Grand Prize of the Russian Academy of Science and published as a monograph. Even at this stage a systematic description of the chromatographic method is given and the results of research into the adsorption characteristics of over one hundred and ten substances and data on a great number of solvents are cited. He also describes research techniques and their facility. The information presented in the book is remarkable for its coverage, exhaustiveness and minuteness of detail.

Going back to the method itself, at first Tswett's discovery caught the public interest. It was discussed in special journals and in the press. Reputable publications in biochemistry which saw light in 1912 carried large articles on the chromatographic method. It was acclaimed everywhere. As an example, it will just suffice to quote the reference about Tswett dated 1908 when he was leaving Warsaw University for the

Warsaw Polytechnical Institute. "Scientific works by Mr. Tswett show him as a mature and competent worker in perfect command of research techniques and with quite a reputation in the scientific community. Also worth mentioning is the prolific number of papers this young scientist has published in the course of the past fourteen years—at least forty. Each of these contains interesting and important observations qualifying him as a mature scientist in the field of morphological and special microscopic analysis, able to make valuable contributions to science".

More than once during his Warsaw period Tswett was sent abroad to study the teaching standards in higher educational institutions, specifically in the Botanical Institutes of Berlin and Kiel. He also attended scientific congresses in Moscow and Petersburg, where he presented his papers. He became a member of the Warsaw Society of Natural Scientists and of the German Botanical Society.

Yet, in spite of all this, Tswett's method did not become popular in his lifetime. Probably, an unfavourable reference to it by a then prominent scientist, Willstätter, is responsible for this. According to Zechmeister, Tswett's work aroused a "tacit distrust" in many a colleague. In later years Tswett's discovery brought him no recognition and, even worse, discredited him as a scientist*.

At the same time Tswett grew more and more uninterested in teaching freshmen at the Polytechnical Institute. He yearned for more new experiments to develop his ideas and prove their validity, but was compelled to teach botany and microbiology to first-year students for whom it was almost optional. He attempted to find a position

Fig. 25. One of the last photos of Tswett.

* One of the few scientists still alive who knew Tswett, S. I. Sokolov, Professor of the Moscow Chemical Engineering Institute, refers to the atmosphere of suspicion which clouded Tswett's report in Warsaw. He recalls Tswett's painful reaction to the groundless attacks on his well-proven results.

in the Botany department of the Samara or Novoalekseyevsk Institutes. He applied to the Ministry of Education and the Ministry of Industry and Commerce. People who knew him well appreciated him highly, but burocratic officials preferred references from "influential" persons. Thus, G. Levitsky, Chief Trustee of educational establishments of the Warsaw District wrote in 1903: "Doctor Tswett has been teaching botany, lecturing and tutoring at the Chemistry Department of the Polytecnic Institute for several years. As an instructor he proved himself to his credit. His teaching of botany has been on a highly scientific footing and is effected in a perfect manner. He has a way of dealing with students and owing to this there has never been even the slightest conflict. His morals are very high; he has an unbiassed and ardent devotion to duty, is modest notwithstanding his prominence in science, industrious, kind and sympathetic, and has lofty and pure ideals".

Fig. 26. The building of Masing's Gymnasium in Moscow where Tswett worked from 1915–1916.

On the other hand, the reference by V. V. Zalessky, an expert of the Tsarist Ministry of Education, reads: "By the standards of his scientific works Mr. Tswett is considerably inferior to all the other claimants to the Chair. Mr. Tswett lacks competence in the techniques of the experimental sciences, which require rigorous research methods, and precision and caution in interpreting the results; his attitude towards facts is not sufficiently critical, thus hasty conclusions are drawn and unsubstantiated ideas may be put forward". It was thus concluded that Tswett would not fit into the position for which he had applied.

How popular became the point of view expressed by Zalessky and other "competent persons" can be seen from a letter by Elena Tswett to the Deputy Minister of Education, which was found in the archives. Here are several quotations from it: "If positions at the departments are not to be obtained on a competitive basis, which gives priority to scientific standards alone, but just by selection, which, alas, more often than not depends on connections, then my husband, who has none, will have only a very slim chance of penetrating the exclusive University community. The only hope which remains in this case is an extraordinary appointment by the Minister himself". And

Fig. 27. The house where Tswett lived in Moscow (Pokrovka 45).

further: "Time is not wholly unimportant for my husband. If he is to be saved from hard conditions and onerous work, which are virtually ruining him, for he has a poor health, it must be done at once." "All his energy is consumed in teaching, which brings no moral satisfaction. Scientific ideas are a dead weight, for there is nobody who cares enough to inherit and take an interest in them. All his personal research is cut down to a minimum. And the best years are being wasted."

Fig. 28. The University of Tartu.

ห้องสมุด ...วิทยาศาสตร์

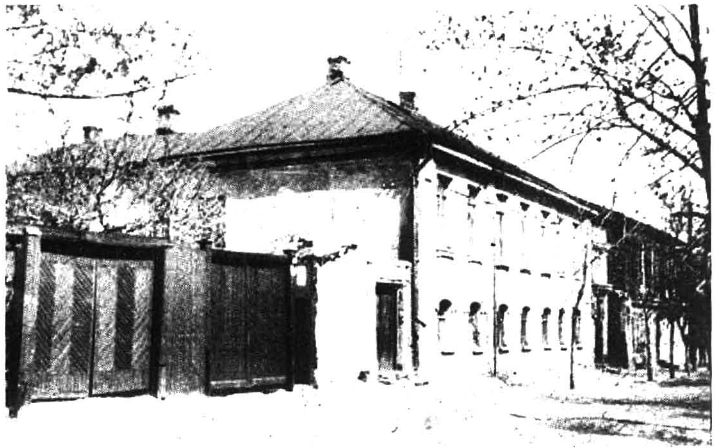


Fig. 29. Halutin street in Voronezh.

His wife's letter gives a very accurate picture of the atmosphere in which Tswett lived before the Revolution, and the spirit of this outstanding scientist could not exist in this atmosphere.

When the German Army approached Warsaw in 1915 Tswett moved first to Moscow and then, in late summer of 1916, to Nizhny Novgorod. All his archives and books were left in Warsaw, where they were lost.

Finally, in 1917, Tswett received the long-hoped-for position as Director of the Botanical Gardens in Tartu (then Yurief). He was already ill at that time. When Tartu was occupied by the Germans in February 1918, Tswett was ill but one month later he left Tartu for Voronezh notwithstanding his German friends' efforts to persuade him to stay, to become one of the first professors of the Voronezh University. In Tartu and in Voronezh his health deteriorated. He gave his lectures sitting at a desk. Though

Fig. 30. The small house where Tswett lived in Voronezh in his last year.

his intellect was as bright as ever and his phrasing strictly consistent, it was felt by everybody that lecturing put a great strain on him. According to the University medical staff, Tswett was suffering from heart disease. He lived his last year in a small room of Professor Verevkin's house in Khalyutinskaya Street (now Baturinskaya), 20. He died on June 26, 1919 in a hospital and was buried in the cemetery in the vicinity of the Alexey monastery. During the Second World War the cemetery was destroyed and Tswett's grave cannot be located now. The memorial plate put up in June 1969 in Baturinskaya 20 reads: "Here lived the prominent Russian scientist Mikhail Semenovich Tswett, 1872–1919".

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CHROM. 4633

HIGH RESOLUTION PARTITION CAPILLARY COLUMNS

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SUMMARY

Various procedures to obtain high resolution glass capillary columns are described. By roughening the glass surface either with a layer of carbon or with polymerized material, a stationary phase of any polarity can be successfully coated. Results obtained with Carbowax, trimer acid and squalane columns are reported; the separation of polar and non-polar compounds from their deuterated homologs ($C_6H_5CD_3$–$C_6H_5CHD_2$–$C_6H_5CH_2D$–C_7H_8; C_2H_6SO–C_2D_6SO) is shown.

INTRODUCTION

It has been reported[1] that high resolution partition capillary columns can be obtained if the liquid phase is uniformly distributed on the wall of glass capillaries. A number of factors affect the spreading of the stationary phase, one of the more important being the wettability of the glass wall.

If ordinary glass tubing is used for the manufacture of the glass capillary a homogeneous and uniform layer is only obtained when the stationary phase has a low surface tension; this characteristic has, so far, prevented the use of a large number of stationary phases.

To overcome this limitation and to be able to use liquids with a high surface energy two techniques have been proposed: the use of surface-active agents which can reduce the surface tension of the liquid phase; and the roughening of the walls of the capillary to decrease the contact angle between the liquid and the glass. The former technique is of limited use as the properties of the stationary phase and its working temperature range may be strongly affected by the addition of surface active agents; the latter procedure can be implemented by building up either a thin layer of carbon or of a polymeric material such as polybutadiene or polytrifluorochloroethylene on the walls of the capillary.

This procedure increases the roughness of the glass surface, eliminates the active sites of the glass and makes the spreading easier.

This work shows that by use of the above procedures columns of great efficiency with any stationary phase can be obtained; as far as polarity is concerned there is no limitation to their application. Experimental results obtained on columns prepared by various procedures are discussed.

SQUALANE COLUMNS

Squalane has a low surface tension (29.95 dynes/cm) and its coating on capillaries is quite smooth providing the walls have been thoroughly cleaned. A uniform layer is obtained by allowing an ether solution of squalane to flow through the capillary. It is easy to obtain high resolution columns of a suitable length. As an example a 200 m column has an efficiency of 250,000 theoretical plates when *n*-heptane is chromatographed and the height equivalent to a theoretical plate is less than 0.1 cm. Such columns can be successfully employed for the analysis of non-polar mixtures.

One application which is related to the high efficiency of these columns is the separation of isotopic molecules such as hydrocarbons from their deuterated homologs. Fig. 1 shows the separation of a mixture of $C_6H_5CD_3$–$C_6H_5CHD_2$–$C_6H_5CH_2D$–

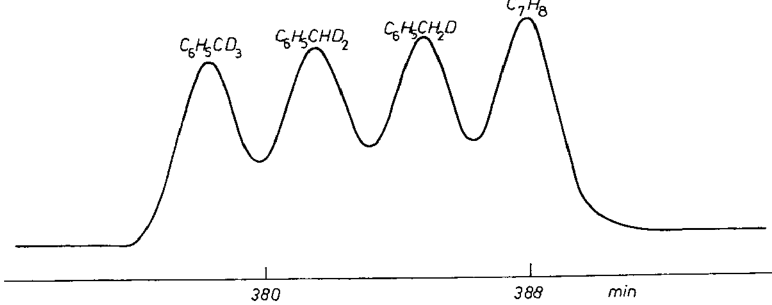

Fig. 1. Gas chromatogram of a mixture of toluene and deuterotoluenes on a 200 m squalane column. Temperature: 16°. Inlet pressure (nitrogen): 1.05 kg/cm^2. Flow rate: 0.57 ml/min.

C_7H_8 carried out at 16°. A reverse isotopic effect is observed; the heavier species being eluted before the lighter ones. It is worth noting that the mass difference among the various species, is about 1%.

TRIMER ACID COLUMNS

Trimer acid, a C_{54} tribasic acid with about 10% C_{36} dibasic acid, can be considered as a stationary phase of medium polarity as it has been found to be more polar than Apiezon and less polar than Emulphor or Triton X (ref. 2). This phase has a large working temperature range (60–200°) and has a number of interesting features as it yields symmetrical peaks with free acids, carbonyl compounds and alcohols.

This liquid phase does not coat the glass walls under ordinary conditions but a uniform layer of trimer acid can be obtained if the capillary is precoated with a carbon layer obtained by pyrolysis of methylene chloride. A methylene chloride–trimer acid solution is then passed through the column. A 77 m trimer acid capillary column has an efficiency of 130,000 theoretical plates using *n*-tetradecane as reference.

Being a stationary phase of medium polarity, it seems to be the most efficient fractionation medium for the analysis of complex mixtures where components with a wide range of polarity are present as, for example, in essential oils. By operating columns of this type under alternatively isothermal and programmed temperature conditions, it has been found that many natural essential oils consist of a very large

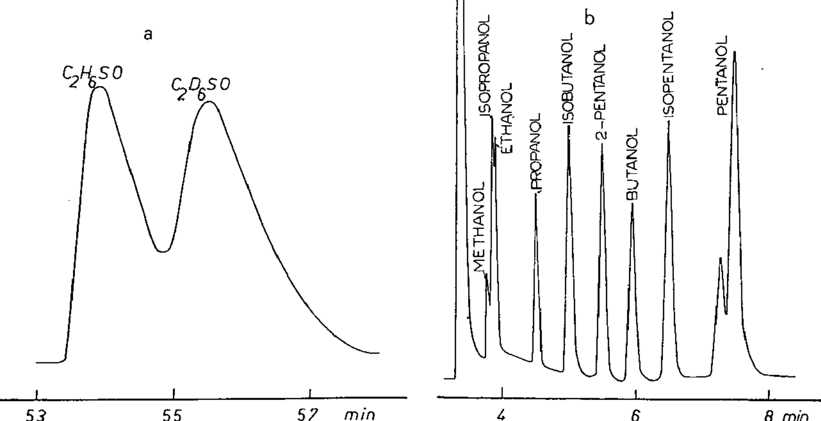

Fig. 2. (a) Separation of dimethylsulfoxide and deuterodimethylsulfoxide on a 28 m Carbowax 1540 glass capillary column. Temperature: 70°. Inlet pressure (nitrogen): 0.5 kg/cm². Flow rate: 1.0 ml/min. (b) Separation of a mixture of alcohols on a 28 m Carbowax 1540 glass capillary column. Temperature: 50°. Inlet pressure: 0.5 kg/cm². Flow rate: 1.0 ml/min.

number of components (about 250 for agrume oils and 400 for coffee oils)[3]. These columns would seem to be very useful for the study and the evaluation of essential oils and perfumes.

Carbowax columns

The Carbowaxes are more polar stationary phases and the preparation of uniform layers of them on the glass walls of the capillary can be realized by roughening the surface with polytrifluorochloroethylene or polybutadiene according to Grob[4]. The capillary, after being coated with polymeric material, is treated with a methanol solution of the chosen Carbowax; columns of great efficiency are obtained. The high resolution of these columns permits their use for the separation of polar isotopic molecules. As an example the separation of dimethyl sulfoxide from deuterium dimethylsulfoxide is shown in Fig. 2(a). The separation of a mixture of alcohols is shown in Fig. 2(b). In both cases Carbowax 1540 was used to coat the 28 m column. A temperature of 70° was used for the first separation and 50° for the second.

Conclusions

Glass capillary column technology has been developed and by means of the procedures described it is shown that stationary phases of any polarity can be successfully used for coating and yield columns of high efficiency. The ability to prepare high resolution glass capillary columns provided a very useful tool for the study of very complex mixtures, for isotopic separations and for separations of geometrical and positional isomers.

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CHROM. 4593

THE INVESTIGATION OF THE STRUCTURE OF MOLECULES BY GAS ADSORPTION CHROMATOGRAPHY

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SUMMARY

The sequence of emergence of substances from the surface of graphitized carbon black principally depends on the energy of the nonspecific interaction, which is connected with the orientation of the molecules on the plane surface. This allows us to use graphitized carbon black successfully, not only for the chromatographic analysis of different isomers but also for the identification of structural isomers. Some examples of identification of the *cis*- and *trans*-configuration of 3-methylpentene-2 and 3,4-dimethylpentene-2 and of the configurations of cyclic hydrocarbons C_6–C_{12} are given.

The energy of adsorption on a flat surface depends upon the geometrical structure of the adsorbate molecules and their orientation on the surface. The influence of the geometrical structure of a molecule on the gas chromatographic separation is especially noticeable when it is adsorbed on graphitized carbon blacks, which are typical of adsorbents with flat surfaces. The adsorption properties of such carbon blacks are principally determined by the properties of the system adsorbate–basal faces of graphite. The adsorption of molecules of different electronic structure on the surface of carbon black is "nonspecific"[1,2] and can be defined by the geometry of the molecule and by the polarizability of its bonds and a special role is played by the number of points of contact with the plane surface of the adsorbent. Therefore, the adsorption energy and the retention volumes determined by gas chromatography depend on the geometrical structure of the molecules. Thus graphitized carbon black can be used successfully not only for the chromatographic analysis of structural isomers and deuterium-substituted molecules, but also for the identification of structural isomers and isotopic molecules.

The interaction of any molecule with the basal face of the graphite depends on dispersion forces. Even in the case of dipole molecules the interaction due to induction is very low. The energy of repulsion is only 35% of the energy of attraction. The additivity of the dispersion interaction and the fact that the molecules of organic compounds are made up of a few atoms or groups allows one to express the potential

energy of interaction with the basal face of graphite by means of a few potential functions of atom–atom interaction or group–atom interaction. For example, for all hydrocarbons the interaction is determined only by two atom–atom potential functions $\varphi_{C\ldots C}$ and $\varphi_{C\ldots H}$ (refs. 3 and 4). These potential functions can be calculated satisfactorily by means of the theory of dispersion interaction[3–5].

The additivity of the energy of adsorption on the basal face of graphite of different molecules is shown in Fig. 1 where we see the calculated values of potential energy of adsorption (for the most advantageous position of molecules) and the measured heats of adsorption at low surface coverages of graphitized carbon black. The theoretically calculated potential functions on graphite Φ are close to the heats of adsorption Q measured by means of gas chromatography, therefore the slope of the curve nears 45°.

In this way, graphitized carbon black is a unique nonspecific adsorbent, and the energy of interaction of all the force centers of the molecule with this adsorbent can be considered additive. The adsorption on such an adsorbent is especially sensitive to the distance of the atoms or molecular links from the plane surface of the adsorbent, *i.e.* to the geometry of the molecule. This property can be used successfully for the analytical separation of structural and stereoisomers on graphitized carbon black[1,3,5–9]. It can, however, also be used for a reverse purpose: that is the determination of the geometrical structure of the molecules by their sequence of emergence and by the values of the retention volumes. This property is of interest in structural chemistry

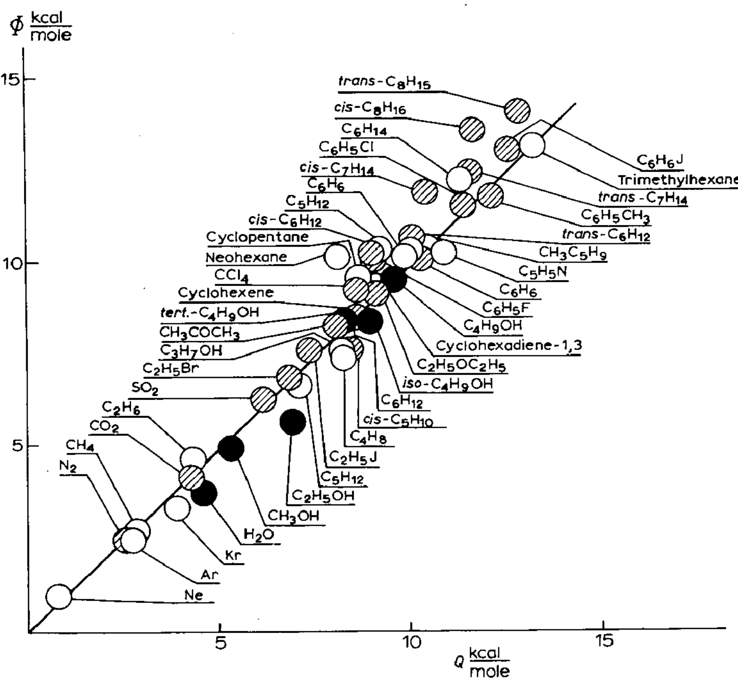

Fig. 1. The potential energies of adsorption (theoretically calculated) Φ for different molecules and heats of adsorption Q of the same compounds on graphitized carbon black obtained experimentally. The slope is 45°.

problems such as the identification and investigation of the structure of *cis*- and *trans*-isomers.

For example, the connection between the structure of the molecules and their adsorption properties on graphitized carbon black can be demonstrated by gas chromatography.

It has already been mentioned that in a series of unsaturated hydrocarbons the *cis*-configurations, having higher boiling points, are retained less strongly than the *trans*-isomers with the same carbon number. Even slight differences in boiling points yield rather different retention times. This is connected with the diverse orientation of the molecules of these isomers. The *trans*-configurations of the olefins have a more advantageous position, energetically, on the surface of graphitized carbon black as compared with the *cis*-isomers. This allowed us to use gas chromatography for the investigation of the structure of *cis*- and *trans*-configurations of 3-methylpentene-2 and 3,4-dimethylpentene-2. In the literature we find contradictory information about the configuration of these compounds and it is difficult to determine which has a *cis*-configuration and which a *trans*. The investigation of the retention volumes and heats of adsorption of these compounds showed that 3-methylpentene-2, with a boiling point of 70.4°, is the first to elute from the column at 65° in 9 min 40 sec and 3-methylpentene-2 with a boiling point of 67.6° in 11 min 34 sec and is the second. This allows us to conclude that the first compound has a *cis*-configuration and the second a *trans*-configuration (Fig. 2). The heat of adsorption of the *cis*-isomer is 9.6 kcal/mole and that of the *trans*-isomer 9.9 kcal/mole.

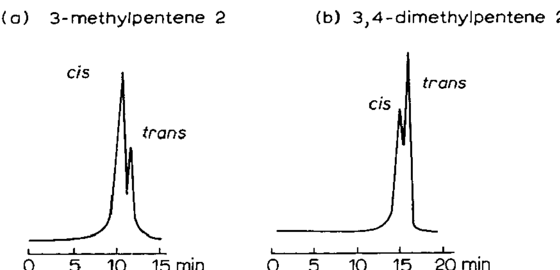

Fig. 2. Chromatogram on graphitized thermal carbon black at 65° of (a) 3-methylpentene-2; (b) 3,4-dimethylpentene-2. Column 120 cm × 4 mm; detector β-ionization.

In the case of 3,4-dimethylpentene-2, the mixture of these compounds with boiling point 87° gives two peaks—the first emerging after 15 min 25 sec is the *cis*-configuration. The *trans*-configuration elutes in 16 min 36 sec at 65°. The heat of adsorption of *cis*-3,4-dimethylpentene-2 calculated from the chromatographic values is 10.0 kcal/mole, and for *trans*-3,4-dimethylpentene-2 10.6 kcal/mole.

The energy of a nonspecific interaction decreases rapidly as the distance between the surface and the force centers increases. Therefore the adsorption on the plane faces of particles of graphitized carbon black is very sensitive to the geometrical structure of the molecules.

We have investigated a number of hydrocarbons with six-membered rings (cyclohexane, cyclohexene, cyclohexadiene-1,3 and benzene), but whose molecules differ geometrically and in the number of their hydrogen atoms, while the number of

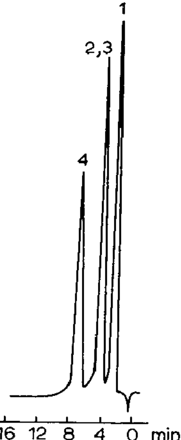

Fig. 3. Chromatogram of separation on graphitized thermal carbon black at 67° of 1 = cyclohexane; 2 = cyclohexene; 3 = 1,3-cyclohexadiene; and 4 = benzene. Column 120 cm × 4 mm; detector β-ionization.

carbon atoms remains the same. Retention volumes, obtained experimentally, and heats of adsorption of these substances show that in this group of cyclic substances an increase of retention volumes and heats of adsorption is observed when we proceed from cyclohexane to benzene (see the chromatogram on Fig. 3). Nevertheless, in this group of substances benzene, although it has both the lowest boiling temperature and the lowest molecular weight, is retained more strongly on the surface of carbon black than the other substances. This confirms once more that the chromatographic behavior of the molecules on the surface of the carbon black depends mainly on their geometrical structure and on their orientation at the surface of the basal graphite face. The molecule of cyclohexane, having a preferential chair configuration, only makes contact with the carbon black surface with three of its carbon atoms, the other three carbon atoms being some distance from the surface. Since cyclohexene and cyclohexadiene-1,3 have flatter molecules, the distance between the carbon atoms and the surface of carbon black becomes shorter, therefore, the energy of interaction with the surface of the adsorbent, which is characterized by the heat of adsorption, increases as the retention volume increases. The differences in retention volumes of cyclohexene and cyclohexadiene-1,3 are not enough for their separation. This allows us to conclude, that cyclohexene and cyclohexadiene-1,3 have similar structural configurations. This sequence of the retention volumes agrees with the values of the potential energy of nonspecific interaction by the adsorption of these substances on the basal face of graphite.

Cyclic hydrocarbons having different configurations are of special interest, even cyclooctane has five configurations. With an increase in the number of carbon atoms the number of configurations increases still further. The information concerning all cyclanes obtained synthetically is far from being available.

Retention volumes of seven cyclic hydrocarbons have been investigated at different temperatures. They were: cyclohexane, cycloheptane, cyclooctane, cyclononane, cyclodecane, cycloundecane and cyclododecane. Their heats of adsorption

TABLE I

HEATS OF ADSORPTION Q AND THE POTENTIAL ENERGY OF ADSORPTION Φ OF C_6–C_{12} CYCLANES ON GRAPHITIZED CARBON BLACK

Compounds	*Structure*	*Mol. wt. M*	*Boiling point (°C)*	*Q kcal/mole*	*Φ kcal/mole*
Cyclohexane		84	81.4	8.0	8.0
Cycloheptane		98	117–118	9.2	9.4
Cyclooctane		112	150.7	10.7	9.3 10.5 12.1 13.5
Cyclononane		126	178.4	12.4	12.1
Cyclodecane		140	70.5/11 mm	14.9	14.0
Cycloundecane		154	105–105.2/21	16.1	15.6
Cyclododecane		168		17.7	16.9

and the theoretical potential energy of adsorption were calculated (see Table I). The heat of adsorption of the cyclanes is linear with respect to the number of carbon atoms. The comparison of the heats of adsorption with the calculated potential energies for all possible configurations is only given in Table I for cyclooctane. The most stable configuration in the case of cyclooctane is the crown configuration. The value of the potential energy of adsorption theoretically calculated for this configuration is 10.5 kcal/mole which is in good agreement with the value of the heat of adsorption obtained experimentally, 10.7 kcal/mole. The calculation of the potential energy of adsorption for other configurations of cyclooctane gives the following values: 9.2; 9.3; 12.1 and 13.5 kcal/mole. These values are rather different from the experimental value for the heat of adsorption. Values of potential energies for the most stable configurations of other cyclic hydrocarbons are given in Table I. As is seen from Table I these values are in good agreement with experimental values of heats of adsorption. In the case of undecane it is still not known which configuration is the most stable. The configuration of undecane chosen which is shown in Table I gives a value for the potential energy of adsorption of 15.6 kcal/mole, which approximates to the value of 16.1 kcal/mole for the heat of adsorption obtained experimentally. Thus one may assume that the configuration chosen is correct. The calculation of potential energies for other configurations shows that the values differ considerably from the heat of adsorption obtained experimentally.

From the above examples we can see that gas chromatography on graphitized carbon black can be used not only for analytical purposes, but can also be used in

conjunction with other methods for the investigation of the geometrical structure of molecules.

ACKNOWLEDGEMENTS

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CHROM. 4631

CONSIDERATIONS FOR THE DESIGN OF A ROTATING UNIT FOR CONTINUOUS PRODUCTION BY GAS CHROMATOGRAPHY AND ITS APPLICATIONS

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SUMMARY

An apparatus for a continuous gas chromatographic process is presented; its principles of operation have already been described by M. TARAMASSO *et al.*[1-6].

The main features of the process are: efficiency at least equal to that of an analytical column, possibility of obtaining several components at the same time or a single peak in many traps, with a high-grade central fraction, and feed rates up to 400 ml/h.

PRINCIPLES OF THE PROCESS

By combining a chromatographic technique with an adequate moving system it is possible to transform a substantially discontinuous process into a continuous one.

In the process described here this principle was used. Unlike SCOTT's[1] process wherein the motions are intimately connected and have opposite sense but the same direction, in this process the two factors are maintained distinct and act in orthogonal directions.

The fundamental idea and the simplest way of putting into practice the principle, may be described as follows: as shown schematically in Fig. 1, let us suppose that a column is moving in a plane at a speed v_s, with respect to the feeding and collecting systems which are stationary. The column assumes successively the positions indicated by dashed lines, whilst the carrier gas flows in the direction indicated by the arrows.

If the length of the chromatographic column is L, and the retention time of the component "i" is t_R, the distance covered by this component in the plane is given by the combination of the two speeds (v_s and L/t_R), and the elution which occurs in the position X_i can be defined as follows:

$$X_i = v_s \cdot t_{Ri}$$

However, owing to longitudinal diffusion, the elution of the component will occur in a range ΔX_i proportional to the band broadening Δt_{Ri}.

If a series of columns is moved into the appropriate positions at successive

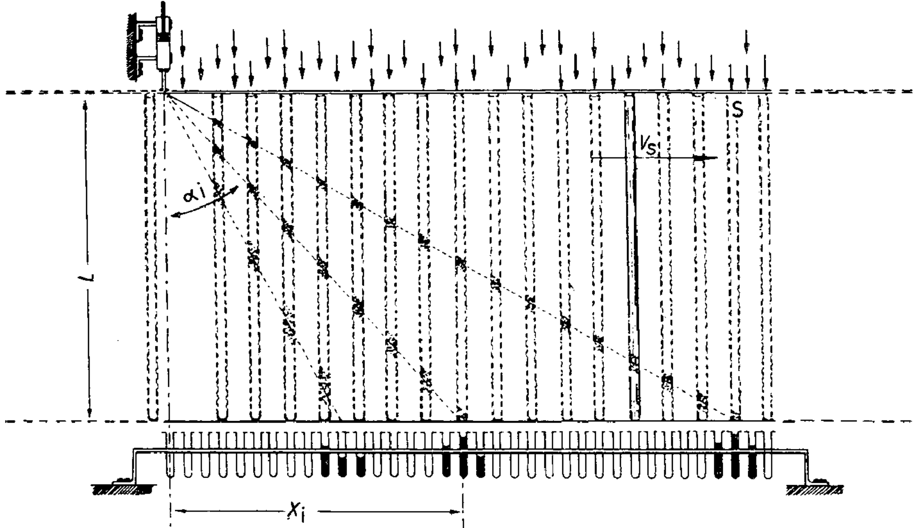
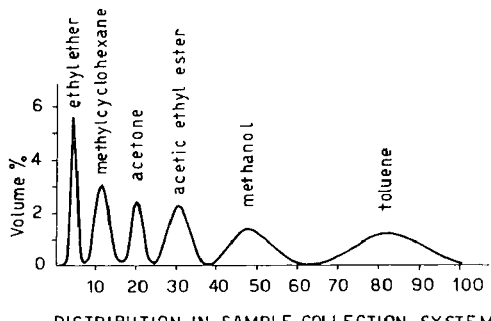


Fig. 1. Schematic diagram of continuous gas chromatography.



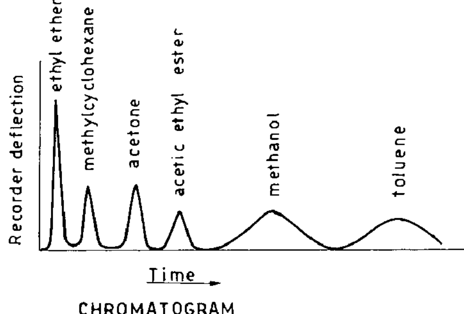
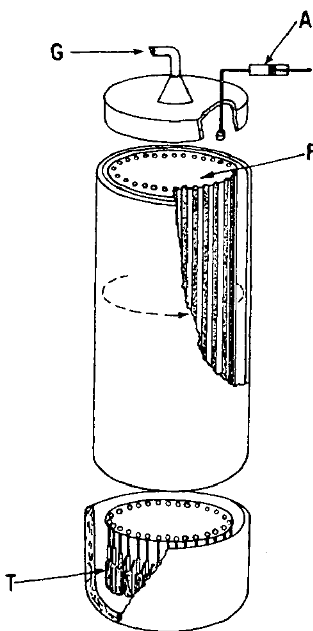


Fig. 2. Equivalence between the chromatogram and space distribution, obtained with a six-component mixture.



Fif. 3. Diagram of the rotating unit for preparative scale gas chromatography. G = carrier gas inlet; A = sample feeding system; F = bundle of columns; T = trapping system.

intervals, the process is made continuous: the component distribution in the trap-system is the space equivalent of the elution diagram given by a single column over a period of time (Fig. 2). Finally, if the columns are placed along the generatrices of a rotating cylinder having a radius r (Fig. 3), the process becomes really continuous and the foregoing equation becomes:

$$X_{\mathrm{i}} = 2\pi r \nu t_{R\mathrm{i}}$$

Where ν is the number of revolutions of the cylinder per unit of time. The conditions corresponding to the maximum utilization are obtained when the last component (retention time t_{R2}) is eluted adjacent to the first component (t_{R1}). The number of revolutions of the series of columns is given by:

$$\nu = \frac{1}{t_{R2} - t_{R1}}$$

ASSEMBLY AND OPERATION FEATURES OF THE APPARATUS

In our second model the number of columns forming the cluster was limited to 36, to facilitate handling and in order to ensure feed rates between 2 and 400 ml/h.

An important feature is the complete automation, thus handling the apparatus does not require skilled personnel. The cluster, consisting of 2 m long, U shaped stainless steel columns, is easily interchangeable. The cluster must be filled in such a way that the carrier gas flow rate in two adjacent columns does not differ by more

than ±1%. Using 6 mm diameter columns, the system will have an efficiency practically equal to that of an analytical one.

As a result of the structural features of the system, the sample introduction occurs in the absence of carrier gas flow, in such a way that preliminary dilution of the sample is avoided.

As the carrier gas enters some moments after the sample, a frontal chromatographic process takes place, so the components are already partially separated when the carrier gas is let in.

The introduction of the samples is effected by a micropump, whose feed rate can be varied continuously from 2 to 400 ml/h, and which has a reproducibility of 1%. This sampling device is capable of feeding volatile liquids or viscous mixtures. The cluster of columns, the feeding system and carrier gas inlets are located in a chamber, the temperature of which may be varied from room temperature up to 220°, with a high accuracy ensured by forced air circulation. The speed of rotation of the cluster can be adjusted continuously from 1 to 9 revolutions/h.

For preparative purposes the criteria for the selection of the stationary phase differ considerably from those valid for analytical gas chromatography. This is particularly true when the separation of a single substance from a complex mixture is required. In this case it is more convenient to elute the components which are not required all together. An example of this is the separation of benzene from all the other hydrocarbons in a cut of petroleum boiling in the range 65–95°; this is shown in Fig. 4. When such a selective stationary phase is not available, one can resort to a mechanical artifice to achieve the same purpose. By suitable selection of the operating conditions, it is possible to allow the tail impurities to elute so that they overlap into the head impurities. This device has been used in the separation of 3-methylpentane from the distillation heads of n-hexane. The analytical chromatogram is shown in

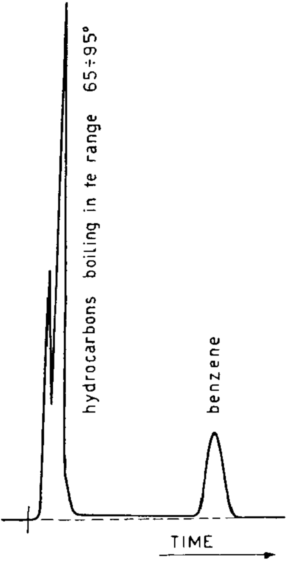


Fig. 4. Separation of benzene from hydrocarbons boiling in the range 65–95°.

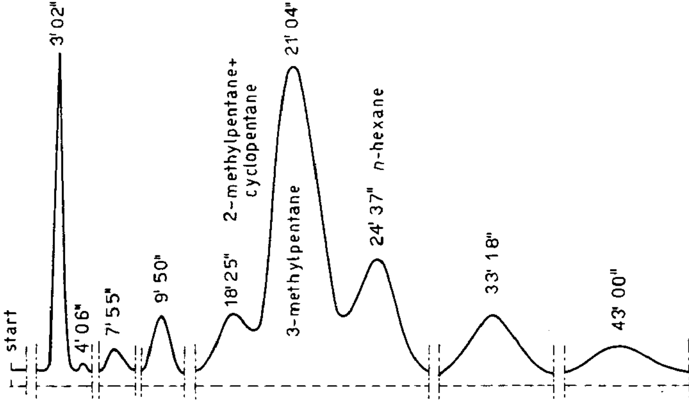


Fig. 5. Chromatogram of the "tops of *n*-hexane distillation".

Fig. 5; Fig. 6 shows the hypothetical chromatogram which the apparatus has been compelled to produce.

Fig. 7, which shows the purity *versus* collecting position, clearly indicates that, while the initial and ultimate fractions collected contain impurities, high purity 3-methylpentane is obtained in the central traps. The same example shows the exceptional purity obtainable by this process: percentages of 99.9% and 99.99% should be considered normal for the central portion of each peak.

FACTORS REGULATING THE PERFORMANCE OF THE APPARATUS

The quantity of a mixture which can be treated per unit of time depends on the number of the columns composing the cluster (N), on the maximum quantity of

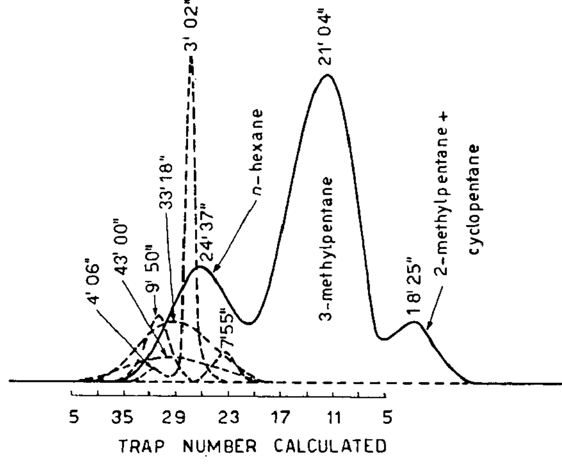


Fig. 6. Apparatus program for the separation of 3-methylpentane.

sample that can be injected into each column for obtaining the desired purity (q_m), and on the number of the rotations of the cluster per unit of time:

$$Q = N \cdot q_m \nu$$

Thus, the maximum quantity that can be injected to obtain the desired degree of purity depends on the resolution R of the two key components, which by definition is expressed as follows:

$$R = 2 \frac{t_{R_2} - t_{R_1}}{W_{b_1} + W_{b_2}} \simeq 2 \frac{\Delta t}{\Delta W_b}$$

where W_b is the width of the peak base for the component eluting last.

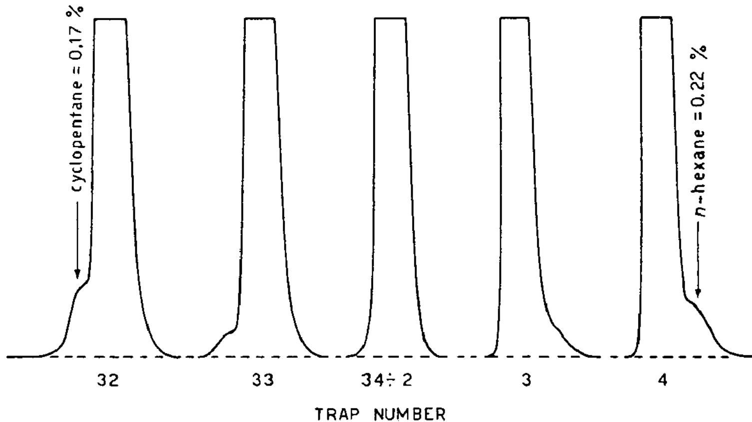


Fig. 7. 3-Methylpentane distribution in the trapping system.

In order to limit the width of the peak, and at the same time obtain high values of q, it is necessary that the variable t has as small a value as possible. Therefore the optimization of q requires a compromise between these two antithetical factors (maximum value of $t_{R_2} - t_{R_1}$ and minimum value of t_{R_2}) since the former is an increasing function of the latter.

On the other hand, a low retention time of the component 2 allows a high revolution rate of the cluster and minimum use of stationary phase per unit weight of separated product.

In practice short columns are preferred ($1 \text{ m} < L < 3 \text{ m}$) and the stationary phase, temperature and flow rate of the carrier gas would be selected so as to obtain low retention times and, at the same time, a satisfactory resolution of the components. The optimization of the above mentioned factors is effected in a preliminary study, defined programming, where the same experiments are made on a "pilot" chromatographic column identical to those forming the cluster and mounted on an analytical chromatograph.

The program makes the process independent of the need to use a detector or any other device to record the eluent peaks.

APPLICATIONS

The method described is primarily applicable to the separation of mixtures which cannot be otherwise easily treated, such as azeotropes, isomers, close boiling point substances.

As an example, it has been possible to separate the *cis-trans* isomers of pentene-2 by means of a diethylene glycol and silver nitrate stationary phase at 30° with a feed rate of 60 ml/h. This is equivalent to a capacity of 1.3 l/day per kg of packing with a consumption of 0.85 m^3 nitrogen. Such a potentiality shows that if the process was applied on a large scale it would be economic. The purity of the *cis–trans* isomers separated was 99.6%. A purity higher than 99.99% has been obtained in the refining of isoprene from a mixture containing the required product mixed with 90% methylbutenes as the main impurities. In this case a production rate of 20 l/week was maintained for three months without any band shifting or other inconvenience.

The feed rate is reduced according to the relative volatility of the components to be separated and to the complexity of the mixture.

An example of a rather difficult separation is that of the methallylchloride from isocrotyl chloride. The starting mixture also contained small amounts of *tert.*-butyl chloride and isobutylene. With a delivery of 21.6 ml/h samples of methallylchloride, 99.99% purity, and isocrotyl chloride, 99.87% purity, have been obtained. Fig. 8

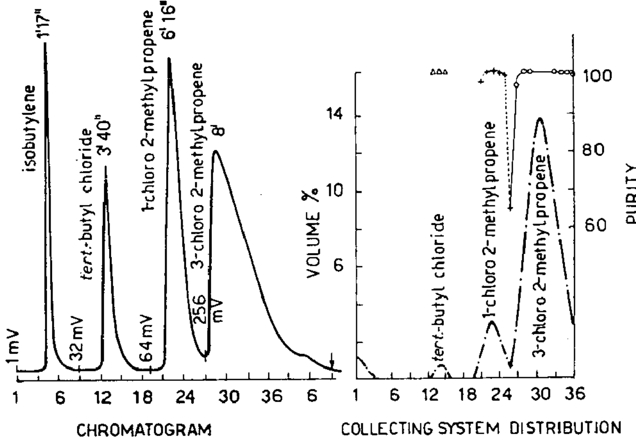


Fig. 8. Chromatogram and collecting system distribution, in the separation of 3-chloro-2-methylpropene and 1-chloro-2-methylpropene.

shows the chromatogram used for programming the apparatus and the distribution obtained in the collecting system, using Carbowax 400 as stationary phase and working at 90°.

The high efficiency of this type of column equalling that of an analytical system is utilized in the following difficult separation, shown in Fig. 9. A separation of the *cis–trans* isomers of piperylene was carried out starting from a trade product having the following composition:

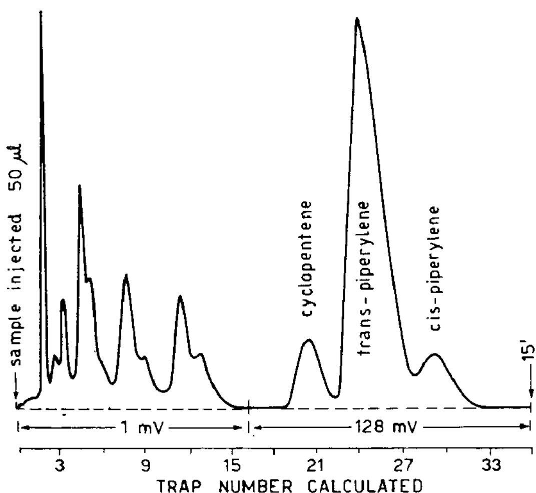


Fig. 9. Program for the separation of *cis–trans* piperylene.

Light impurities	0.31%
Isoprene	0.76%
Cyclopentene	9.28%
trans-Piperylene	79.27%
cis-Piperylene	10.38%
Cyclopentadiene	(0.007%)

1,2-Bis(2-cyanoethoxy)-ethane was found to be a suitable stationary phase for this mixture. The rotating unit was run with a feed rate of 7.2 ml/h. The distribution obtained in the collecting system was equivalent to that of the chromatogram (Fig. 9) and the purity of the *trans*-piperylene fraction collected was 99.9%; the *cis* fraction obtained had a purity of 81.9% with 18.0% of *trans* isomer, after treatment with maleic anhydride it gave a product of 99.9% purity. The recovery of these volatile compounds, made with dry ice and Dowanol was about 70% by vol. based on the feed.

Mixtures of terpenes (namely camphene, myrcene, and α,β-pinenes), which present similar difficulties, have been separated on Carbowax 400 at 104° with a feed rate of 10 ml/h; purities of 99.8–99.9% for single components, and in some cases even higher than 99.99%, have been obtained.

The applicability of the method is far from being limited to the examples cited above. The high feed rates possible, the very high purities obtainable and the compactness of the apparatus render the method an effective alternative to the fractional distillation. Proof of this was given by the separation of *iso*- and *n*-pentane the result of which was very competitive as far as the delivery and purity realized were concerned when compared to the fractionation obtainable with an 85 plates Oldershaw column. For the reasons mentioned, the prospects for producing hydrocarbons of very high purity are remarkable. In addition to the 3-methylpentane already mentioned, cyclopentane has been obtained from the bottoms of an *n*-pentane distillation in a similar way by using a stationary phase of 1,2-bis(2-cyanoethoxy)-ethane at 39° with a feed rate of 45 ml/h. The purification of *n*-hexane of 95% purity was effected

with a feed rate of 30 ml/h; a final product of 99.99% purity was obtained. In a purification of cyclohexane we obtained a product whose chromatogram showed no impurity traces.

A special feature of the process is the possibility of obtaining a number of components from a mixture simultaneously. This is shown by the separation of reaction crudes, which normally require a long fractionation procedure and separation analysis.

1,1-Dichloro-2-vinylcyclopropane and 4-vinylcyclohexene-1 with purities of 99.9% have been obtained from the mixture resulting from the reaction between sodium trichloroacetate and butadiene. The feed rate in this case was 30 ml/h.

The reaction mixture between cyclopentadiene and isoprene was fed into the apparatus at a rate of 4 ml/h at 110° and in a single working step 1,4-dimethyl-1-vinylcyclohexene-3, limonene and methyltetrahydroindene were separated with a purity of 99.9% for each component.

We could separate the dihydrodicyclopentadiene with a purity higher than 99.9% from a mixture of hydrogenation products of the dicyclopentadiene, with a rate of 6 ml/h at 140° and a stationary phase of methylsilicone polymer.

The various features of the preparative rotary chromatograph and its possible applications render it a valuable tool for laboratory research, and for small scale industrial production.

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CHROM. 4634

GAS CHROMATOGRAPHY OF FLUORINATED COMPOUNDS ON POROUS POLYMERS

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SUMMARY

The porous polymers Chromosorb-101 and Chromosorb-102 are weakly specific adsorbents. Complete separation of organic compounds and their fluorinated analogues is possible on sorbents of this type; the fluorinated compounds usually elute first. The heats of adsorption of some substances and their fluorinated analogues on the weakly specific adsorbents Chromosorb-101 and Chromosorb-102 and the specific adsorbent Porapak T were measured.

INTRODUCTION

It has been shown earlier[1,2] that the porous polymers Chromosorb-101 and Chromosorb-102 are weakly specific adsorbents of the III-d type[3] as π-bonding phenyl groups exist on their surfaces. However, the contribution of nonspecific dispersion interactions to the total energy of the molecules of different compounds on these polymer surfaces predominates. This can be seen if the retention volumes of organic compounds of different groups (A,B,D) are compared with the retention volumes of their fluorinated analogues. When the energy of dispersion interactions is evaluated the values of the electron polarizability and the Van der Waals radii of groups of interacting molecules are very important. The polarizabilities of hydrogen and fluorine are slightly different, but the hydrogen Van der Waals radius is less than that of fluorine. Therefore the value of a/r^6 characterizing the dispersion interaction is less for F than for H. Since there is a partial or complete change of hydrogen atoms for fluorine atoms in the organic molecules studied, this must result in a decrease in the retention volumes and heats of adsorption (especially in the case of the predominant appearence of nonspecific dispersion interactions). This effect was noticed earlier[4] in calorimetric analyses of heats of adsorption of methylcyclohexane and perfluoromethylcyclohexane (molecules of group A) on the nonspecific adsorbent graphitized carbon black and the specific adsorbent Zeolite.

EXPERIMENTAL

Measurements were made on a Zvet-3 gas chromatograph with a thermal conductivity detector. Samples were injected with a 1-μl syringe. Chromosorb-101, Chromosorb-102 (Johns Manville) and Porapak T (Waters Associates) were used as adsorbents.

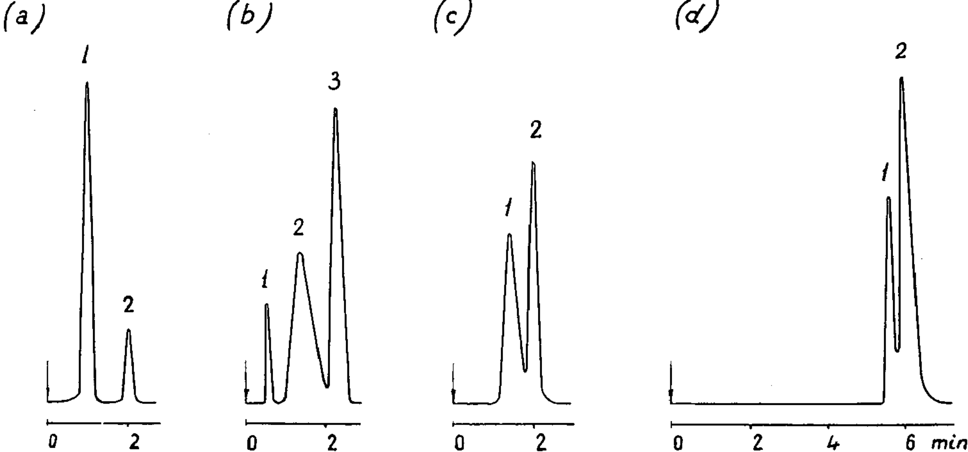


Fig. 1 Chromatograms obtained on a column, length 0.37 m, with Chromosorb-101: (a) 1 = trifluoromethyl acetate, 2 = methyl acetate; temperature 130°; carrier gas (helium) rate 50 ml/min; (b) 1 = water, 2 = perfluoropropanol, 3 = propanol; temperature 130°; carrier gas (helium) rate 50 ml/min; (c) 1 = *n*-perfluoroamyl alcohol, 2 = *n*-amyl alcohol; temperature 190°; carrier gas (helium) rate 36 ml/min; (d) 1 = *p*-fluoroacetophenone, 2 = acetophenone; temperature 220°; carrier gas (helium) rate 30 ml/min.

RESULTS AND DISCUSSION

Fig. 1 shows chromatograms of the separation of compounds of different classes and their fluorinated analogues on the porous polymer Chromosorb-101. In all four cases (alcohols, ester and ketone) the substitution of hydrogen atoms by fluorine atoms results in a decrease of the retention volumes (even in the case of one atom of fluorine in acetophenone). Thus, it can be seen that the contribution of the nonspecific dispersion interaction to the total energy of adsorption on Chromosorb-101, at fixed temperatures, predominates. In the case of molecules of group D, orientation interaction takes place especially at lower temperatures. In fact, the order of elution of amyl and perfluoroamyl alcohol on Chromosorb-102 is reversed in the temperature interval 100–250° (Fig. 2). The chromatogram for the methyl acetates and acetophenone on this Chromosorb is analogous to that on Chromosorb-101 (Fig. 3). It was interesting to compare the behaviour of fluorinated compounds on weakly specific and specific adsorbents. Fig. 4 shows a chromatogram of the seperation of propanol and perfluoropropanol on the polar porous polymer Porapak T. In this case the nonfluorinated alcohol elutes much earlier than the fluorinated one owing to the large influence of the orientation interactions. Heats of adsorption were calculated from the temperature dependences. The results are shown in Table I.

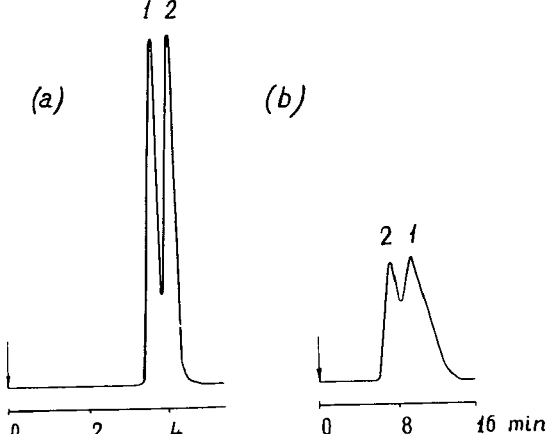
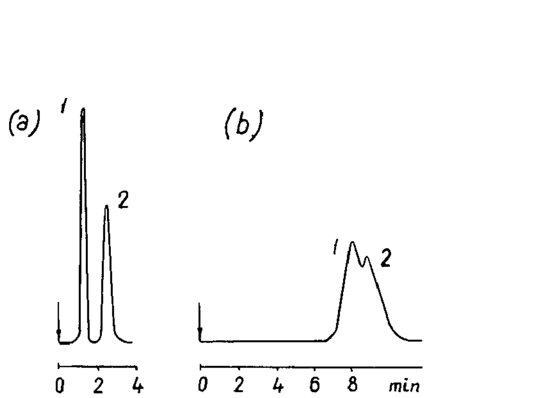


Fig. 2. Chromatograms obtained on a column with Chromosorb-102: (a) 1 = *n*-perfluoroamyl alcohol, 2 = *n*-amyl alcohol; column length 2 m; temperature 230°; carrier gas (helium) rate 30 ml/min; (b) 1 = *n*-perfluoroamyl alcohol, 2 = *n*-amyl alcohol; column 0.375 m; temperature 120°; carrier gas (helium) rate 50 ml/min.

The heats of adsorption of the fluorinated compounds on weakly specific adsorbents are generally less than those of the nonfluorinated compounds. The exception is amyl alcohol for both Chromosorbs. The fact that heats of adsorption of fluorinated compounds are less than those of nonfluorinated ones shows that the predominant contribution to the total energy of interaction is made by the nonspecific dispersion interaction which is proportional to α/r^6.



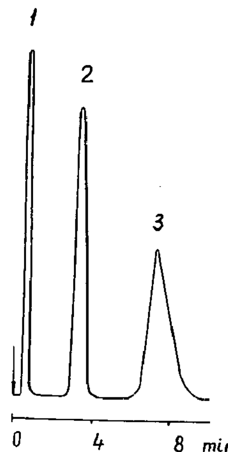


Fig. 3. Chromatograms obtained on an 0.375 m column with Chromosorb-102: (a) 1 = trifluoromethyl acetate; 2 = methyl acetate; temperature 100°, carrier gas (helium) rate 60 ml/min; (b) 1 = *p*-fluoroacetophenone, 2 = acetophenone; temperature 190°; carrier gas (helium) rate 43 ml/min.

Fig. 4. Chromatograms obtained on an 0.375 m column with Porapak T: 1 = water, 2 = *n*-propanol, 3 = perfluoropropanol; temperature 150°; carrier gas (helium) rate 45 ml/min.

TABLE I

HEATS OF ADSORPTION *(Q)* OF SOME ORGANIC COMPOUNDS AND THEIR FLUORINATED ANALOGUES ON THE POROUS POLYMERS CHROMOSORB-101, CHROMOSORB-102 AND PORAPAK T

	Q (kcal/mole)		
	Weakly specific		*Specific*
	Chromosorb-101	*Chromosorb-102*	*Porapak T*
C_3H_7OH	10.2	—	11.4
$C_3H_2F_5OH$	9.7	—	13.7
CH_3COOCH_3	9.3	10.5	11.1
CF_3COOCH_3	8.3	9.9	10.3
$C_5H_{11}OH$	12.1	11.0	15.1
$C_5H_2F_9OH$	12.8	13.2	20.8
$CH_3COC_6H_5$	13.6	15.3	—
p-$CH_3COC_6H_4F$	13.2	14.4	—

ACKNOWLEDGEMENTS

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CHROM. 4594

RESINS AND GELS FOR CHROMATOGRAPHY

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SUMMARY

Ion exchangers and gels for chromatography synthesized by the authors are described. Resins and gels for chromatography have been prepared based on copolymers of styrene and *p*- or *m*-divinylbenzene. They are characterized by their high purity, homogeneity of granular composition, high mechanical strength and stability, and the reproducibility of their properties.

The properties of some organomineral ionites and ion-exchange cloths which show promise in their use in chromatographic processes are presented.

Ion exchangers and gels have been widely applied in different branches of science and technology but only recently has there been a tendency to classify them according to their purpose. In the early stages of development of ion-exchange methods the same resins were used both in the laboratory and on a commercial scale for quite different purposes. Neither analytical chemists nor engineers considered the situation satisfactory. As a result special ion exchangers for chemical analysis, for chromatography, and for preparation and purification of highly pure substances etc. were produced.

The main differences in the properties of resins for chromatographic use and of those for standard ion exchangers are in their:

1. Stability and reproducibility of their physico-chemical properties (capacity, swelling, selectivity, etc.);
2. Low impurity content;
3. Negligible quantity of elutable organic compounds;
4. Highly homogeneous granular composition;
5. Increased mechanical strength.

Ion exchangers that do not have stable properties with respect to capacity, exchange constants, partition and separation coefficients and other important characteristics evidently cannot be used for chromatography. The presence of impurities or soluble organic compounds in ion exchangers excludes their use for the chromatographic preparation of high purity substances and for precision analysis. Heterogeneity of the granular composition hampers the efficient performance of chromato-

graphic processes, especially of ions with similar properties. Resins for chromatography should therefore be of high mechanical strength and should contain a minimal quantity of defective beads which can be destroyed during use, thus causing the deterioration of chromatographic and hydrodynamic processes.

Along with ion-exchange resins, hydrophobic and hydrophilic gels are also of great interest. They are widely used in gel chromatography and also as packings in gas chromatography. Gel chromatography has greatly progressed lately. It is used in biochemistry, in analysis, in the chemistry of high molecular compounds and in other branches of science for molecular weight determination and fractionation of polymeric compounds; and for the identification, separation, purification and concentration of different compounds. It should be mentioned that all the requirements described above for chromatographic resins apply to materials for gel chromatography as well.

In our report we shall examine the properties of the ion exchangers and gels that have been prepared in our laboratory for chromatographic use.

The main lattices for the preparation of polymeric resins and hydrophobic gels are copolymers of styrene and divinylbenzene (DVB). Up to the present commercial DVB has been used as a cross-linking agent in the preparation of polymeric ion exchangers. The compound contains more than twelve components, their composition and contents being different from batch to batch. Some of these components are telogens or inhibitors of the process, others are polymerized themselves. To obtain ion exchangers and gels for chromatography possessing reproducible and strictly controllable properties, one naturally needs monomers containing no by-products which could influence the polymerization or participate in it. Having this in mind we developed a procedure for the separation of *m*- and *p*-isomers of DVB from the commercial mixture. The purity was 99.0–99.5%.

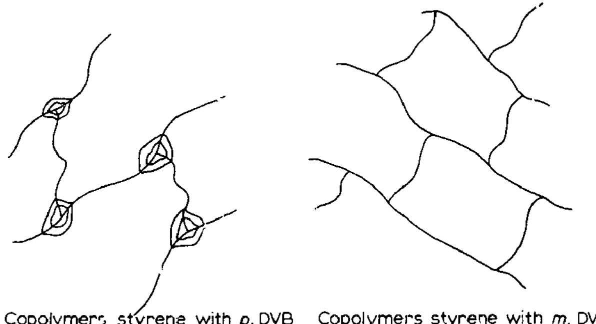

Fig. 1. Structure of copolymers of styrene with *p*- and *m*-DVB.

Kinetic investigation showed that styrene copolymers with the two isomers of DVB had completely different structures (Fig. 1). The copolymer structure of styrene and *p*-DVB may be considered as a heterogeneous micro-gel, containing strongly cross-linked areas, which are bound by polystyrene chains. Quite another structure is observed for the copolymer of styrene and *m*-DVB. In this case the structure is sufficiently homogeneous for one to see the regular interchange of styrene and DVB groups. Therefore, depending on the type of isomer, one can prepare ion exchangers and gels with a certain permeability, selectivity, or swelling properties, etc.

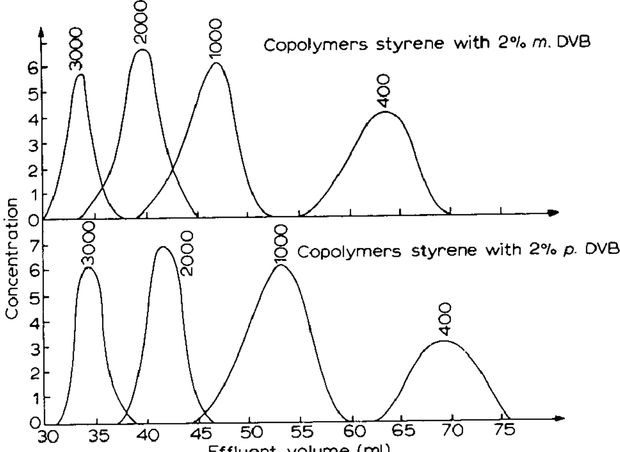

Fig. 2. Separation of polyethylene glycols of different molecular weight; dependence of gel chromatographic properties on copolymer structures.

The more homogeneous structure of copolymers based on *m*-DVB in comparison to that of copolymers based on *p*-DVB was confirmed by gel chromatography. Though the SDV-2*p** gel swells somewhat less than the SDV-2*m* gel, its permeability due to its heterogeneous structure is higher. Therefore in the case of SDV-2*p*, larger quantities of eluent are required than in case of SDV-2*m* (Fig. 2).

It should be mentioned that the copolymers of styrene with *p*-, *m*- or commercial DVB each have different swelling properties so they also have different chromatographic properties.

We studied the preparation of gel filtration materials including lattices of other ionites synthesized from the copolymers of styrene and DVB-isomers. Copolymerization was performed in the presence of certain solvents which functioned either as telogens (carbon tetrachloride) and diluents (toluene, dodecane and others) or as effective transmitters (ethyl thioglycolate). By varying the type and quantities of the solvents and their mixtures and the amount of the cross-linking agent, we obtained gels and resins of different permeabilities and structures.

It was shown that for the separation of compounds with a molecular weight up to 10^4 it was necessary to prepare the gel in the presence of the solvents that completely dissolve the monomer and cause the polymer to swell (toluene). To obtain a gel with a permeability higher than 10^4, one should use a solvent in which the monomer is completely dissolved and the polymer only swells a little (heptane), that is one should use practically macroporous structures. Similar structures are also required as packing for columns in gas chromatography. An example of the separation of polyethyleneglycols of different molecular weights by means of a styrene divinylbenzene gel is shown in Fig. 3.

We have developed a procedure for preparing macroporous gels based on the copolymerization of styrene and DVB in silica gel pores. After the copolymer formation

* The figure following the symbol of the copolymer or ionite indicates per cent content of DVB in the styrene divinylbenzene mixture. The letters *m* and *p* indicate the type of isomers used.

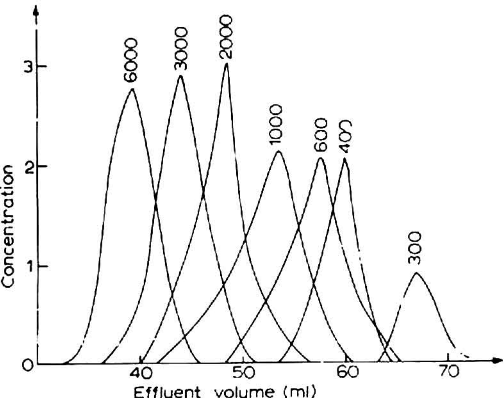

Fig. 3. Separation of polyethylene glycols of different molecular weight on styrene divinylbenzene gel.

the inorganic lattice was removed by dissolving it in NaOH or HF. The copolymer obtained had a specific surface area equal to that of the silica gel used. By varying the properties of the silica gel (specific surface area, pore volume and dimensions) one can prepare copolymers with a particular porosity.

For the preparation of ion exchangers and gel filtration materials of low impurity content, deionized water is necessary as well as pure monomers. Impurities in the prepared products are as follows: Fe–$2 \cdot 10^{-5}$; Cu–$2 \cdot 10^{-5}$; Al–$5 \cdot 10^{-5}$; Mn–10^{-5}; Ni–10^{-5}; Pb–$1 \cdot 10^{-5}$; Cr–$5 \cdot 10^{-5}$%.

The number of defective beads (broken, cracked etc.) in the resins did not exceed 2–3 per 100.

Precision chromatographic procedures require ion exchangers and gels of reliably similar dimensions. That is why double fractionation was carried out: firstly

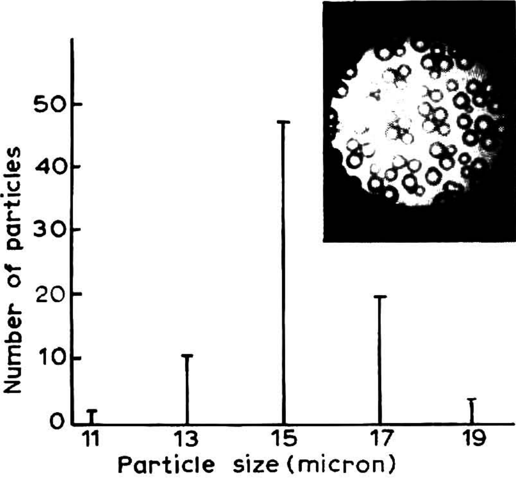

Fig. 4. Fractional composition and appearance of microspherical sulforesin (KRS-8*p*) for an automatic amino acid analyzer.

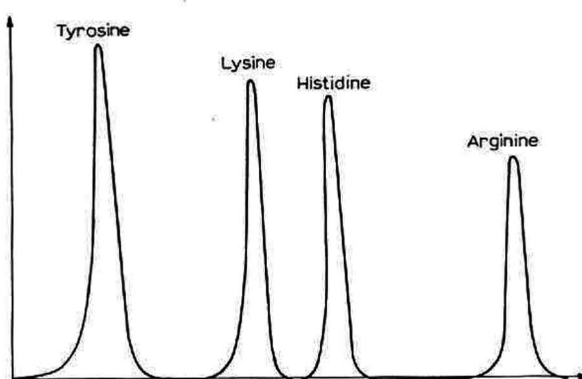

Fig. 5. Chromatogram of tyrosine and basic amino acids (10^{-8} M) obtained on the column (0.9 × 5 cm) packed with resin KRS-8*p*. The time of analysis is 1 h.

of the copolymers (before the polymer analogous transformation) and secondly of the ion exchangers. Before fractionation by dimension, the copolymers and ion exchangers had been fractionated according to their specific weight in organic liquids or in salt solutions with an accuracy of ± 0.001 g/c.c. The resins for chromatography had the following fractional compositions when air dried: 10 ± 2, 15 ± 3, 20 ± 5, 50 ± 10, 100 ± 20 μ. Over 96–98% of the ion exchangers (by weight) are within the range.

Microspherical ionites (10 ± 2; 15 ± 3; 20 ± 5 μ) were prepared specially for our automatic amino acids analyzers. The outward appearance of the resins and their fractional composition are shown in Fig. 4; the values of the composition indicate high homogeneity of the resins. Chromatograms of tyrosine and some basic amino acids are shown in Fig. 5.

Ion exchangers for chromatography have been prepared from copolymers of styrene and *p*- or *m*-DVB, containing the following groups: $-SO_3H$; $-PO_3H_2$; $-COOH$;

$-(CH_2)_nCOOH$; $-N(CH_2)_nCOOH$; $-SH$; $-N(CH_3)_3$; $-N(CH_2CH_2OH)_3$; $-N$(pyridine ring)

TABLE I

MAIN PROPERTIES OF ORGANO-MINERAL SORBENTS

Type of sample	*Exchange capacity (mequiv./g)*	*Swelling (%)*	*Pore volume c.c./g*	*Surface m²/g*
White soot–sulfonated polystyrene	1.1	5	—	40
Aerosil–sulfonated polystyrene	1.8	10	—	200–250
Zirconium oxide–polyvinylphosphinic acid	3.0	10	—	10–100
Macroporous silica gel–polyacrylic acid	1.0	5	0.4–0.6	200
Macroporous silica gel–alkylated polyvinylpyridine	0.6	15	0.2–0.3	300
Macroporous silica gel –aminated polystyrene	1.2	5	0.4–0.6	200

TABLE II

MAIN ION-EXCHANGE PROPERTIES OF CLOTHS

Type of polymer on cloth	*Amount of inoculated polymer in the material obtained (%)*	*Type of functional group*	*Capacity by 0.1 N NaCl (mequiv./g)*	*Capacity by 0.1 N NaOH (mequiv./g)*
Polyacrylic acid	32	–COOH	0.5	4.2
Polyacrylic acid	43	–COOH	0.6	5.7
Polyacrylic acid	54	–COOH	0.7	7.3
Polystyrene	70	$-SO_3H$	3.1	3.9
Polystyrene	70	$-CH_2 \cdot N(CH_3)_3$	1.7	2.1

Capacities and swelling properties of the ion exchangers are reproducible with an accuracy of $\pm$ 5%, which is rather valuable.

By using different solvents in the preparation of the copolymers we obtained ion exchangers with a high permeability for large organic ions (for ions of antibiotics in particular).

We have also prepared some organo-mineral ionites which show promise for thin-layer chromatography, and in some cases for column chromatography. These substances are prepared by radiation induced polymerization of the different monomers from the gaseous phase on inorganic carriers. The main properties of the ionites are shown in Table I. They have a highly developed specific surface and increase the speed of ion exchange.

Ion exchange cloths instead of ion-exchange papers are also proposed. The ion exchange textile materials were prepared by the same method of radiation-induced polymerization. Their main properties are shown in Table II. Unlike the papers, these can be used repeatedly for the separation of substances by ascending and descending chromatography.

CHROM. 4609

CHARACTERIZATION AND GAS CHROMATOGRAPHIC DETERMINATION OF ACTIVE PRINCIPLES OF BIOLOGICAL INTEREST IN PHARMACEUTICAL PRODUCTS (GASTRIC MUCOPROTEIN AND THIOCTIC ACID)

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SUMMARY

The assay of thioctic acid in pharmaceutical preparations and that of *l*-fucose in gastric mucoprotein are described here as two representative examples of the application of gas–liquid chromatography in analytical work with biologically active products, natural or synthetic.

In both these cases, gas–liquid chromatography gives qualitative and quantitative analytical results such as could not be obtained by traditional techniques; these are limited both in terms of specificity and sensitivity.

The quantitative results obtained are completely specific, reproducible and accurate, as required in quality control and assay of substances being prepared for therapeutic use.

INTRODUCTION

The application of gas–liquid chromatography in analytical work in the pharmaceutical field is of considerable importance. The assay of thioctic acid in pharmaceutical products and that of *l*-fucose in gastric mucoproteins used in the pharmaceutical industry are reported here as two highly representative examples of this method of analysis.

ASSAY OF THIOCTIC ACID IN PHARMACEUTICAL PRODUCTS

Thioctic acid (α-lipoic acid) occurs naturally both in the vegetable and animal kingdoms. The substance was first isolated in the crystalline state by REED *et al.*[1] in 1951 and called "α-lipoic acid" because of its behavior in fat solvents. Later, BROCKMAN *et al.*[2] identified its chemical structure as 6,8-dithiooctanoic acid in a cyclic form;

these same authors called the substance thioctic acid. In the following years, thioctic acid was made synthetically by various methods.

Pharmacological investigations carried out in view of the biochemical properties of thioctic acid revealed that the substance had liver-protecting qualities in various hepatic diseases and antitoxic properties in poisoning from various substances (*e.g.* mercuric chloride, arsenobenzene).

The quantitative assay of thioctic acid in compound formulations of some complexity is extremely difficult by means of the classical colorimetric and spectrophotometric methods. In pharmaceutical formulations with liver-protecting action, thioctic acid is usually associated with other active substances that are not always completely separable (*e.g.* liver extract, cysteine, methionine) and interference from such substances makes these analytical techniques unreliable. Further, because of the low specific extinction of thioctic acid (6.80 in methanol), one must work with relatively large quantities of the substance.

Our work is concerned with the more commonly used pharmaceutical products containing thioctic acid; these include tablets and injectable solutions. We have developed a quantitative method of analysis by gas chromatography after the substance has been extracted from the preparation and converted to its methyl ester[3]. Benzyl benzoate is used as an internal standard.

Thioctic acid is extracted with benzene from aqueous solutions or dispersions of the test product after slight acidification with hydrochloric acid. The benzene extracts are evaporated to a small volume; the extract is then esterified to methyl thioctate with anhydrous methanol–HCl (10% solution in methanol). Methylation is carried out in a refluxing apparatus in a water bath for about one hour. After the esterification, a suitable amount of benzyl benzoate (internal standard) is added; the solution is concentrated in vacuum and then diluted with ethyl ether. The ether solution is washed with a 5% solution of sodium bicarbonate in water, then filtered over anhydrous sodium sulfate; finally the solution is concentrated to a small volume in a stream of nitrogen. A standard solution of pure thioctic acid in methyl alcohol, which is then processed in exactly the same way as the test sample, is prepared in parallel. These two solutions, sample and standard, are examined by gas–liquid chromatography. Table I shows the details of the gas chromatographic technique.

The retention time of methyl thioctate relative to the internal standard, under the experimental conditions described, is 1.19.

TABLE I

ASSAY OF THIOCTIC ACID IN PHARMACEUTICAL PRODUCTS: EXPERIMENTAL CONDITIONS OF GAS CHROMATOGRAPHY

Apparatus	"Fractovap" Carlo Erba Model C
Detector	Flame ionization (FID)
Column (glass)	Length 80 cm; diameter 6 × 4 mm
Stationary phase	SE-52 5% on Gas-Chrom P 100–120 mesh, silanized
Column temperature	165°
Evaporator temperature	200°
Carrier gas	Nitrogen (25 ml/min)
Auxiliary gases	Hydrogen (20 ml/min), air (300 ml/min)
Chart speed	$\frac{1}{2}$ in./min
Introduction	$\sim$ 1 μl.

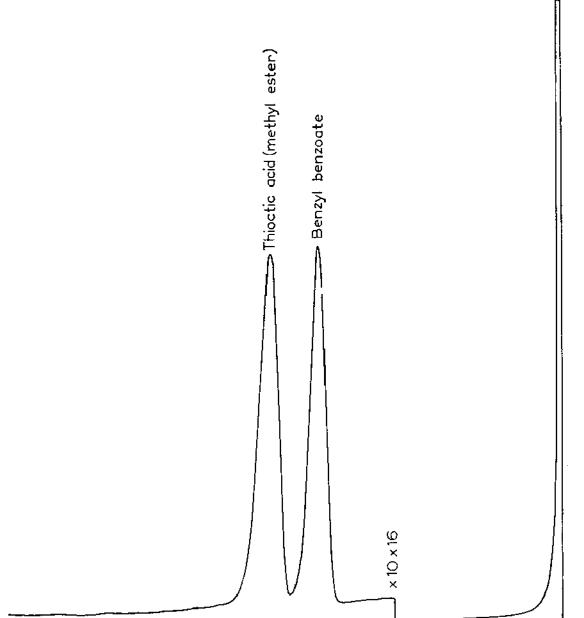

Fig. 1. Chromatogram of thioctic acid from injectable solutions. Internal standard: benzyl benzoate.

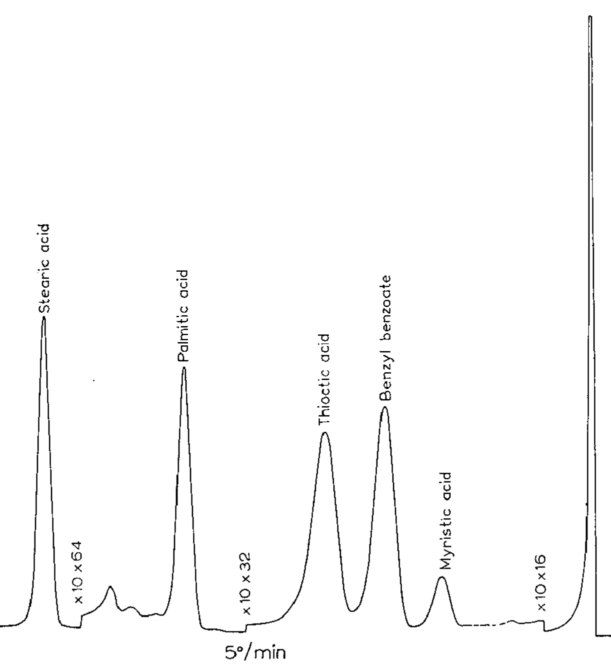

Fig. 2. Chromatogram of thioctic acid extracted from tabletted products. Internal standard: benzyl benzoate. Fatty acids contained in the tablet excipient are also visible .

The areas of the methyl thioctate and benzyl benzoate peaks are measured from the gas chromatographic tracings. By comparison of the areas of the sample and standard methyl thioctate, with reference to benzyl benzoate, the amount of thioctic acid contained in the aliquot of the test product can be calculated.

Figs. 1 and 2 show the chromatograms obtained. The analysis of the ampoule product shows two peaks, one for methyl thioctate and one for benzyl benzoate. In the chromatogram from the tablet product, in addition to the said two peaks, one can also see peaks due to myristic, palmitic and stearic acids from the tablet excipients which consist of saturated fatty acids; these peaks, however, do not interfere with those of methyl thioctate and benzyl benzoate.

Fig. 3 shows the calibration curve constructed by assaying various amounts of thioctic acid by gas chromatography relative to constant amounts of internal standard. It can be seen that the curve follows a linear course over fairly wide limits.

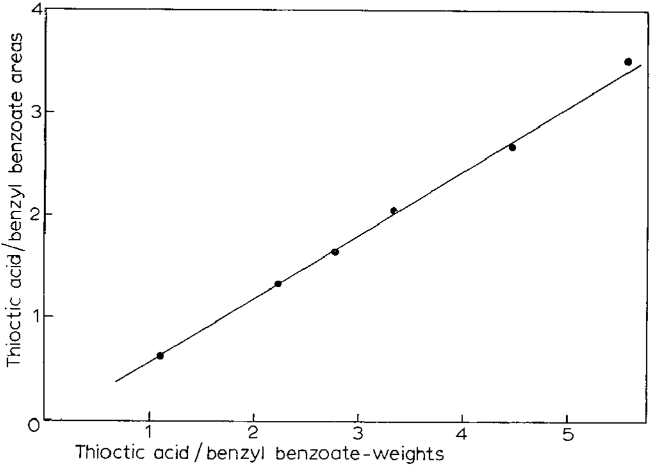

Fig. 3. Calibration curves for thioctic acid with an internal standard of benzyl benzoate.

ASSAY OF *l*-FUCOSE CONTAINED IN GASTRIC MUCOPROTEIN

The mucoproteins are extracted by suitable methods from the gastric mucosa of pigs and are used in the preparation of pharmaceutical products and of special powdered milk for infant feeding. The biological activities of mucoproteins are multifarious; it will suffice to mention their activity as an "intrinsic factor" (or binding factor) facilitating the intestinal absorption of vitamin B_{12}, their liver-protecting and fat-dispersant activity, and their action in improving the utilization of protein nitrogen.

From the analytical point of view it is very expedient to characterize this mucoprotein in terms of a particular carbohydrate it contains, namely *l*-fucose.

After an acidic hydrolysis of the polysaccharides the monosaccharides produced were usually identified and estimated by paper and column chromatography and by colorimetric reactions which are not very sensitive or specific.

TABLE II

ASSAY OF *l*-FUCOSE IN GASTRIC MUCOPROTEIN: EXPERIMENTAL CONDITIONS OF GAS CHROMATOGRAPHY

Apparatus	"Fractovap" Carlo Erba Model C
Detector	Flame ionization (FID)
Column (glass)	Length 2 m; diameter 6 × 4 mm
Stationary phase	SE-30 1 % on Gas-Chrom P 100–120 mesh, silanized
Column temperature	Programmed from 150° to 220° (3.5° /min)
Evaporator temp.	290°
Carrier gas	Nitrogen (25 ml/min)
Auxiliary gases	Hydrogen (20 ml/min), air (300 ml/min)
Chart speed	½ in./min

The application of gas chromatography to carbohydrate analysis has made it possible to investigate the carbohydrate composition of glycoproteins both qualitatively and quantitatively[4–7].

Our aim in the present investigation was the quantitative assay of *l*-fucose in the mucoprotein; accordingly, we deliberately omitted assaying or even separating the other component sugars. We hydrolyzed the glycoproteins with methanol, then converted the methyl glycosides to TMS derivatives. The assay was carried out with *d*-sorbitol as internal standard. Table II shows the experimental conditions for gas chromatography.

Methanolysis is carried out with anhydrous methyl alcohol in an ampoule containing about 100 mg of mucoprotein, in the presence of *p*-toluenesulfonic acid, for 12 h, at 80°–85°, with continuous agitation. A suitable amount of *d*-sorbitol is then added to the solution containing the methyl glycosides; the mixture is transferred to a 10-ml vial and evaporated until dry in a stream of nitrogen. The residue is desiccated for 6 h under vacuum, then redissolved in 2 ml of anhydrous pyridine and treated with 1 ml of hexamethyldisilazane and 0.5 ml of trimethylchlorosilane. The

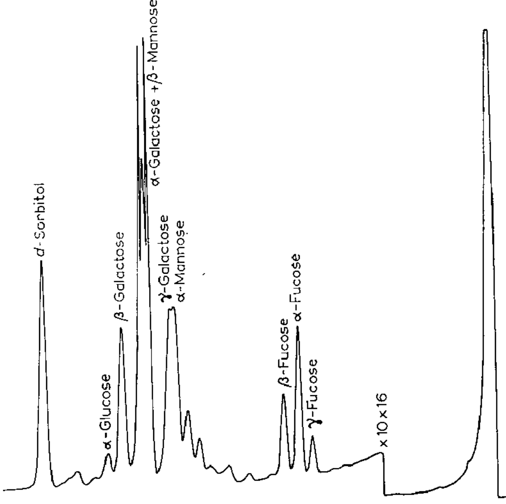

Fig. 4. Chromatogram of mucoprotein monosaccharides as methyl glycoside TMS ethers. Internal standard: *d*-sorbitol.

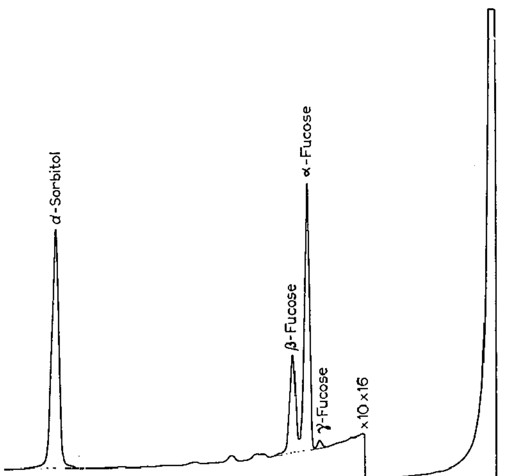

Fig. 5. Chromatogram of the standard *l*-fucose (as methyl glycoside TMS ether) together with the internal standard.

reaction mixture so obtained is shaken vigorously for at least 1 min; after 10–15 min it is ready for chromatography. A known amount of standard *l*-fucose (approximately 3 mg for 100 mg of mucoprotein assayed) is processed in parallel.

Fig. 4 is a gas chromatographic tracing showing the saccharide content of the mucoprotein. Each monosaccharide is represented by two or three peaks, corresponding to the anomeric forms, α, β, and γ (as methylglycoside TMS ethers). The peak of the internal standard (*d*-sorbitol) is also visible.

Fig. 5 shows a gas chromatogram of the standard *l*-fucose with the internal standard added.

Quantitative assay is carried out by calculating the sums of the areas of the *l*-fucose peaks in the sample and standard chromatograms. The percentage fucose contained in the mucoprotein being tested is calculated by comparing the values obtained, with reference to the *d*-sorbitol added.

Table III shows the results obtained on the testing of 5 specimens of mucoprotein. In addition to each *l*-fucose value, the corresponding protein nitrogen value is shown. An inversely proportionate relationship between the two values is apparent.

TABLE III

l-FUCOSE VALUES (BY GLC) AND CORRESPONDING PROTEIN NITROGEN VALUES OBTAINED IN THE TESTING OF SOME MUCOPROTEINS

Sample	*l-Fucose (%)*	*Nitrogen (%)*
1	4.00	7.59
2	3.80	8.35
3	3.30	8.54
4	2.95	9.03
5	4.90	7.12

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CHROM. 4595

ANION-EXCHANGE CHROMATOGRAPHY OF GLYCOLYSIS INTERMEDIATES

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SUMMARY

A purification procedure for glycolysis intermediates is described. The separation of each compound was achieved by means of anion-exchange chromatography on a column of AG-1 X4 (Cl^-) and an eluting gradient of ammonium chloride, containing sufficient alkaline borate to ensure complexing of the sugar phosphates.

The eluting buffer was afterwards removed by evaporating to dryness with methanol and subsequent chromatography on an AG-50W X4 (H^+) column. Under such conditions the quantitative separation, identification and recovery of the components of the glycolytic pathway can be obtained.

INTRODUCTION

The chromatographic separation of the intermediates of glycolysis is of great interest as a tool for solving the problems of metabolic interrelationship[1], flux rates[2] and compartmental situations[3] within the EMBDEN-MEYERHOF pathway.

Several methods have been developed in which borate complexes of phosphate esters could be separated by ion-exchange chromatography[4-8]. Procedures using thin-layer chromatography[9,10], paper[11] and thin-layer electrophoresis[12] as well as gas chromatography[13] have been described. All these methods however, while yielding good results, allow the separation of only a few glycolysis intermediates.

The present paper describes a method in which sugar phosphates complexed with borate are eluted from an anion-exchange resin by means of a concave gradient of ammonium chloride. This method gives reproducible results and allows the separation, with good recoveries, of phosphorylated glycolysis intermediates.

EXPERIMENTAL

Materials

[U-^{14}C]Glucose; [U-^{14}C]glucose-6-phosphate; [U-^{14}C]glucose-1-phosphate; [U-^{14}C]lactate and [U-^{14}C]pyruvate were obtained from the Radiochemical Centre, Amersham, Bucks., Great Britain. [U-^{14}C]Fructose-1,6-diphosphate and [U-^{14}C]-phosphoenolpyruvate were purchased from Boehringer, Mannheim, G.F.R. [U-^{14}C]-

Fructose-6-phosphate was from Sorin, Saluggia, Italy, while [U-^{14}C]3-phosphoglycerate and [U-^{14}C]2,3-diphosphoglycerate were from Calbiochem, Los Angeles, Calif., U.S.A.

Labelled dihydroxyacetone phosphate and glyceraldehyde-3-phosphate were prepared from [U-^{14}C]fructose-1,6-diphosphate according to BEISENHERZ *et al.*[14]. Uniformly labelled 1,3-diphosphoglycerate was prepared from [U-^{14}C]3-phosphoglycerate by KRIMSKY's procedure[15] as modified by ALPERS[16].

Unlabelled compounds and enzymes were obtained from Boehringer. Ion-exchange resins were from Bio-Rad Laboratories, Richmond, Calif., U.S.A.

Analytical procedures

Phosphorus was assayed by the method of BARTLETT[17] as modified by MARINETTI[18]. Phosphoglyceric acids were determined with pyrogallol in sulphuric acid[19]. Anthrone was used as reducing agent[20]. Borate was determined spectrophotometrically with curcumin reagent[21].

After elution and removal of buffer, glycolysis intermediates were determined enzymatically according to BERGMEYER[22].

Column chromatography

The columns (1 × 50 cm) used were packed with AG-1 X4 (Cl^-) (200–400 mesh) until a 30-cm-high bed was obtained. After applying the mixtures of sugar phosphates, the column was washed with 100 ml of 0.001 N ammonium hydroxide to remove glucose and any other free sugar that may be present. Sugar phosphates were eluted, at room temperature, with a concave gradient made up of 0.0025 M NH_4OH + 0.25 M NH_4Cl in the reservoir (1.95 l in a 2.5-l Erlenmeyer flask) and of 0.0025 M NH_4OH + 0.005 M $K_2B_4O_7$ + 0.03 M NH_4Cl in the mixing chamber (2 l in a 4-l Mariotte bottle). The flow rate was maintained at 0.5 ml/min by means of an LKB 10200 peristaltic pump. Radioactivity in the effluent was monitored with a Nuclear Chicago 6770 Chroma/Cell; 10-ml fractions were collected. Individual column runs were carried out in order to establish the elution behaviour.

Fractions, under each peak, were pooled separately and evaporated to dryness in a Büchi evaporator (about 30° inside the flask).

Borate was easily removed by three to four evaporations with methanol. The residue, dissolved in 3–5 ml of water, was passed down a column (1 × 10 cm bed) of the resin AG-50W X4 (H^+) (200–400 mesh) to ensure the removal of the ammonium ion. The column was washed with water to neutrality and the effluent evaporated to dryness.

After this treatment the amount of borate was less than 0.01 μmoles per peak and did not interfere in the successive enzymatic analyses.

RESULTS AND DISCUSSION

The results obtained with a mixture of glycolysis intermediates are shown in Fig. 1. Similar patterns have been obtained by KHYM AND COHN[4] with a stepwise elution, utilizing a combination of borate complexing and pH.

The anion-exchange behaviour of the compounds examined is, as expected, the resultant effect of the pK values of the phosphate groups and the stability constants

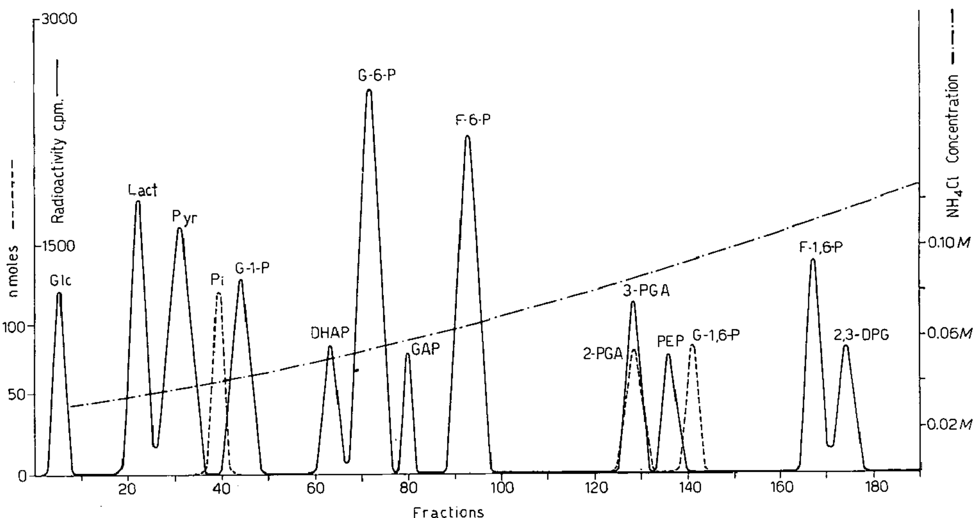

Fig. 1. Anion-exchange chromatography of glycolysis intermediates. The mixture applied to the column was: glucose (Glc); glucose-6-phosphate (G-6-P); glucose-1,6-diphosphate (G-1,6-P), glucose-1-phosphate (G-1-P); fructose-6-phosphate (F-6-P); fructose-1,6-diphosphate (F-1,6-P); dihydroxyacetone phosphate (DHAP); glyceraldehyde-3-phosphate (GAP); 3-phosphoglyceric acid (3-PGA); 2,3-diphosphoglyceric acid (2,3-DPG); 2-phosphoglyceric acid (2-PGA); phosphoenolpyruvate (PEP); pyruvate (Pyr); lactate (Lact). (———) c.p.m.; (— — —) unlabelled compounds assayed for inorganic phosphate (Pi); (— · — · —) gradient from 0.03 *M* to 0.12 *M*.

of the borate complexes, so that large differences were found among members of the same series such as glucose-1-phosphate, glucose-6-phosphate and glucose-1,6-diphosphate. Steric hindrance of the phosphate group can, in fact, make the borate complex less stable, which is reflected as lower binding to the anion exchanger. The latter hypothesis, together with the participation of the hydroxyl group arising from the furanosic ring formation in reactions with boric acid, can also explain why aldoses are eluted ahead of ketoses.

TABLE I

AMOUNT OF GLYCOLYSIS INTERMEDIATES RECOVERED

Glycolysis intermediates	*nMoles added to column*	*nMoles recovered*	*Percent recovery*
Glucose	743	691	93
Glucose-6-phosphate	110	105	95
Glucose-1,6-diphosphate	100	88	88
Glucose-1-phosphate	180	152	84
Fructose-6-phosphate	160	144	90
Fructose-1,6-diphosphate	142	122	86
Dihydroxyacetone phosphate	100	78	78
Glyceraldehyde-3-phosphate	100	66	66
3-Phosphoglyceric acid	380	330	87
2,3-Diphosphoglyceric acid	75	68	90
2-Phosphoglyceric acid	95	83	87
Phosphoenolpyruvate	120	113	94
Pyruvate	170	155	91
Lactate	630	554	86

In different experiments 85–95% of the initial samples were recovered (Table I). Recoveries for dihydroxyacetone phosphate and glyceraldehyde-3-phosphate were, however, between 15–30%; but if the two peaks were lyophilised as fast as possible after elution and the deionising procedure was carried out in a cold room, recoveries rose to 70–80%.

In our system separation of 2-phosphoglyceric acid and 3-phosphoglyceric acid could not be achieved, and therefore their specific radioactivities must be measured as a whole after enzymatic conversion of the former in the latter[22].

Under the conditions employed 1,3-diphosphoglycerate was always recovered as 3-phosphoglycerate and inorganic phosphate because of spontaneous decomposition. However, the method can equally be applied to biological material; in the living cell the concentration of 1,3-diphosphoglycerate is so low that it can hardly be estimated.

As the same recoveries were found both by measuring the radioactivity of the sample and its amount, the method is suitable for obtaining valid specific radioactivities of most glycolytic intermediates in experiments with labelled substrates.

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CHROM. 4600

SANDWICHED COLUMNS IN GAS–LIQUID CHROMATOGRAPHY

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SUMMARY

Sandwiched capillary columns consisting of one or more carbon threads inserted in a glass capillary and coated with a stationary phase have been prepared and the dependence of the performance characteristics on the capillary diameter and on the number of carbon threads has been investigated. Van Deemter equation constants, gas and liquid phase mass transfer resistance terms, performance indices and performance parameters have been calculated. A comparison between sandwiched columns and other types of columns shows that regarding permeability, performance index, performance parameter and loading capacity, the former have intermediate values between open tubular and classical packed columns. The advantage of the sandwiched columns with respect to packed capillary columns lies in the ease of their preparation and the lower pressure drop corresponding to a minimum plate height. Large columns with more than a hundred threads offer no advantage over classical packed columns.

INTRODUCTION

Sandwiched capillary columns are the latest type of column to be introduced in gas chromatography. So far they have been used only as adsorption columns[1]. This investigation deals with the performance of these columns in gas–liquid partition chromatography.

The solid support in a sandwiched column is represented by any material available as a thread and consists therefore of a bundle of fibres, inserted in a glass capillary.

With regard to their geometrical features, these columns differ from any other type of column and consequently have different chromatographic properties.

A sandwiched column of ideally regular structure would be a good approximation to a column consisting of a bundle of parallel capillaries. A regular structure requires that no free space should be left between the surface of the threads and the wall of the capillary and that the fibres should be in a uniformly loose contact with each other. With respect to classical open tubular columns, this structure affords the possibility of using larger quantities of liquid phase without at the same time increasing the film thickness. On the other hand, sandwiched columns could be expected to offer

a higher permeability as compared with classical columns packed with a granular support having a particle size similar to the diameter of the fibres.

The present investigation deals with the influence of geometrical characteristics on the performance of liquid coated sandwiched columns. A comparison is made with other columns, such as classical packed, open tubular and packed capillary columns, on the basis of data reported in the literature[2,3].

EXPERIMENTAL

The sandwiched columns were prepared according to the method described by LIBERTI *et al.*[1]. The carbon yarn designated WYG from Union Carbide had the following characteristics: diameter of a filament: 7.5 μ, specific surface: 1 m^2/g, weight of yarn: 0.00375 g/m.

A 1.5% solution of squalane, dissolved in pentane, was passed through the

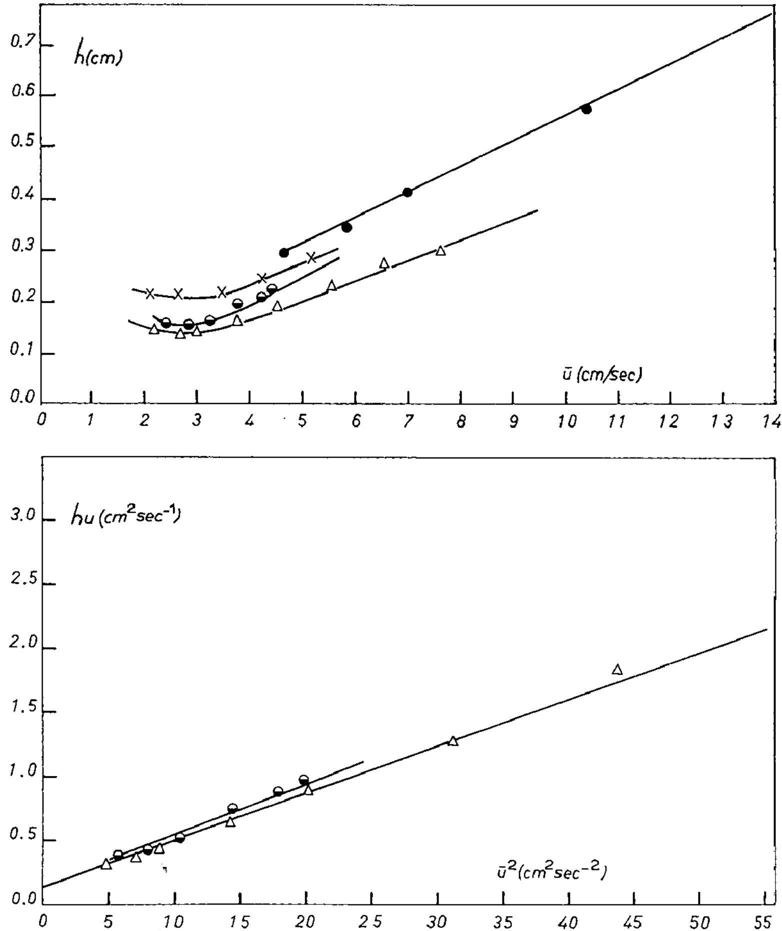

Fig. 1. Plots of h *vs.* $\bar{u}$ and $h\bar{u}$ *vs.* $\bar{u}^2$ for n-octane on sandwiched columns. Carrier gas: N_2. ● column No. 1; × column No. 2; △ column No. 3; ◒ column No. 4.

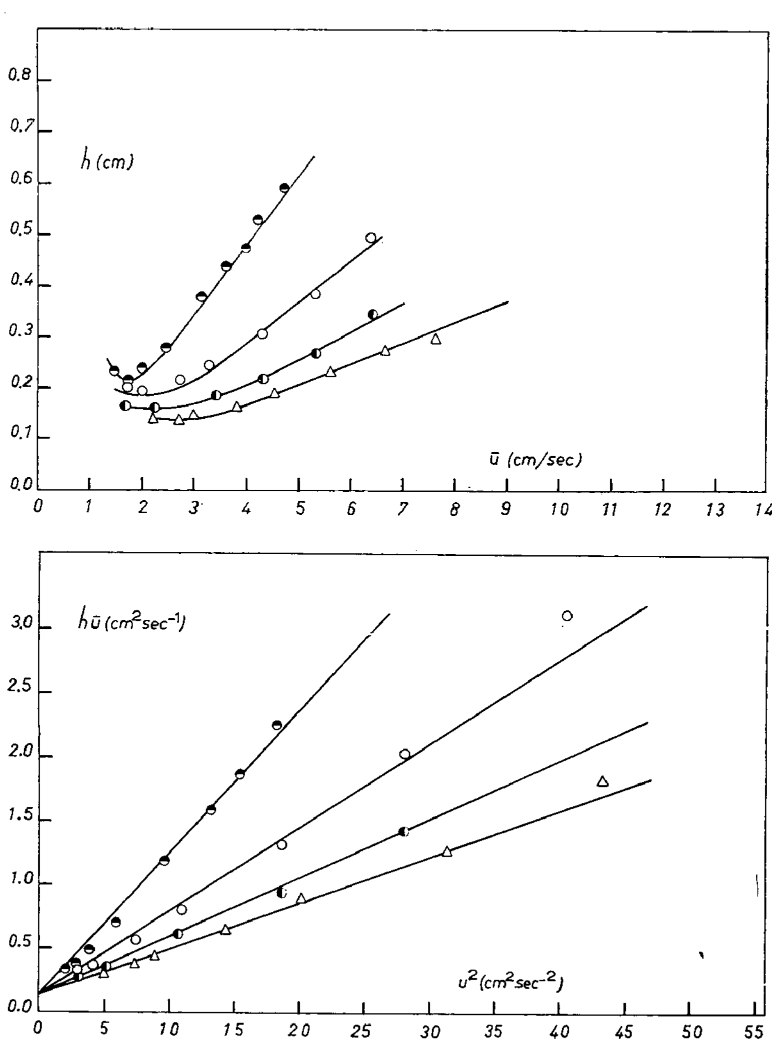

Fig. 2. Plots of h *vs.* $\bar{u}$ and $h\bar{u}$ *vs.* $\bar{u}^2$ for n-octane on sandwiched columns. Carrier gas: N_2. △ column No. 3; ◐ column No. 5; ○ column No. 6; ◓ column No. 7.

column. The solvent was evaporated slowly at room temperature by passing air through the column and subsequently nitrogen at 50°. Columns prepared with a more dilute solution of squalane gave rise to peak tailing, indicating interference due to adsorption phenomena on the solid surface.

All the columns showed a considerably increased efficiency after heating them at 200° and maintaining a slow stream of nitrogen through them for 2 h. In the course of this operation the amount of stationary phase did not vary significantly; however, a slight decrease of permeability could be observed. These effects can be attributed to a more uniform redistribution of the stationary phase on the support surface and to the expansion of the bundle of filaments constituting the yarn.

Two series of columns were prepared. In the first series, columns were prepared

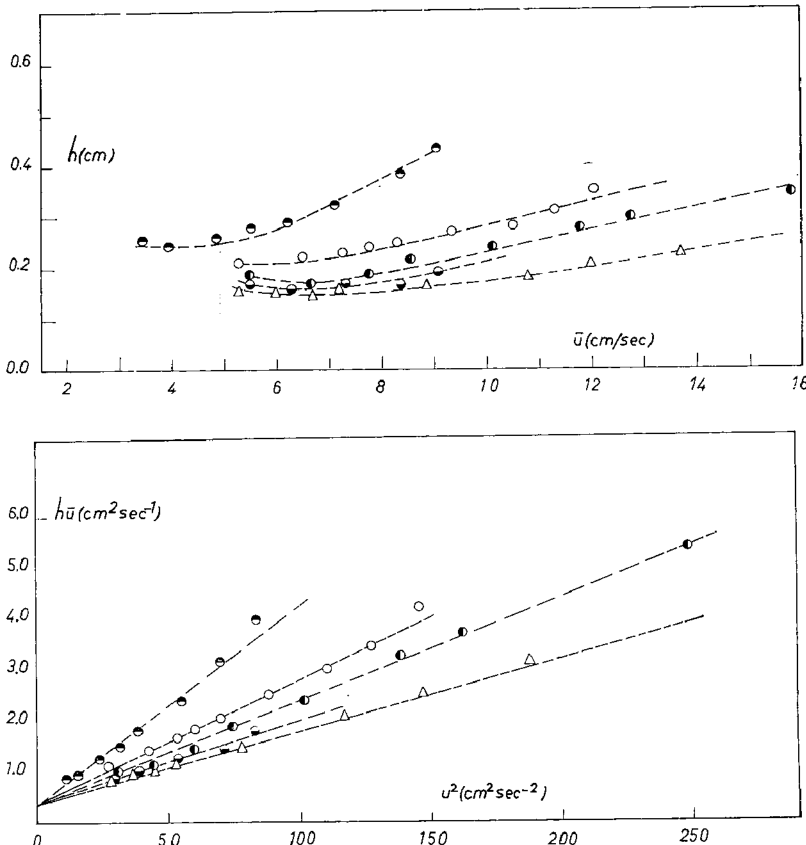

Fig. 3. Plots of h *vs.* $\bar{u}$ and $h\bar{u}$ *vs.* $\bar{u}^2$ for *n*-octane on sandwiched columns. Carrier gas: H_2. △ column No. 3; ◒ column No. 4; ◐ column No. 5; ○ column No. 6; ◓ column No. 7.

containing one thread of carbon and the internal diameter of the glass capillary was varied. In the second series, the columns were prepared in such a way as to maintain the permeability constant, by increasing the internal diameter of the column together with the number of the threads.

A hydrocarbon mixture containing *n*-pentane, *n*-hexane, *n*-heptane and *n*-octane was run on each column. The samples were taken from the vapour phase of the mixture and a small quantity of methane was added to each sample. The methane peak was used for the evaluation of the dead volume. All measurements were made at 50°, using nitrogen and hydrogen as carrier gas. The apparatus was a Carlo Erba Fractovap model C gas chromatograph. HETP values for *n*-heptane and *n*-octane were determined and h *vs.* $\bar{u}$ curves were plotted for all columns (Figs. 1, 2 and 3).

The constant terms A, B and C of the VAN DEEMTER equation for *n*-octane were determined graphically from the h–$\bar{u}$ plots (Figs. 1a, 2a, 3a) and where the term A was 0, from the $h\bar{u}$–$\bar{u}^2$ plots (Figs. 1b, 2b, 3b). The mass transfer resistance terms in the gas and in the liquid phase, C_g and C_l were determined on the basis of plate height measurements made with two carrier gases (hydrogen and nitrogen).

RESULTS AND DISCUSSION

These values are reported together with the constants for the Van Deemter equation and with the geometrical characteristics of the columns in Table I. The following method was employed for the calculation of C_g and C_l terms. According to the Golay equation we have for the same column, but for two different carrier gases:

$$h_{N_2} = \frac{B_{N_2}}{u} + C_{g,N_2} + C_l u \quad (1)$$

$$h_{H_2} = \frac{B_{H_2}}{u} + C_{g,H_2} + C_l u \quad (1a)$$

Since the difference between B_{N_2} and B_{H_2}, as well as between C_{g,N_2} and C_{g,H_2}, is only caused by the difference of the diffusion coefficients, we can write:

$$B_{H_2} = cB_{N_2}$$

and

$$C_{g,H_2} = \frac{C_{g,N_2}}{c}$$

Then

$$h_{H_2} = \frac{cB_{N_2}}{u} + \frac{C_{g,N_2}}{c} + C_l u \quad (2)$$

B_{N_2} and B_{H_2} as well as C_{N_2} ($= C_{g,N_2} + C_l$) and C_{H_2} ($= C_{g,H_2} + C_l$) were determined graphically from the $h\bar{u}$–$\bar{u}^2$ plots, and the ratio of B_{H_2}/B_{N_2} yielded c. Knowing C_{N_2}, C_{H_2} and c, we are able to resolve the system:

$$C_{N_2} = C_{g,N_2} + C_l$$

$$C_{H_2} = \frac{C_{g,N_2}}{c} + C_l$$

and thus obtain the values of C_{g,N_2} and C_l.

The amount of stationary phase was calculated for each column from measurements made at 80° by comparing the retention volumes of n-octane with the values reported by McReynolds[4].

Values of the film thickness d_l and the volume ratio of the phases V_g/V_l were calculated from the weight of the stationary phase, the specific weight of squalane and the surface of the carbon thread.

All these data are collected in Table II. The same table also contains calculated values of permeability, P., the capacity ratio k' for the four hydrocarbons, the height equivalent for an effective plate H, the performance index $P.I.$ and the performance parameter PP, all calculated for n-octane, at the minimum of the $h\bar{u}$– curve.

The load capacity of the sandwiched capillaries was examined on two columns, No. 3 and No. 7, by injecting increasing quantities of a mixture of hydrocarbons, containing 28.3% of n-hexane, 35.4% of n-heptane and 36.3% of n-octane. The samples were introduced by means of a 10 μl Hamilton microsyringe and the quantities of n-heptane and n-octane passing through the column were calculated from the volume

TABLE I

GEOMETRICAL CHARACTERISTICS AND VAN DEEMTER EQUATION CONSTANTS OF SANDWICHED COLUMNS

Column No.	I.D. (mm)	Length (m)	Number of threads	Carrier gas N_2				Carrier gas, H				C_l (sec)
				A (cm)	B (cm^2/sec)	C (sec)	C_g (sec)	A (cm)	B (cm^2/sec)	C (sec)	C_g (sec)	
1	0.43	14.0	1	0.07	0.05	0.0470	—	—	—	—	—	—
2	0.37	15.0	1	0.07	0.10	0.0520	—	—	—	—	—	—
3	0.35	11.5	1	0.00	0.13	0.0360	0.0345	0.00	0.40	0.0136	0.0115	0.0021
4	0.32	10.0	1	0.00	0.13	0.0400	0.0367	0.00	0.40	0.0155	0.0122	0.0033
5	0.45	7.5	2	0.00	0.13	0.0469	0.0408	0.00	0.40	0.0197	0.0136	0.0061
6	0.55	10.4	3	0.00	0.13	0.0690	0.0675	0.00	0.40	0.0240	0.0227	0.0015
7	0.69	9.4	5	0.00	0.13	0.1116	0.1091	0.00	0.40	0.0385	0.0364	0.0025

TABLE II

DATA AND RESULTS OBTAINED WITH VARIOUS SANDWICHED COLUMNS

Column No.	Permeability $P \cdot 10^{-7}$ cm^2	Squalane (mg/m)	V_g/V_l	Liquid film thickness (mμ)	k′				H_{min} (cm)	u_{min} (cm/sec)	ΔP_{min} (atm)	P.I. (poise)	P P (nC_8–nC_7) (atm/sec)
					nC_5	nC_6	nC_7	nC_8					
1	84.5	0.93	105.6	30.6	0.37	1.04	2.80	7.50	—	—	—	—	—
2	25.2	0.68	95.8	27.1	0.45	1.20	3.30	8.92	0.265	2.62	0.32	115	0.44
3	8.6	0.90	65.3	29.6	0.58	1.56	4.31	11.45	0.167	2.70	0.70	146	0.68
4	4.6	1.31	43.6	44.0	0.70	1.74	5.30	14.36	0.177	2.86	1.30	334	0.47
5	8.3	1.92	54.3	31.5	0.76	2.10	5.83	15.50	0.188	2.25	0.40	202	1.17
6	7.2	2.96	45.2	32.0	0.80	2.20	6.20	16.60	0.218	2.03	0.60	333	1.97
7	4.5	5.59	38.6	37.3	1.08	3.00	8.00	21.55	0.230	1.73	0.70	587	4.39

injected, the split ratio, the composition and the density of the mixture. The base width of the peaks was determined by measuring the distance between the intersections of the base line and the tangents drawn to the inflexion points of the peak. Fig. 4 shows a plot of peak base width *vs.* quantity of hydrocarbon. The variation of the base width was chosen as the characteristic value indicating column overloading because neither the peak width at half height nor the number of theoretical plates showed any significant variation even when peak broadening became quite apparent.

Comparing columns 1, 2, 3 and 4 in Table II, all containing a single thread inserted in glass capillaries of different diameter, it can be seen that the permeability decreases with decreasing internal diameter according to Poiseuille's law. At the same time the ratio V_g/V_l also decreases, which explains the increasing values of the capacity ratio.

The height equivalent to an effective plate attained for each column a minimum in the region of 2.6–2.8 cm/sec of linear velocity of carrier gas. The data in Table II also demonstrate the decrease of the height of an effective plate with decreasing

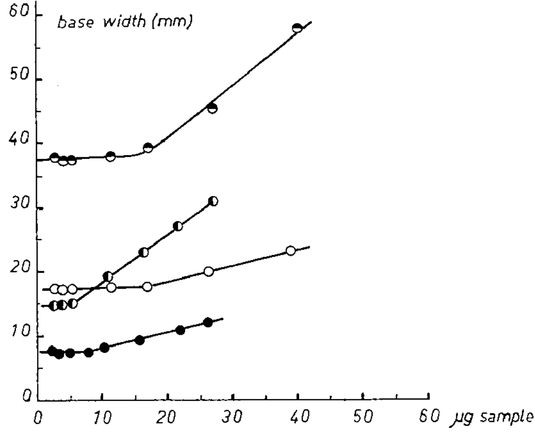

Fig. 4. Plots of peak base width *vs.* sample quantity. ◓ *n*-octane on column No. 7; ◐ *n*-octane on column No. 3. ○ *n*-heptane on column No. 7; ● *n*-heptane on column No. 3.

internal diameter and the increase of the pressure corresponding to the minimum plate height.

Comparing the data of columns 2, 3 and 4 in Table II, it can be seen that the pressure corresponding to the minimum plate height varies almost like a geometrical progression. The height of an effective plate, on the other hand, displays a considerable decrease when passing from column 2 to column 3, while the decrease is less significant when passing from column 3 to column 4. Consequently column 3 seems to represent a favourable compromise with respect to column efficiency and permeability.

Performance indices and performance parameters of all sandwiched columns have values intermediate between open tubular columns and classical packed columns. They might be considered and classified as regularly packed capillaries. This statement is supported by values of the constant terms of the Van Deemter equation, reported in Table I. The term A is 0, and B becomes almost independent of the column diameter, as soon as the capillary becomes narrow enough to straighten the thread and to make the filament packing smoother.

If the threads are not slightly compressed by the wall of the capillary, the structure and the orientation of the thread become less regular, which is a possible explanation for the increment in term A for columns 1 and 2.

Another series of experiments served for the examination of columns prepared with more than one carbon thread. The columns were prepared in such a way as to maintain nearly constant the ratio of the internal diameter to the number of threads thus obtaining columns all having a similar permeability.

A comparison of columns 3, 5, 6 and 7 shows that the minimum heights of an effective plate for *n*-octane show slightly increasing values as well as performance indices and performance parameters, while the carrier gas velocities corresponding to H_{min} slightly decrease when the number of threads increases.

An examination of the Van Deemter equation constants shows that there is only a slight alteration in the regularity of the packing, the term A being 0, and B constant for all these columns. The term C shows a pronounced increase on increasing the number of threads.

As shown by the data of Table I, the rather high values of C are mainly due to C_g, the mass transfer resistance in the gas phase, while C_l is much lower and nearly constant for columns 3, 6 and 7, in spite of the increasing quantity of liquid phase. The higher value of C_l in column No.5 is very probably due to the irregular distribution of the liquid phase in this column.

The increment of the gas phase mass transfer resistance term seems to be a limiting factor in increasing the number of threads. Even the column with 5 threads showed a rapidly decreasing separation efficiency at a carrier gas velocity higher than that corresponding to the minimum of the h–$\bar{u}$ curve. In fact, columns consisting of 120 and 180 threads inserted in a tube of 4 mm I.D. showed very poor separation efficiency, the column of 180 threads having a permeability of $20 \cdot 10^{-7}$ cm^2.

An examination of the graphs in Fig. 4 shows that the critical sample quantity causing column overloading is somewhat larger than 7 μg for the column with one thread and about 17 μg for the column with five threads which are doubtlessly larger quantities than those for classical capillary columns.

In Table III some characteristics of the sandwiched columns are reported together with those of other types of columns (data taken from refs. 2 and 3) for the sake

TABLE III

COMPARATIVE DATA FOR VARIOUS TYPES OF COLUMNS

Column type	*Reference*	*Column length (m)*	H_{min} *(mm) n-heptane*	ΔP_{min} *(atm)*	$\Delta P/l$ *(atm/m)*	*Squalane mg/m*	*P* $(10^{-7}\ cm^2)$	V_g/V_l	*k'*
Classical packed	2	4	1.11	2.11	0.50	337.6	1.94	22.6	18.4
Classical packed	3	2	1.59	0.98	0.49	185.4	2.1	54.2	7.8
Packed capillary	2	7	0.89	2.7	0.39	2.6	7.9	48.0	6.0
Packed capillary	2	21.5	1.37	3.8	0.18	1.8	14.0	89.6	6.4
Packed capillary	2	17.5	2.54	2.2	0.13	2.2	28.5	89.0	6.8
Packed capillary	3	10.0	2.53	0.75	0.07	0.25	18.5	267.0	1.6
Open tubular	2	140.0	1.37	3.9	0.03	0.26	124.5	150.0	2.7
Sandwich No. 2		15.0	3.00	0.32	0.02	0.68	25.2	95.8	3.3
Sandwich No. 3		11.5	2.04	0.60	0.05	0.89	8.6	65.3	4.2
Sandwich No. 4		10.0	1.86	1.30	0.13	1.31	4.6	43.6	5.4
Sandwich No. 5		7.5	2.27	0.40	0.05	1.92	8.2	54.3	5.6
Sandwich No. 6		10.4	2.51	0.60	0.06	2.96	7.2	45.2	6.2
Sandwich No. 7		9.4	2.7	0.70	0.07	5.59	4.5	38.6	8.0

of comparison. These values are the minimum height of an effective plate for *n*-heptane, the pressure gradient of the column, the amount of the stationary phase contained in one meter of the column, the ratio V_g/V_l and the permeability. All these reported values correspond to the minimum of the h–$\bar{u}$ curve.

A comparison of these data demonstrates that the sandwiched columns have an intermediate value of V_g/V_l, ranging between that of the classical packed columns and the packed capillary columns, and a permeability comparable with that of the latter type.

The amount of the stationary phase is of the same order of magnitude as that of a packed capillary, *i.e.* it is higher than the quantity contained in an open tubular column. Also with respect to efficiency, they are comparable to the packed capillaries but require a lower pressure in the zone of the minimum plate height, providing the possibility of preparing and using columns of considerable length without the use of inconveniently high pressures.

The advantages of the sandwiched columns coated with a liquid phase may be summarized by underlining the ease of their preparation, their rather good separation efficiency even with a low pressure gradient, and the possibility of coating them with a rather high amount of stationary phase.

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CHROM. 4630

DETERMINATION OF POLYNUCLEAR HYDROCARBONS IN ATMOSPHERIC DUST BY A COMBINATION OF THIN-LAYER AND GAS CHROMATOGRAPHY

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SUMMARY

Polynuclear hydrocarbons contained in the atmospheric dust are analyzed by a combination of thin-layer and gas chromatography. The dust as such, or a cyclohexane extract, is applied on a silica gel plate and developed with cyclohexane–benzene (1:1.5).

In this way polynuclear hydrocarbons are separated from other classes of components such as paraffins, heterocyclics, etc. The part containing polynuclear hydrocarbon is extracted again and examined by gas chromatography on a glass capillary column.

INTRODUCTION

The analysis of polynuclear hydrocarbons contained in atmospheric dust is usually carried out by the following methods:

(1) Separation of the compounds by column chromatography and determination of each constituent in the eluted fractions by spectrophotometry or fluorimetry.

(2) Separation and quantitative determination by gas chromatography after solvent extraction.

(3) Separation by thin-layer chromatography and approximate determination of the compounds on the spots or elution of the substances and determination by spectrophotometry or fluorimetry.

Detailed examination of each of these methods showed that there are many difficulties and that none are satisfactory for use in routine analysis at various air pollution control stations.

In all these methods the sample has to be extracted for many hours and large volumes of solvents have to be used. Consequently for the subsequent steps it is necessary to concentrate the large volumes of solution with the risk of losing or destroying some of the compounds.

In the first method the separation by column chromatography takes a long

time; many fractions need to be collected and many measurements with the spectrophotometer or fluorimeter are required to obtain the complete analysis.

In the second method, although the gas chromatographic procedure is more sensitive and faster, there are many preliminary steps in the preparation of the sample before its injection into the gas chromatograph. A large part of the time required to complete the analysis is taken up by the Soxhlet extraction, separation of the paraffins from the polynuclear hydrocarbons by partition between two solvents (cyclohexane and nitromethane) and evaporation of a large volume of solvent to concentrate the sample to a very small volume.

The third method, using thin-layer chromatography alone, is very simple for the separation of the compounds. However, this separation is not complete; there are too many compounds in the dust to be separated by a single chromatogram and the quantitative determination is very approximate.

As a result of these difficulties we are now studying a procedure which is based on gas chromatography for the separation of the polynuclear hydrocarbons where we try to eliminate all the preliminary steps by using thin-layer chromatography for the preparation of the sample to be injected in the gas chromatograph. Gas chromatography carried out with glass capillary columns and temperature programming give a satisfactory separation for many of the most interesting compounds[1]. The retention index system is used for the identification of the peaks and by means of an internal standard it is possible to obtain the relative quantities.

The thin-layer chromatography was only used as a preliminary step to separate the various classes of compounds. Silica gel and alumina plates were tried. With the former the separation achieved was better. Solvents of different polarity were used: cyclohexane, hexane, benzene, ether. With cyclohexane or hexane alone the polynuclear hydrocarbons have low R_F values and are not resolved from the other constituents of atmospheric dust. With the other solvents the R_F values were too high. By

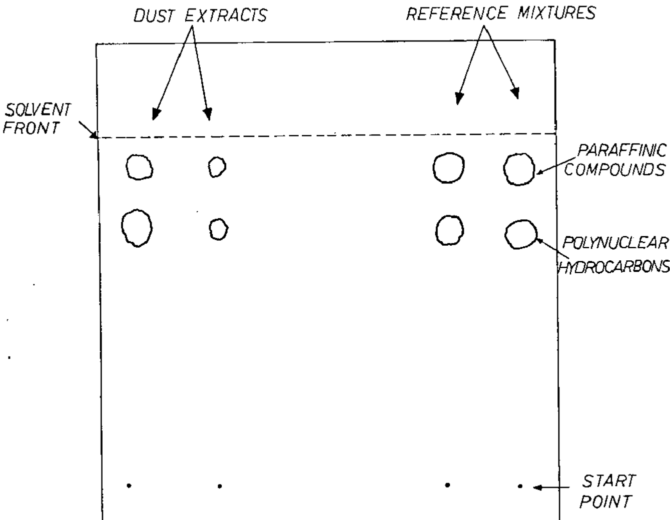

Fig. 1. Separation of paraffinic compounds from polynuclear compounds by thin-layer chromatography. Silica gel plate 500 μ thick; solvent, benzene–cyclohexane (1.5:1).

using a mixture of cyclohexane–benzene in the ratio 1:1.5, it was possible to obtain R_F values in the range 0.65–0.75 for all the polynuclear compounds tested. At the same time the paraffins are all found together very near the front, while more polar compounds, such as oxygen or nitrogen heterocyclics, are very strongly retained near the starting point (Fig. 1).

In Table I are reported the R_F values of some polynuclear hydrocarbons and paraffins on Silica Gel G and alumina plates developed with different solvents.

TABLE I

R_F VALUES OF SOME TYPICAL POLYCYCLIC AND PARAFFINIC HYDROCARBONS

Compounds	*R_F values on silica gel*				*R_F values on Alumina G*			
	Cyclohexane	*Hexane*	*Benzene*	*Ether*	*Cyclohexane*	*Hexane*	*Benzene*	*Ether*
Polycyclic								
Anthracene	0.31	0.25	0.74	0.95	0.55	0.42	0.95	1.00
3,4-Benzofluoranthene	0.17	0.12	0.64	0.95	0.30	0.078	0.71	1.00
1,2-Benzopyrene	0.20	0.13	0.75	0.95	—	—	0.89	1.00
3,4-Benzopyrene	0.20	—	—	0.95	0.38	0.078	0.86	1.00
Fluoranthene	0.23	0.19	0.70	—	0.46	0.34	0.85	1.00
3-Methylpyrene	0.26	0.20	0.66	0.93	0.49	0.27	0.93	1.00
Pyrene	0.27	0.18	0.67	0.95	0.51	0.30	0.91	1.00
Paraffins								
C_{18}	0.72	0.72	0.79	1.00	0.98	0.94	0.94	1.00
C_{20}	0.73	0.72	0.79	1.00	0.98	0.94	0.94	1.00
C_{22}	0.72	0.72	0.79	1.00	—	—	—	—
C_{24}	0.72	0.72	0.79	1.00	0.86	0.94	1.00	1.00
C_{28}	0.72	0.72	0.79	1.00	0.98	0.94	0.94	1.00

PROCEDURE

Dust is collected from the air by means of a high volume air pump (Staplex) for the time necessary for about 100–200 mg of dust to be collected in the filter. The filter with the dust is extracted in a Soxhlet with 100 ml of cyclohexane for about 8–10 h. The cyclohexane is concentrated to a small volume (about 0.1 ml) and transferred onto a thin-layer plate. A standard mixture of polynuclear hydrocarbons is run alongside the cyclohexane extract which is applied repeatedly as a 10-cm-long line till the whole extract is on the plate. The plate is then developed with the solvent mixture (cyclohexane–benzene, 1:1.5), dried and observed under a UV lamp. The fluorescent part, containing the polynuclear hydrocarbons with an R_F range 0.65–0.75 is separated and scraped from the plate into a small test tube. The compounds are then extracted 2–3 times with small portions of ether, the ether fractions are collected in a test tube with a conical end and the solvent is evaporated to a few microlitres. To obtain quantitative results it is necessary to add an internal standard to this solution. 1,3,5-Triphenylbenzene was used for these determinations.

The gas chromatographic separations are carried out with a Carlo Erba Fracto-

TABLE II

RECOVERIES OF SOME POLYCYCLIC HYDROCARBONS

Compounds	*Added* (*μg*)	*Found* (*μg*)
Phenanthrene	9.4	8.4
Fluoranthene	2.9	2.4
3-Methylpyrene	2.8	2.5
3,4-Benzofluoranthene	2.6	2.0
3,4-Benzpyrene	2.2	1.9

vap Model C. The column used is a glass capillary 30 m long with an efficiency of about 50,000 theoretical plates.

The silicone rubber SE-52 which is slightly polar and has a good thermal stability, is used as liquid phase.

In Table II are reported the quantitative results obtained for some polynuclear hydrocarbons analyzed with the procedure reported above.

TABLE III

QUANTITATIVE DETERMINATION OF POLYNUCLEAR HYDROCARBONS IN ATMOSPHERIC DUST (μg/g)

Compounds	*Cyclohexane*	*Nitromethane*	*Dust*
Phenanthrene	3.35	4.0	7.25
Fluoranthene	5.5	5.5	8.75
Pyrene	13.0	7.15	11.25
11,12-Benzfluoranthene / 3,4-Benzfluoranthene	21.5	20.5	23.0
1,2-Benzpyrene / 3,4-Benzpyrene	21.5	10.0	21.0

CONCLUSIONS

In Table III the values obtained from the determinations of some polynuclear hydrocarbons identified in atmospheric dust are compared. The analysis has been carried out on the same dust sample using three different methods: applying the cyclohexane solution from the Soxhlet extraction or the nitromethane fraction prepared according to the procedure described in previous papers to the plates[1-3] and directly applying a small amount (50–100 mg) of the atmospheric dust on a thin line at the starting point on the plate.

This system which combines thin-layer and gas chromatography takes advantage of the best properties of both these techniques: the fast and simple procedure of thin-layer chromatography to isolate and purify the sample and the high sensitivity and resolution of gas chromatography to separate and evaluate each single constituent.

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CHROM. 4596

THE ORGANIC COMPLEXES OF THE CLAY MINERALS AND THEIR USE IN GAS CHROMATOGRAPHY

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SUMMARY

The physical properties, selectivity, efficiency, and preparation of organic derivatives of clay minerals is discussed and the possible applications in gas chromatography are studied. The paper is a review of some unpublished work and also refers to work already published (*Chromatographia*, (1969) 239-242 and 551-553).

The more interesting clay minerals, from a gas chromatography point of view, are the so-called 2:1 phyllosilicates. These minerals have a lamellar structure built up from two planar layers of silica tetrahedrons and alumina octahedrons. A single unit consists of two tetrahedral sheets connected by an octahedral one. In most cases a part of the Al and Si is isomorphically substituted by ions of lower valency; for example the Si in the tetrahedral sheet can be replaced by Al, and the Al by Mg, Fe^{2+} or other bivalent, and sometimes even monovalent, ions in the octahedral sheet. To render the structure electrically neutral, the negative charges within the lattice are compensated by inorganic cations held on the basal surface of the lamellae. Using a suitable procedure, these inorganic cations can be replaced by long-chain alkylammonium ones. The interlamellar complexes obtained in this manner show an alternating sequence of inorganic and organic layers. The long alkyl chains have a more

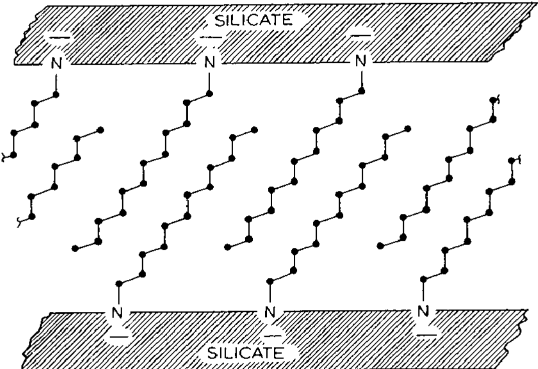

Fig. 1. Idealized model for organic complexes of clay minerals.

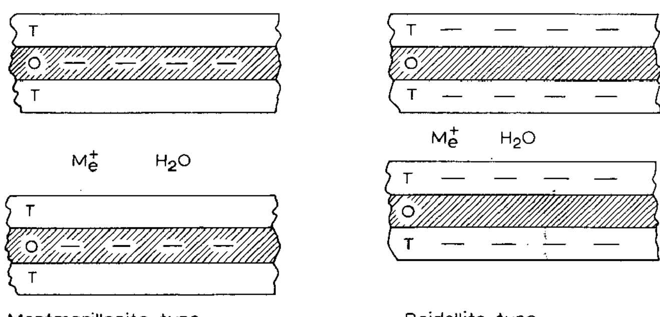

Fig. 2. Two types of clay minerals.

or less regular arrangement, due to the orientating effect of the electrical field existing in the interlamellar space as well as to the dispersion forces acting between the alkyl chains. An idealised model of structure for these complexes can be seen in Fig. 1.

The first representative of these complexes used in gas chromatography was the well-known Bentone-34 which is the dimethyldioctadecylammonium complex of montmorillonite. This substance has been used as a stationary phase because of its high selectivity towards aromatic hydrocarbons and in particular towards *meta* and *para* isomers. In the montmorillonite family of the clay minerals, the isomorphic substitutions are mainly in the octahedral sheet, that is the charge deficit is located at a discrete distance from the basal surface. Recently investigations were carried out with clay minerals belonging to the beidellite-vermiculite family in which the isomorphic substitutions are located mainly in the tetrahedral sheet; the latter position is nearer the basal surface than in the case of the montmorillonites. The difference between the two types of clay minerals is illustrated by Fig. 2.

Investigations carried out with clay minerals having cation exchange capacities ranging from 24 to 150 mequiv./100 g mineral have shown that the gas chromatographic performance of the "beidellite type" complexes was superior to that of the "montmorillonite type" clay mineral complexes. This may be due to the fact that these minerals have a greater charge density near the basal surface, which, in turn, may ensure a more regular arrangement of the alkyl chains, at the same time making the external surface more homogeneous. This differentiation can be made very easily by gas chromatography, while it is a difficult task by other methods.

Accordingly, we shall deal mainly with the complexes of the beidellite-vermiculite type clay minerals, as they are more effective stationary phases for gas chromatography.

The selectivity shown by the interlamellar complexes is mainly due to two facts. Firstly, they are able to establish specific interactions with the molecules having a mobile electron cloud or a permanent dipole, and, secondly, owing to their structural and surface properties, they are able to separate molecules which differ only in their geometric arrangement. In this latter case the interlamellar complexes behave like a crystalline adsorbent (*e.g.* graphitised carbon black). These two effects appear together in most cases, and sometimes it is rather difficult to separate them. The application of the retention indices may be a useful tool for this purpose; for example, in this case reference is made to the normal paraffins which are unable to enter specific interactions with any adsorbent. Therefore, if the retention index of a component

investigated is substantially higher than the value corresponding to a normal paraffin with the same boiling point, the existence of specific interactions is verified. On the other hand, if this difference does not exist or is negligible while the components in question are separated, we can state that it is due to steric effects. If one takes into consideration the difference in the indices from the value valid for the corresponding normal paraffin, and also eventually the difference between the differences, the effects of specific and steric interactions in most cases can be separated. This is demonstrated with a few data collected in Table I.

TABLE I

DIFFERENCES BETWEEN RETENTION INDICES FOR SOME HYDROCARBONS

	Retention index	*ΔI on the basis of "C" numbers*	*ΔI on the basis of boiling points*	*Difference of the differences*
trans-1,2-Dimethyl cyclohexane	800	—	—	
				20
cis-1,2-Dimethyl cyclohexane	820	20	20	
trans-1,3-Pentadiene	680	180	150	
				20
cis-1,3-Pentadiene	700	200	170	
p-Xylene	1070	270	220	
				50
m-Xylene	1120	320	270	

The selectivity which is most interesting, from a practical point of view, is that shown toward the aromatic hydrocarbons. The separation of aromatic hydrocarbons from other types of hydrocarbons is due mainly to the strong specific interactions existing between the complex and the mobile electron cloud of the aromatics; the separation of the aromatic hydrocarbons, one from another, however, can only be explained by assuming that specific and steric effects act together. This can be demonstrated by the classical example of the *m* and *p*-xylene pair. The difference between the retention indices and that of the corresponding normal alkane is 200 to 300, showing that strong specific interactions are acting. On the other hand the difference between the differences is about 50. This value cannot be explained only by the differences in polarisability but by taking into account the steric effects too.

The relative retentions of the individual aromatic hydrocarbons are slightly different for the various clay mineral complexes, so it is possible, by using sufficiently long columns packed with different minerals, to separate practically any mixture containing aromatic hydrocarbons. Fig. 3 shows the separation of a complex mixture on a column composed of two parts, each containing different clay mineral complexes.

The efficiency obtainable with the interlamellar complexes, if the complex is prepared correctly, is quite good although somewhat inferior to that of columns containing partition liquids. The difference can be seen in Fig. 4, where the van Deemter curves are compared for two columns, one of which contains the alkylammonium complex of Nontronite Garfield (a beidellite-type clay mineral), the other the alkylammonium halide employed as a conventional stationary phase.

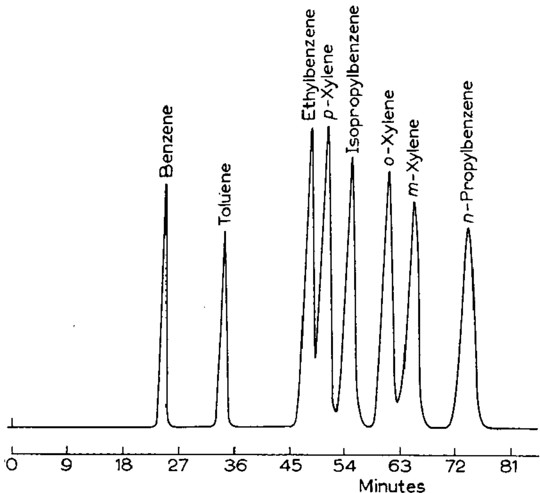

Fig. 3. Separation of aromatic hydrocarbons on a combined column.

Analysing the van Deemter curves, the following conclusion can be drawn:

1. There is no significant difference between the behaviour of cyclohexane and *p*-xylene. Taking into consideration that, as is known from the literature, only aromatic hydrocarbons are able to penetrate the interlamellar space of the clay mineral complexes, this observation indicates that under gas chromatographic conditions this penetration does not occur in the case of aromatics. This hypothesis can also be supported by the isotherms obtained for these complexes using aromatic and alicyclic hydrocarbons as adsorbates.

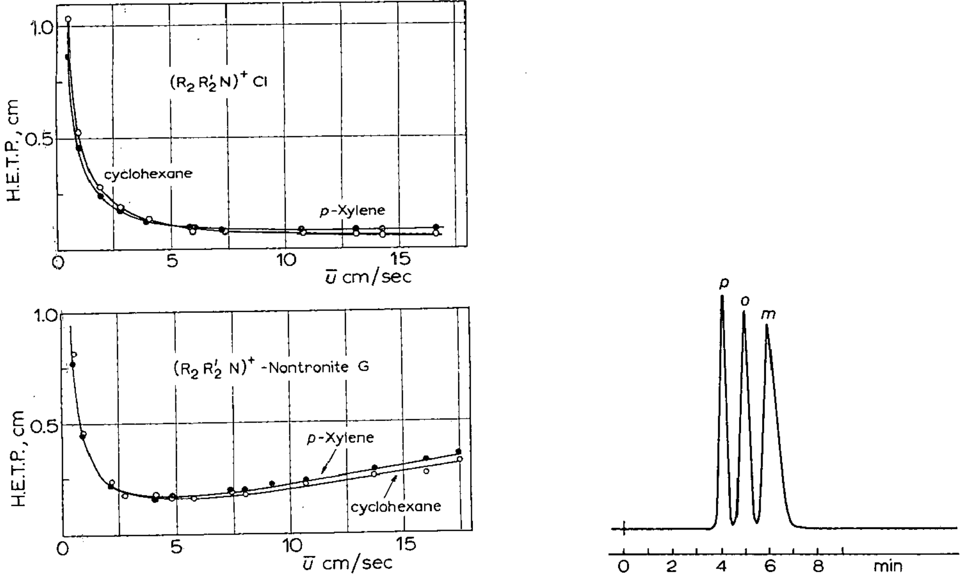

Fig. 4. Van Deemter curves for the two different types of columns.
Fig. 5. Fast separation of the three xylenes.

2. Comparing the van Deemter coefficients obtained for the two types of columns, it can be seen that only the "*C*" coefficients differ substantially. This indicates a greater resistance to mass transfer in the case of the interlamellar complexes.

3. The van Deemter curves are rather flat, permitting the use of flow rates very much higher than the optimum, with only a slight loss in efficiency which, in turn, makes it possible to accomplish analyses in a very short time while the separation remains almost unaffected. Fig. 5 shows the complete separation of the three xylenes carried out in seven minutes.

The preparation of these interlamellar complexes is a very easy process and will not be reported here, but some remarks must be made concerning it. With the minerals of the montmorillonite family it is possible to prepare complexes containing more than the equivalent quantity of organics; the amount can exceed the equivalent value by as much as 100%. The organic substance (both in the form of free base and unreacted halide) present in excess does not greatly affect the performance of the column, and acts rather as a modifying stationary phase, which decreases both the selectivity and the HETP values a little. This overloading is not possible with the minerals of the beidellite family; with these minerals, as a rule, not even the equivalent quantity can be reached, probably because of steric hindrance. If the quantity of the organic substance is significantly lower than the equivalent value, the resulting complex shows the behaviour of a heterogeneous adsorbent, the peaks are very asymmetric and, as a consequence, the efficiency is very low. For this reason it is advisable to use complexes with an organic content near the cation exchange capacity value. Since the interlamellar complexes show a high retentivity toward aromatic hydrocarbons, and the selectivity in separating them is fairly high, a rather short column (1–2 m) is usually sufficient. Of course, if the mixture to be separated is more complex, a longer, and in some cases combined, column can be used.

The interlamellar complexes are used as stationary phases deposited on the surface of a convenient support, 10 to 15% by weight. The column is filled in the same manner as in the case of other stationary phases, the only difference being that in this case the solvent used is benzene.

The interlamellar complexes can be used between the temperature limits of 50 and 150°; above 150° some of the clay mineral complexes begin to decompose, though some are able to withstand a higher temperature. Up to 150° all the complexes investigated could be used, even with the very sensitive ionization detectors. At temperatures below 50° there is a transition temperature at which, within one or two degrees, all the parameters of the column show a sharp change, the selectivity and retentivity of the column decrease and also the efficiency deteriorates to a great extent. This transition temperature is slightly different for the various clay minerals investigated and its value can be found in the range of 32–50°.

In order to separate mixtures of aromatic hydrocarbons which are very strongly retained by the complexes, the highest temperature possible is usually advisable in order to obtain good efficiency and reasonable retention times. For the separation of the lower boiling geometric isomers (*e.g.* dimethylcyclohexanes) a lower temperature may be employed, however, it is still best to apply the highest temperature possible because the high temperature favours the efficiency of the interlamellar complexes.

As a conclusion, it can be stated that the organic complexes of the clay minerals investigated can be used as stationary phases in gas chromatography, in particular

for the separation of aromatic hydrocarbons. The selectivity of the various clay mineral complexes is somewhat different, so it is possible by using combined columns to solve almost any problem in the field of the aromatic hydrocarbon analysis. The complexes also show a selectivity for geometric isomers. In this case their behaviour is similar to that of graphitised carbon black.

The columns of interlamellar complexes correctly prepared show a satisfactory efficiency, particularly at higher temperatures. The usable temperature range is between 50–150°; these limits being determined by the decomposition of the complexes and by their transition temperature.

The investigations have shown that the complexes of the beidellite type clay minerals are more effective than those of the montmorillonite type. The preparation of these interlamellar complexes does not present any particular difficulty; care must be taken, however, about the quantities used. The materials employed are very cheap and this fact is another advantage.

CHROM. 4597

THE CAPACITY OF LABORATORY-SCALE PREPARATIVE CHROMATOGRAPHY COLUMNS

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SUMMARY

It was found that a relationship exists between the capacity of gas chromatographic columns and the different experimental parameters for the linear part of the sorption isotherm.

Preparative gas chromatography is finding ever increasing applications not only in purely laboratory preparative work but also in the semi-industrial production of pure substances. The problem of the capacity of preparative units is of decisive importance here. It is expedient to characterise the capacity as the amount of the mixture separated per unit time at a given degree of separation (or the degree of purity of the substances obtained). The latter is usually determined by the resolution value R

$$R = \frac{2(V_2 - V_1)}{\Delta S_1 + \Delta S_2} \tag{1}$$

where V_1 and V_2 are the retention volumes of the first and second components, respectively; ΔS_1 and ΔS_2 are widths of the peaks corresponding to these components.

An expression for the capacity of greatly overloaded columns under the conditions of stepwise chromatography at $R = 1$ has been derived by ALEKSEEVA *et al.*[1]. To correlate the capacity and the experimental parameters, use can be made of the theory of VAN DEEMTER *et al.*[2]. According to this theory, when introducing a vapour-like sample of volume A, the band width is

$$\Delta S = A + v\sqrt{2\pi n_0} \tag{2}$$

and the retention volume is

$$V = n_0 v + \tfrac{1}{2} A \tag{3}$$

where n_0 is the number of theoretical plates in the absence of overloading; v the effective volume of a theoretical plate. These equations hold provided that the sorption isotherm is linear and the sample is introduced by the "plug injection" method. If

the value for A is represented as the product $V_s K_m$, where V_s is a volume of a sample undiluted by a carrier gas and K_m is a dilution factor[3], then by substituting (2) and (3) into eqn. 1, the expression for the peak resolution value under overloading conditions will be

$$R = \frac{2\, n_0\, K_s}{\dfrac{2\, V_s K_m}{v_1 + v_2} + \sqrt{2\pi\, n_0}} \tag{4}$$

where $K_s = \dfrac{v_2 - v_1}{v_2 + v_1}$ is a selectivity factor.

Making use of the apparent relation $v_1 + v_2 = \dfrac{SL\,(\Gamma_1 + \Gamma_2)}{n_0}$ where S is the cross-section, L is the column length, and Γ is Henry's general coefficient, we obtain

$$R = \frac{2\, K_s}{\dfrac{2\, V_s K_m}{SL\,(\Gamma_1 + \Gamma_2)} + \sqrt{\dfrac{2\pi H_0}{L}}} \tag{4a}$$

where H_0 is height of an effective theoretical plate (HETP) in the absence of overloading. One can see from this equation that resolution under the overloading conditions is proportional to K_s. To a lesser degree, it depends on effective-diffusion washing-out characterised by H_0. The first term in the denominator is defined by the volume of the dose introduced, $K_m V_s$, and by the column volume, SL, and its sorption holding capacity, Γ.

From eqn. 4a, the volume of the mixture, V_s, separated at a given value R can be expressed as

$$V_s = \frac{SL\,(\Gamma_1 + \Gamma_2)}{K_m R}\left(K_s - \tfrac{1}{2} R \sqrt{\frac{2\pi H_0}{L}}\right) \tag{5}$$

By definition, the capacity of the unit is

$$P = \frac{V_s}{\tau} \tag{6}$$

where τ is the separation cycle time calculated from the moment of introduction up to complete elution of the second peak

$$\tau = \frac{n_0 V_2 + K_m V_s + \frac{1}{2} V_2 \sqrt{2\pi\, n_0}}{W} \tag{7}$$

where W is the gas flow-rate.

Substituting (5) and (7) into formula 6 and making some simplifications, we obtain

$$P = W \frac{\dfrac{\alpha + 1}{\alpha}}{R + \frac{1}{2}\dfrac{\alpha - 1}{\alpha}}\left(K_s - \tfrac{1}{2} R \sqrt{\frac{2\pi H_0}{L}}\right) \tag{8}$$

where $\alpha = \dfrac{V_2}{V_1}$ is a separation factor.

Formula 8 holds for the case when the mixture is supplied to the chromatographic column as a squared pulse with concentration C_0 corresponding to a linear region of the sorption isotherm. But in most preparative chromatographs, the mixture rapidly evaporates in the overheated evaporator and is supplied to the column at high concentration. Therefore, a check on the validity of eqn. 2 for these conditions was made. A column, 14 mm in diameter and 3 m in length, was filled with INZ-600 as column packing and 20% of dinonyl phthalate. Increasing volumes of liquid mixtures containing diethyl ether, 2,2-dimethyl butane, hexane, benzene, pentane and dichloroethane were introduced through an evaporator into a steady carrier gas flow. A metallic cylinder with a spiral packing heated externally serves as an evaporator. The evaporator temperature was higher by 30 to 40° than the average boiling point of the mixture introduced.

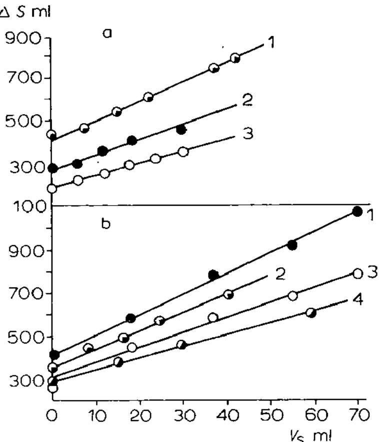

Fig. 1. Peak width dependence (in gas ml) on sample volume. (a) Column temperature 70°: 1 = cyclohexane; 2 = hexane; 3 = 2,2-dimethyl butane. (b) Column temperature 95°: 1 = dichloroethane; 2 = benzene; 3 = carbon tetrachloride; 4 = cyclohexane.

In all the cases, the proportionality between the peak width and sample volume was satisfactory (Fig. 1). The slope of the straight line, *i.e.* the K_m factor did not change as the gas velocity increased from 50 up to 500 ml/min and the column length increased from 2 up to 10 m. However, K_m was dependent on the distribution coefficient, increasing with its growth. In fact, K_m decreased with increasing column temperature and became practically constant at temperatures close to the boiling point of the substance. K_m is different for various substances, increasing with the retention volume. At temperatures close to the boiling point of the substance, this difference is small. Inconstancy in K_m, probably due to non-linearity in the sorption isotherm, leads one to regard it as a semi-empirical factor. Furthermore, it was found experimentally that the intercepts cut off by the straight lines on Fig. 1 are practically equal to the peak width at the introduction of a dose of several microlitres in volume when the column is not as yet overloaded. As a result of this, the numerical factor in eqn. 8 is equal to 4, rather than to $\sqrt{2\pi}$.

The deviations from eqn. 2 discussed, particularly the dependence of K_m on the nature of the substance, hinder the use of formula 8 in quantitative or even semi-quantitative calculations of the capacity. It was, however, found by us that the ratio

TABLE I

THE DEVIATIONS OF THE CHROMATOGRAPHIC PEAK SHAPE FROM THE GAUSSIAN CURVE

Experimental conditions				*Sample volume (ml)*	*Asymmetry factor*	a_2/a_1	*% deviation*	a_3/a_1	*% deviation*
Substance	*Column diameter (mm)*	*Column length (m)*	*Temperature (°C)*						
Cyclohexane	30	1.9	77	1.0	0.75	1.37	3.5	1.86	7.0
				3.0	0.61	1.37	3.5	1.81	9.5
				5.0	0.48	1.33	6.0	1.73	13.5
n-Pentane	30	3.5	20	0.6	0.92	1.36	4.0	1.89	5.5
				1.0	0.66	1.38	3.0	1.89	5.5
				2.0	0.61	1.35	5.0	1.82	9.0
				10.0	0.34	1.35	5.0	1.77	11.5
Benzene	15	1.9	95	4 μl	0.8	1.42	0	2.0	0
				0.2	0.36	1.36	4.0	1.77	11.5
Heptane	15	1.9	95	4 μl	0.6	1.40	1.5	2.0	0
				0.4	0.38	1.34	5.5	1.79	10.5

of K_m values obtained on preparative and analytical columns is practically the same for various substances. Having determined this ratio experimentally, one can then calculate K_m for a preparative column, based on the value determined for an analytical column.

Also of interest is a relationship between R and the purity of the fraction. The literature contains the methods for calculating the fraction purity for peaks described by Gaussian equations[4–6]. Under overloading conditions, the shape of a peak deviates from this equation. From the Gaussian equation of the curve, it follows that its width at a distance $e^{-0.5}$ (0.607) of the whole height (a_2) is to the width at a distance e^{-1} (0.367) of the whole height (a_1) as 1.42:1. Accordingly, the width at a distance e^{-2} (0.134) of the whole height (a_3) is to a_1 as 2:1.

We have calculated the peaks of various hydrocarbons obtained on columns with dinonyl phthalate. Table I lists the results of some measurements, *viz.* the a_2/a_1 and a_3/a_1 ratios, their deviations from the theoretical values as a percentage, and the asymmetry factors equal to the ratio of the front and the back peak half-widths (measured at the half-height). For a not too large overloading (the sample volume is of the order of 0.5 ml for a column of 15 mm in diameter; for a 30 mm column, it is of the order of 2–3 ml), the deviation from the Gaussian curve is not too great, *viz.* 5–6% for the a_2/a_1 ratio and about 10% for the a_3/a_1 value. Under these conditions, satisfactory agreement can be expected between the calculated and experimentally determined purity of a fraction. With a large R value, the peak "tail" has a decisive influence on the purity of the fraction and the assumption of the Gaussian character of the distribution seems to be poorly founded. If traps are switched midway between the maxima of the peaks, the impurity of fractions with a mixture of equimolecular composition is determined by the formula (see ref. 6)

$$\eta = \frac{1}{2.82\sqrt{\pi}R} e^{-2R^2} \tag{9}$$

and varies directly with the proportion of the components in the mixture.

To check the accuracy of the calculation of fraction purity, a model mixture of heptane and benzene was separated on a column 15 mm in diameter and 1.9 m in length packed with dinonyl phthalate on a solid carrier INZ-600; the fractions selected were analysed using the Pan-Chromatograph. The traps were switched at a point equidistant from maxima of both the peaks. The experimental data and results of calculations by means of formula 9 are presented in Table II, which also gives the results of the calculation with a correction for the symmetry of the peaks. Although the calculation of fraction purity is only semiquantitative, usually a certain range or an upper level is preset rather than the determination of the exact impurity content; such a calculation is of use in most cases. As could be expected, the greatest error is related to the peak asymmetry.

TABLE II

EXPERIMENTAL AND CALCULATED DEGREES OF FRACTION PURITY (%)

Mixture of heptane and benzene.

Component proportion	*Sample volume (ml)*										
	0.5				0.3				0.1		
	R	*η exp.*	*η theor. 1*	*η theor. 2*	*R*	*η exp.*	*η theor. 1*	*η theor. 2*	*R*	*η exp.*	*η theor. 1*
50:50	0.9	0.5	4	1.0	1.25	0.1	0.8	0.07	1.7	0.04	0.05
		2.0	4	—		—	—	—		—	—
70:30	1.0	1.0	1.0	—	1.35	0.1	0.15	—	1.8	0.03	0.01
		1.5	5.0	—		0.9	0.6				
80:20	1.05	0.8	0.6		1.4	0.15	0.07				

Note: 1. The upper line: purity of the first fraction; the lower: purity of the second fraction.
2. $\eta_{t \cdots 1}$ is the impurity calculated neglecting peak asymmetry;
$\eta_{t \cdots 2}$ is that taking account of peak asymmetry.

THE INFLUENCE OF THE SEPARATION CONDITIONS ON THE CAPACITY

Eqn. 8 can be used for the qualitative estimation of the effect of the experimental parameters on the capacity. The capacity was experimentally determined as follows: a dose which provides a prescribed R value was determined from the plot of R *versus* the volume of mixture to be separated. The capacity was further calculated by dividing the dose value by the separation time.

First of all, let us consider the influence of the column length. The second term in parentheses of eqn. 8 decreases to zero with increasing column length, while the capacity approaches the asymptotic limit P_{as}. Given the ratio P/P_{as}, the limit above which it is not advantageous to increase the column length can be determined. If it is assumed that the ratio equals 0.9, then

$$L_{\max} = 50\pi \frac{R^2}{K_s^2} H_0 \tag{10}$$

With a decrease in the selectivity factor and an increase in R, the maximum

length of the column increases. Thus, the more difficult the mixture to be separated and the higher the requirements of product purity, the longer the column that must be used.

With a simultaneous increase in gas velocity and column length, the capacity should increase proportionally with length. With great pressure drop, however, it also will tend to an asymptotic limit.

The experiments on the influence of column length on the capacity were performed at room temperature on the column 15 mm in diameter, packed with INZ-600 as solid carrier and dinonyl phthalate. The model mixture consisted of isopentane and *n*-pentane with nitrogen as carrier gas. The dependence of capacity on column length at constant average gas velocity is given in Fig. 2. As seen from Fig. 2, with increasing column length, the capacity increases up to a definite limit only. It is of interest to note that with increasing diameter, H_0 increases as a rule, moderating the capacity increase. However, if the column length is increased simultaneously with diameter so that the ratio is constant, then the capacity can be increased in proportion with the column cross-section. In this case, the simultaneous increase in column length and diameter may be advantageous.

With an increase in the flow rate of the carrier gas, the capacity should pass through a maximum according to eqn. 8 provided that $H_0 = CW$. The position of the maximum will be determined by the relationship

$$w_{\max} = \frac{8}{9\pi} \frac{K_s^2 L}{R^2 C} \qquad (11)$$

w is the gas flow rate per unit column cross-section.

With increasing R and decreasing K_s, the maximum rapidly shifts in the direction of low flow rates. This is confirmed by the experimental data obtained on the column 30 mm in diameter and 3 m in length which are given in Fig. 3.

It follows from formula 8 that the capacity is nearly proportional to $K_s = \alpha - 1/\alpha + 1$, as the expression before the parentheses containing α only slightly affects this dependence. To check the dependence of P on K_s, binary mixtures of hydrocarbons and chlorohydrocarbons with various K_s values were separated on a column with dinonyl phthalate. As seen from Fig. 4, the capacity is proportional to K_s, though dispersion of the points is rather wide. The use of high-selective phases is one of the most promising techniques for increasing the capacity of gas chromatographic columns. Using such phases for separating substances with close boiling points, one may obtain a capacity similar to that of a rectifying column. For instance, the capacity of the laboratory rectifying column with a spiral packing is 2.1 ml/h cm^2 for a mixture of benzene and carbon tetrachloride with the composition varying from 24 to 92% of benzene, while the capacity of a gas chromatographic column of 6.7 m in length with dinonyl phthalate is 1.3 ml/h cm^2. For the benzene–cyclohexane mixture, where the difference in boiling temperatures of the components is 0.5°, the capacity is 2.5 to 3.0 ml/h cm^2 on a chromatographic column of 1.8 m in length with dinonyl phthalate, while in the stationary phase bis(propionitrile)-ethylene glycol, the capacity calculated from the experimental data by Bayer is 12 g/h cm^2. When purifying reagents to 99.90–99.995% purity, the capacity of chromatography usually is 2 to 3 ml/h cm^2, and sometimes up to 6 ml/h cm^2 according to our data. These examples show that it is possible to use eqn. 8 for qualitative estimation of the effect of experimental param-

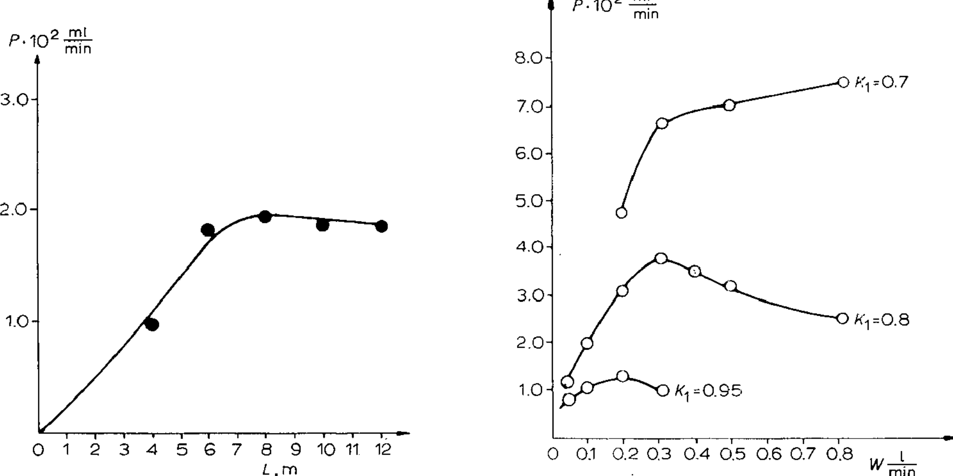

Fig. 2. Capacity dependence on column length. Mixture of isopentane and *n*-pentane.

Fig. 3. Changes in capacity with increasing flow rate of carrier gas at various values. Mixture of isopentane and *n*-pentane.

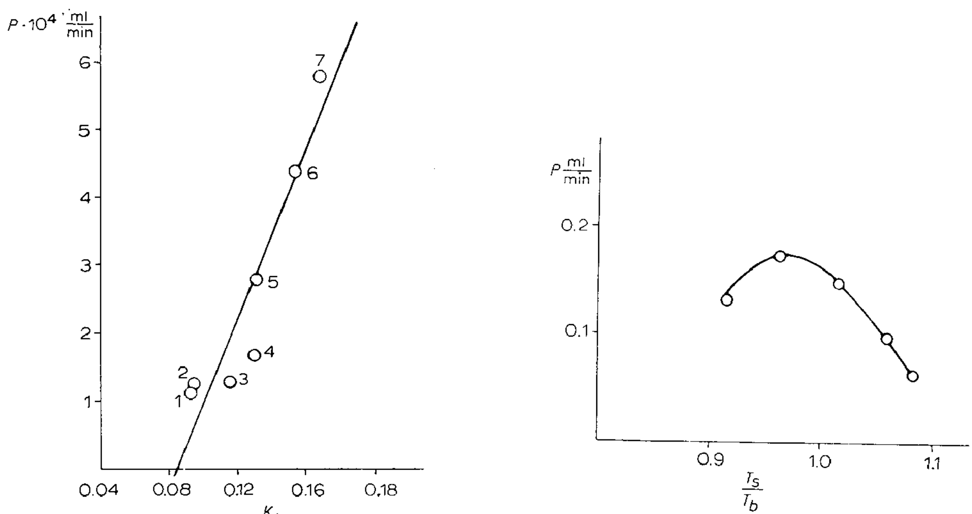

Fig. 4. Capacity dependence on the selectivity factor. Mixtures separated: 1 = carbon tetrachloride–benzene; 2 = carbon tetrachloride–cyclohexane; 3 = chloroform–cyclohexane; 4 = benzene–heptane; 5 = chloroform–cyclohexane; 6 = carbon tetrachloride–dichloroethane; 7 = heptane–methyl cyclohexane.

Fig. 5. Capacity dependence on column temperature. Mixture of hexane and cyclohexane; stationary phase—dinonyl phthalate; column diameter 30 mm. T_s, column temperature; T_b, boiling point of the mixture.

eters on the capacity. In cases where experimental parameters affect the distribution factor, such a consideration becomes more difficult.

In Fig. 5 are given the experimental data on the effect of temperature on the capacity. Maximum capacity is obtained at a temperature close to the boiling point of the mixture.

In conclusion, the possibility of quantitative estimations by means of eqn. 8 is considered. The ratio between K_m for the preparative column used, 14 mm in diameter, and that for the analytical column of the Griffin chromatograph was determined initially. For various hydrocarbons and chlorohydrocarbons, this ratio differed by not more than 30% and on the average was equal to 9.3 when using silicone rubber SKTFT -50 and silicone oil 2a/300 as the stationary phases. The capacity was calculated for the mixture of benzene and cyclohexane using dinonyl phthalate as a stationary phase. The values of α and K_m were determined on the analytical column as $\alpha = 1.42$ and $K_m = 35$, from which, using the ratio 9.3, the K_m value for the preparative column was calculated. H_0 was determined by introducing 5 μl of the mixture into the preparative column. Calculated by formula 8, with $R = 1.9$ and $W = 230$ ml/min the capacity proves to be 3.34 ml vapour/min, the experimental value being 3.8 ml/min. For the mixture of benzene and heptane, the capacity calculated in a similar way for $R = 1.5$ and $W = 230$ ml/min was 2.4 ml vapour/min; the experimental value was 1.6 ml/min. Thus, eqn. 8 can apparently be used for semiquantitative evaluation of the capacity.

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CHROM. 4601

PROBLEMS OF MOLECULAR ADSORPTION CHROMATOGRAPHY

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SUMMARY

In this review we have tried to show that molecular gas and liquid chromatography on adsorbents have a number of advantages for analytical and preparative applications. The vast amount of experimental data obtained on adsorbents with known and reproducible chemical and geometrical structure, together with the combined use of static and chromatographic methods, makes the development of a molecular theory of gas adsorption possible on the basis of molecular-statistics and semi-empirical, or effective, potential functions of intermolecular interactions. Advances made in this field so far warrant further applications of this theory to gas adsorption chromatography. Of practical interest is the fact that forecasts of certain parameters and phenomena can be made on the basis of this theory. The theory of adsorption from solutions and liquid chromatography has been developed to a lesser degree. However, here, too, the main qualitative aspects of this theory have been established. Further efforts should be made to develop the theory of adsorption from liquid solutions and apply this theory to equilibrium, non-equilibrium, molecular and macromolecular sieve liquid chromatography.

INTRODUCTION

About ten years ago molecular adsorption chromatography was mainly used in the separation of gas mixtures. Today this method is used on a much larger scale. It has been applied to the separation of various mixtures from hydrogen isotopes and isomers, to proteins and even to viruses. This development is related to a number of achievements in science and technology: (1) greater control of the homogeneity and specificity of the molecular adsorbents by means of the individual synthesis of adsorbents and the chemical modification of their surface; (2) expansion of the working range of the gas chromatographic columns, up to 500°; (3) employment of strongly adsorbable carrier gases at high pressures, the result of which is a narrowing of the gap between gas and liquid chromatography; (4) advances in liquid molecular adsorption chromatography on the basis of adsorbents whose nature and degree of porosity can be controlled; (5) the development of molecular and macromolecular sieves, especially of the non-swelling type; (6) the development of highly sensitive detection methods in liquid chromatography.

The following factors—the relatively simple physicochemical basis of the chromatographic separation of molecules and macromolecules on adsorbents, the possibility of controlling the geometrical structure of the adsorbents and the chemical nature of their surface, and the fact that most of the adsorbents are non-volatile and have a high thermal and chemical stability—all make an adsorbent particularly valuable for use in analytical temperature-programmed columns as well as for preparative gas and liquid molecular-adsorption chromatography and its use on an industrial scale.

In addition to the more usual applications of gas adsorption chromatography, it is now an important tool in physicochemical investigations of the surface chemistry of solids, in studies of isotherms, the heat and entropy of adsorption, and the heat capacity of adsorption systems. Owing to the fact that the equipment is simple to operate and that it can be used over a wide temperature range, gas chromatography has made possible the achievement of rapid advances in the investigation of the adsorption of a large variety of organic, inorganic, and elemento-organic compounds. The investigation of the adsorption of many of these compounds is practically impossible using the usual vacuum techniques. Both gas chromatographic analysis of solutions which are in contact with solid surfaces and direct liquid molecular adsorption chromatography have opened wide possibilities for investigating such adsorption processes. All these developments provide the necessary experimental basis for studying adsorbate–adsorbent and adsorbate–adsorbate molecular interactions of the most diverse types of molecules. Such interactions take place during adsorption from the gas phase and from solutions.

A good deal of highly reproducible experimental data has been obtained by using homogeneous and chemically well-defined adsorbents whose surface has a known geometrical structure. These results may serve as a basis for the development of a quantitative statistical molecular adsorption theory and provide a theoretical basis for chromatographic separation of complex molecules. With the aid of modern electronic computers, such a theory derived from the properties of the surface and the adsorbed molecule would make it possible to select adsorbents with properties optimal for a definite purpose as well as to calculate adsorption equilibria and retention indices for a given system. Besides their theoretical significance, such calculations would facilitate the identification of the components of the mixture being separated and give data necessary for designing industrial-scale chromatographic equipment.

Such are the general aspects of the theoretical and applied problems under further development of molecular adsorption chromatography. The complete solution of these problems will require considerable effort on the part of many scientists. The purpose of the present paper is to analyse some of the results achieved in this field and to indicate the difficulties that may be encountered in future work.

MOLECULAR THEORY OF ADSORPTION AND GAS ADSORPTION CHROMATOGRAPHY

Problems of molecular adsorption theory

At the present time an adsorption theory for adsorption from the gas phase on homogeneous crystal surfaces is being developed on the molecular level, *i.e.* by considering the geometrical and electronic structure of the solid body surface and of the molecule being adsorbed (see refs. 1–5). On the whole, this theory includes

molecular-statistical calculation of equilibrium constants, virial coefficients, retention volumes at zero, low and average levels of surface coverage, as well as other thermodynamic constants, *e.g.*, heat and entropy of adsorption and the heat capacity of the adsorption systems. The calculation of configurational integrals, which are part of these thermodynamic terms, is carried out with the aid of semi-empirical expressions for the potential energies of the adsorbate–adsorbent and adsorbate–adsorbate interactions.

For analytical applications of this theory, especially in the case of highly sensitive detectors together with very low concentrations of the adsorbed molecules and fairly high temperatures, the adsorbate–adsorbate interaction is insignificant, and thus one only needs to consider the adsorbate–adsorbent interaction, *i.e.* the Henry constants and their dependence on temperature. At higher surface concentrations, one should introduce into the equation the next virial coefficient which takes into account the paired adsorbate–adsorbate interaction in the field of the adsorbent, etc., or take into account in some other way the adsorbate–adsorbate interaction.

The calculation of the potential energy of the adsorbate–adsorbent interaction from semi-empirical potential functions is a separate problem. The potential energy of adsorption of the molecule, in its most favourable orientation on the surface, equals the heat of adsorption at absolute zero (including the correction for the zero energy). Owing to the relatively slight dependence of the heat of adsorption on temperature, the magnitude of the potential energy of adsorption also approximately expresses the heat of adsorption at the temperature of the gas chromatographic column. At fairly low temperatures the order of the retention volumes usually corresponds to that of the heats of adsorption. Thus the first approximation in the molecular adsorption theory is already of interest, *i.e.* the definition of the potential energy of adsorption for the most favourable orientation of the molecules on the adsorbent surface. In this section we shall consider some data obtained from the calculation of the potential energy of adsorption for adsorption of different molecules on a series of crystalline adsorbents. An analysis will also be made of the Henry constants obtained by molecular-statistical calculations and of the retention volumes at zero sample size.

Up to now molecular-statistical calculations of retention volumes have only been made for a few compounds, whereas calculations of the potential energy of adsorption have been carried out for a relatively large number of compounds. To compare the calculated values with experimental ones requires calorimetric, isosteric, or gas chromatographic determinations of the heat of adsorption. The most convenient method, especially for compounds of low volatility, consists of the gas chromatographic determination of the retention volumes at different temperatures. However, the precision of such determinations is somewhat low, and thus it is necessary to carry out repeated measurements and make a statistical analysis of the data obtained.

The calculated potential energy of adsorption and a comparison of this calculated energy with the measured heat of adsorption

Graphitised thermal carbon black. The surface of this adsorbent is almost entirely formed of the basal faces of large crystals of graphite[5,6]. Therefore calculations were made for adsorption on the basal face of a graphite crystal. For adsorption of simple molecules on graphite the potential functions of the paired molecule–carbon atom interaction were used, *e.g.* $\varphi_{C\ldots Ar}$ or $\varphi_{C\ldots NH_3}$. For the adsorption of complex hydro-

carbon molecules and their derivatives the atom–atom potential functions $\varphi_{C...C}$, $\varphi_{C...H}$, $\varphi_{C...O}$, or group–atom potential functions $\varphi_{C...CH_3}$, $\varphi_{C...CH_2}$, $\varphi_{C...CH}$, etc. were used. The summation of the energy of paired interactions was made with respect to all atoms or atomic layers on the semi-infinite lattice of a graphite crystal (see refs. 1–3,5), and for complex molecules, with respect to the atoms or linkages which form these compounds, so that the potential energy of the molecule is:

$$\Phi = \sum_{C}\sum_{i} \varphi_{C...i} \tag{1}$$

where $\varphi_{C...i}$ is the potential energy of interaction of a C atom of the lattice with an atom or linkage i of the molecule. The equation for $\varphi_{C...i}$ was written in the form of the Buckingham or Lennard–Jones potential augmented by the term for dipole–quadrupole dispersion–attraction. In the first case we obtain:

$$\varphi_{C...i} = -C'_{Ci}\, r_{Ci}^{-6} - C''_{Ci}\, r_{Ci}^{-10} + B_{Ci} \exp\left(-\frac{r_{Ci}}{\varrho_{Ci}}\right) \tag{2}$$

The constants C'_{Ci} and C''_{Ci} were calculated with the aid of the KIRKWOOD–MÜLLER equation[7,8] or an analogous equation[9] for the polarisability and diamagnetic susceptibility of C and i linkages. The constant B_{Ci} was determined from the equilibrium conditions and was expressed in terms of the equilibrium distance z^0_{Ci} of the atom or linkage centres i from a plane passing through the centres of the C atoms of the outer basal plane, for the most favourable orientation of the molecules relative to this plane (*i.e.* the maximum number of molecular linkages which are in contact with the basal plane). The constant ϱ_{Ci} was taken to be equal to 0.28 Å, as for the molecular crystals. In the case of adsorption of a dipole molecule a term was introduced into eqn. 2 for the energy of the inductive attraction of the dipole to the polarised C atom in the

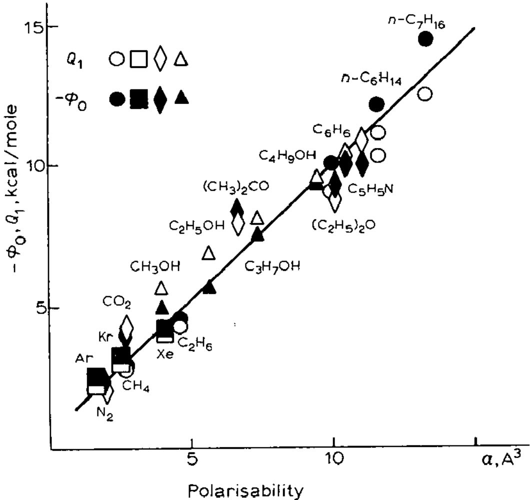


Fig. 1. Dependence of the calculated potential energy of adsorption, $-\Phi_0$, and the experimental heat of adsorption, Q_1, for different substances adsorbed on the basal plane of graphite and on graphitised thermal carbon black, respectively, at low surface coverage as a function of the adsorbate polarisability, α.

lattice[10] or for the energy of the reflected force of the dipole in the carbon layer of the lattice[11]. In both cases the contribution due to this energy is small. Detailed calculations of the constants, the summation of the expression (2) in eqn. 1, and the calculation of $-\Phi_0$ for the equilibrium distances z^0_{Ci} have been described in original studies which have been reviewed[3,5]. Fig. 1 shows a comparison of some results obtained by AVGUL AND KISELEV[5], CROWELL *et al.*[12], and CURTHOYS AND ELKINGTON[13] of the calculation of Φ_0 together with the calculated heat of adsorption Q_1 (for zero surface coverage) derived on the basis of experimental measurements (calorimetric, isoteric, and in many cases gas chromatographic). The values $-\Phi_0$ and Q_1 in Fig. 1 are plotted as the function of the molecule polarisability α. The calculated values agree well with the experimental data. Furthermore, the values of $-\Phi_0$ and Q_1 fall approximately on the same line as those obtained by plotting the data for *n*-alkanes. This holds true for the molecules of group A (noble gases, saturated hydrocarbons) which are incapable of specific molecular interaction, group B which have π-bonds or lone electron pairs, and group D which contains functional HO- or HN-groups; groups A, B, and D are according to the classification by KISELEV[1,5,14,15]. This indicates that graphitised thermal carbon black is non-specific in nature[1,5,14-16]; *i.e.* it belongs to the adsorbents of the first type according to the above-mentioned classification.

Boron nitride. The lamellar structure of BN crystals is analogous to that of graphite except that at the intersection of the hexagonal basal planes there are alternate B and N atoms instead of C atoms. When the composition of the crystal corresponds exactly to the BN formula and the lattice is practically free of defects, the basal plane BN exhibits properties of a non-specific adsorbent. CROWELL AND CHANG[17] have carried out calculations of the potential energy of adsorption and measurements of the heat of adsorption for noble gases, and CURTHOYS AND ELKINGTON[13] for adsorption of hydrocarbons. As can be seen from the plots based on these data in Fig. 2,

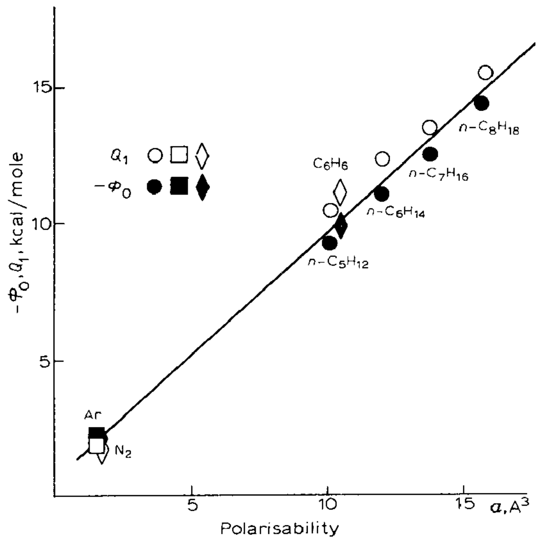
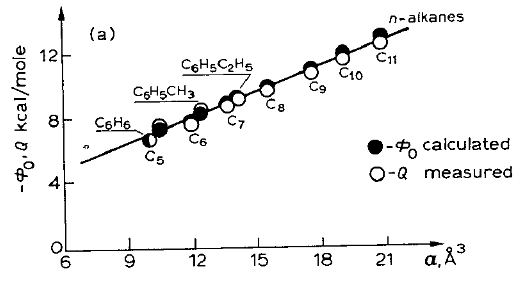


Fig. 2. Dependence of the calculated potential energy of adsorption, $-\Phi_0$, and the experimental heat of adsorption, Q_1, on boron nitride at low surface coverage as a function of the adsorbate polarisability, α.

Fig. 3. Diagram showing the packing of phthalocyanine molecules on the surface of graphitised thermal carbon black[18].

the values of $-\Phi_0$ and Q_1 are in good agreement (for hydrocarbons Q_1 was obtained by a gas chromatographic method).

Phthalocyanine. The basal plane of the phthalocyanine crystal shown in Fig. 3 is an example of a specific adsorbent, since it contains different functional groups: =C=, -CH=, -N=, HN=[18]. Although these groups are conjugated and thus facilitate a more regular distribution of the electron density, the resulting surface of the adsorbent is chemically heterogeneous. At the edge of the plane corresponding to the plane of the molecule there is a higher electron density locally. Thus this adsorbent belongs mainly to the third type according to the classification by KISELEV[1,5,14,15], even though it contains HN= centres which belong to the second type of adsorbent. The planes in a phthalocyanine molecule formed by CH linkages possess a weaker molecular field. KOUZNETSOV *et al.*[18] have obtained, by gas chromatographic methods, the heat of adsorption for different compounds including those from group A (n-alkanes), which are incapable of specific molecular interactions, group B (acetone, ethers, aromatic hydrocarbons) and group D (phenols and amines) which are capable of such interactions[1,5,14,15]. The Φ_0 values for n-alkanes were calculated by the method described above with the use of the Lennard-Jones equation for calculating $\varphi_{j...i}$. The force centres j of the adsorbent were taken as =C=, -CH=, -N=, and =NH groups. The calculation of $\varphi_{j...i}$ for i = CH_3 and CH_2 at different distances, z, of the linkage centres in the molecule i from the basal plane containing adsorbing centres j was carried out for points I–VI in the plane $x,y,z = 0$ (Fig. 3). The absolute value of Φ_{0i} reaches its maximum above the centre of the phthalocyanine molecule (above point I in Fig. 3). It decreases linearly with the increase in the distance away from this point and parallel to the x and y axes, which are at a 45° angle to each other, the potential being somewhat greater for the x axis than for the y axis. Between these axes the change of potential was slight and could be easily compensated for. In Fig. 4 the calculated $-\Phi_0$ values for C_5–C_{11} n-alkanes and some aromatic hydrocarbons are compared with the experimental values for the heat of adsorption Q_1 for graphitised thermal carbon black coated with phthalocyanine (10% by wt.); such an amount of phthalocyanine is sufficient to provide almost complete shielding of the carbon black. From Fig. 4a it can be seen that for the non-specific adsorption of molecules of group A (n-alkanes) and weakly specific adsorption of molecules of group B with conjugated



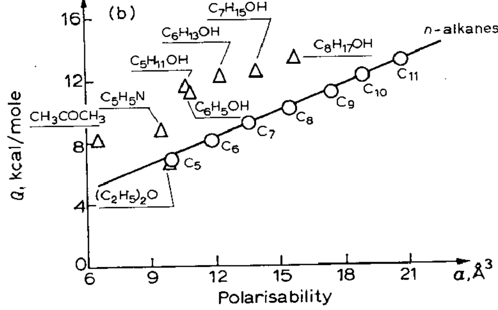


Fig. 4. Dependence of the calculated potential energy of adsorption, $-\Phi_0$, and the experimental heat of adsorption, Q_1, on graphitised thermal carbon black coated with 10% by wt. phthalocyanine as a function of the adsorbate polarisability α. (a) $-\Phi_0$ (solid points) and Q_1 (open points) for n-alkanes and aromatic hydrocarbons; (b) Q_1 for n-alkanes (solid line) and for a number of specifically adsorbing substances.

π-bonds (aromatic hydrocarbons), the calculated values for the potential energy of a non-specific interaction, $-\Phi_0$, are close to the measured Q_1, values, as shown in Figs. 1 and 2.

Fig. 4b shows the values for the heat of adsorption Q_1 for dipole molecules of group B (acetone, ether, pyridine) and also for molecules of group D (alcohols). These values are considerably larger than the known heats of adsorption[1,5,14–16] of the corresponding molecules of group A (n-alkanes) which do not undergo specific molecular interaction. The distance between the point for Q_1 in the case of specifically adsorbable molecules and the corresponding point (for the same polarisability value α (ref. 16)) on the straight line for n-alkanes indicates the contribution to the total heat of adsorption Q_1 due to the energy of specific molecular interaction $Q_{\text{specif.}}$; see refs. 1,5,14–16.

$$Q_{\text{specif.}} = Q_{1(\text{B,D})} - Q_{1(\text{A})} \tag{3}$$

where, $Q_{1(\text{A})}$ is the heat of adsorption of the corresponding molecule used for the comparison, the molecule being incapable of specific interaction. A theoretical calculation of the specific interaction energy has not yet been made in this case.

Some non-porous ionic adsorbents. The surface of ionic adsorbents is often heterogeneous not only because of the presence of growth steps or dislocations on individual planes but also because of sharp differences in these planes with respect to their crystallographic indices, *i.e.* because of the difference in the population of cations and anions. The simpler cases are those of laminar and cubic lattices (the latter

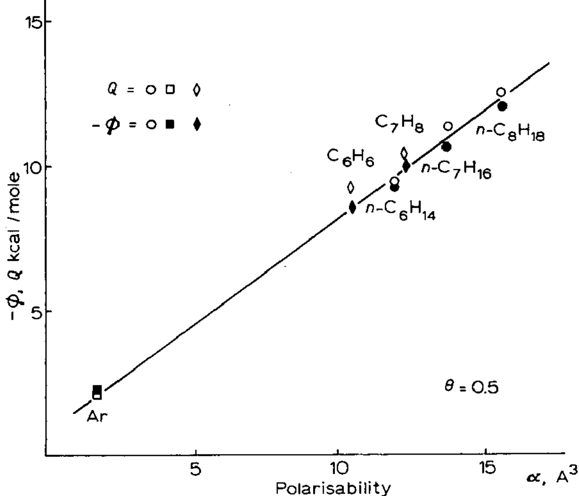


Fig. 5. Dependence of the calculated potential energy of adsorption , $-\Phi$, and the experimental heat of adsorption, Q, for different substances adsorbed on magnesium oxide at the level of surface coverage $\theta = 0.5$ as a function of the adsorbate polarisability α.

being of the NaCl type). In the latter case all (100) planes are identically populated by cations and anions. If the dimensions of these cations and anions are small and similar to one another, the electrostatic field changes periodically parallel to the surface in a rapid sequence. Owing to this, the contribution of the specific interaction energy to the total heat of adsorption of relatively large-size molecules is small. Fig. 5 shows a comparison of the calculated values for the potential energy of adsorption, $-\Phi$, (with compensation for the induction potential) and the measured heat of adsorption, Q, (for MgO cubic crystals whose surface was half-filled by molecules of noble gases[19] and hydrocarbons[9]). Here, the values for $-\Phi$ and Q are similar, and the specific adsorption of benzene is low.

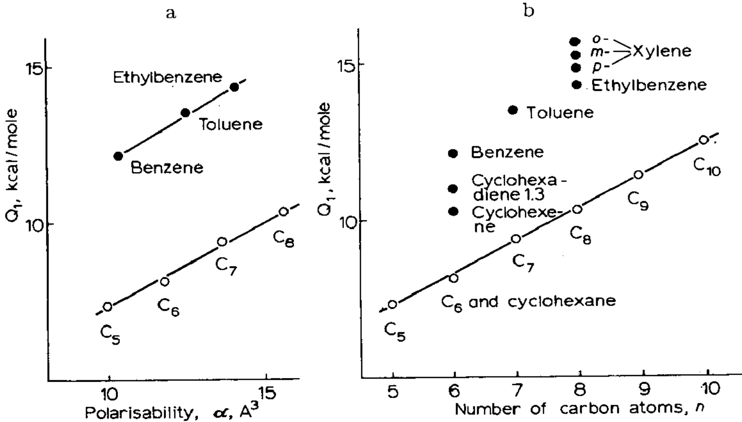


Fig. 6. Dependence of the heat of adsorption Q_1 for different substances separated on barium sulphate at low surface coverage as a function of the adsorbate polarisability α (a) and of the number of carbon atoms n in the molecules (b).

In a similar case, when the cations and especially multi-charged cations protrude on the surface of the crystal and the negative charge is distributed within large complex anions, the adsorbent exhibits very strong specific adsorptions (specific adsorbent of the second type according to the classification of KISELEV[1,5,14,15]). This can be seen from Fig. 6 which shows the comparison of the heats of adsorption, Q_1, for *n*-alkanes and cyclohexane, cyclohexene, cyclohexadiene, benzene, toluene, ethylbenzene and *o*-, *m*-, *p*-xylene with barium sulphate as the adsorbent. The data were obtained by a calorimetric method[20] and a gas chromatographic method[21]. The contribution to the heat of adsorption of benzene due to specific interaction energy, $Q_{\text{specif.}}$, amounts to ~ 5.0 kcal/mole. The heats of adsorption on salt surfaces depend to a great extent on the methods of preparation and treatment of the sample.

Zeolites. The cavity surface of porous crystals of cationised zeolites carries positive charges concentrated within the exchange cations. The negative charges are distributed among many weakly charged oxygen ions surrounding Al and Si atoms. Because of this, zeolites are classified as specific adsorbents of the second type. From Fig. 7 it can be seen that the contribution to the heat of adsorption, Q, of the B- and D-type molecules due to specific interaction energy is rather large. The heats of adsorption of the A-, B-, and D-type molecules shown in Fig. 7 were mainly measured by the calorimetric[22,23] and isoteric methods[24,25], but for small molecules gas chromatography[26] was also used.

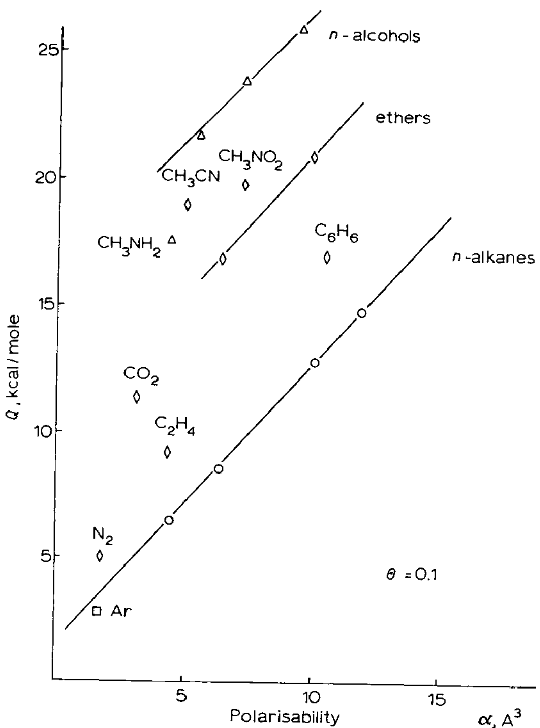


Fig. 7. Dependence of the heat of adsorption Q for different substances adsorbed on zeolite NaX with a degree of surface coverage $\theta = 0.1$ as a function of the adsorbate polarisability α.

Owing to the local distribution of the cations and the specific structure of the porous crystal network, the molecular field inside the zeolite cavity, and especially near its surface, is quite heterogeneous (here, the dispersion and electrostatic attraction forces must be taken into account). However, this heterogeneity can be allowed for[27], and thus we can obtain the average statistical value for the potential adsorption energy. This has been done for the adsorption of some gaseous compounds and a number of *n*-alkanes[28] on zeolites of the A-type[27]. The calculated $-\Phi_0$ values for the potential adsorption energy and the measured heat of adsorption in this case are also similar.

Pure silica and silica containing impurities. The surface of non-porous and porous silica, as a rule, is covered by hydroxyl groups (see refs. 1, 14, 15, 29–33). The concentration of these groups on the surface and within the bulk of the particles depends on the nature and crystalline structure of the silica (for amorphous silica, on the nature of the sample). The concentration of hydroxyl groups also depends on whether the sample has been subjected to hydrothermal or simple thermal treatment or thermal treatment *in vacuo*. The concentration of hydroxyl groups on the surface can be determined by means of the deuterium-exchange method (see ref. 33) or by means of IR spectroscopy[32]. Reproducible values for the concentration of hydroxyl groups on the silica surface (*e.g.* aerosil, aerosilogel[34], silica gel) can be obtained when reproducible conditions are used for the hydroxylation procedure or the dehydroxylation of the surface. The concentration of hydroxyl groups is independent of the specific surface area[33].

The presence of hydroxyl groups on the silica surface determines the specific nature of this adsorbent, which is classified as one of the second types of adsorbent. This specific behaviour is demonstrated by hydrogen bonding with molecules of the B- and D-group[1,14,15,35]. The removal of HO-groups from the surface by heating the sample causes a decrease in the degree of specific molecular interaction. This behaviour of the silica surface is to a great extent determined by the chemical purity of the sample.

The presence on the silica surface of impurities which form strong Lewis acid centres (such as Al and B) causes an increase in the bond energy formed with electron-donor molecules. The effect is especially noticeable upon dehydroxylation when strong Lewis acid centres become exposed. This can be directly observed by comparing the heat of adsorption of electron-donor molecules on pure aerosilogels and aerosilogels containing aluminium before and after dehydroxylation at high temperatures (the surface area of the macroporous aerosilogels in these experiments remains unchanged). Fig. 8 shows some calorimetric data obtained by Ash *et al.*[36] for the heat of adsorption of vapours of N,N,N-triethylamine, a strong organic base. Relatively pure aerosilogel and aerosilogel containing 0.36% by wt. aluminium served as the adsorbent. The samples were kept *in vacuo* at 200° and 1100°. Both samples containing HO-groups following the 200° treatment showed, with respect to triethylamine, only molecular specificity on the surface (weak Brönsted acid centres). After gradual coverage of the monolayer on the hydroxylated silica surface, the heat of adsorption of triethylamine was about 20 kcal/mole. This value corresponds to a specific molecular adsorption leading to the formation of a $SiOH...N(C_2H_5)_3$ hydrogen bond. Strong dehydroxylation of the surface, when the sample is treated at 1100° (without altering the surface area) in the case of pure aerosilogel, leads to the formation of

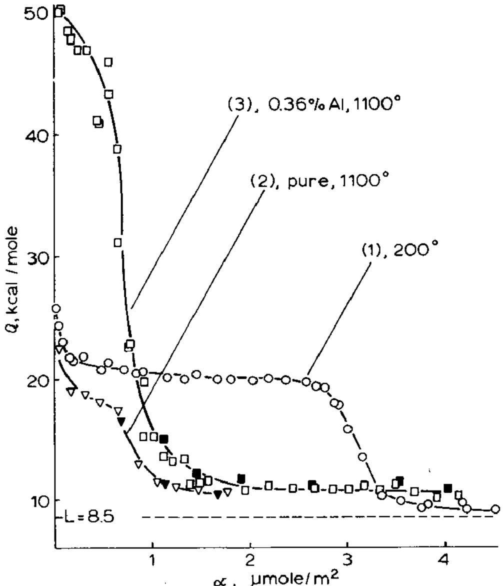


Fig. 8. Heat of adsorption of triethylamine on silica. 1 = on silica gel with the hydroxylated surface stored *in vacuo* at 200°; 2 = on pure aerosilogel with dehydroxylated surface stored *in vacuo* at 1100°; 3 = on aerosilogel containing 0.36% by wt. Al with a dehydroxylated surface stored *in vacuo* at 1100°.

siloxane groups which are incapable of specific molecular interaction. In this case the heat of adsorption of triethylamine decreases to 10 kcal/mole. This value corresponds to the energy for the non-specific interaction of triethylamine with a silica surface. In the case of the aerosilogel containing an aluminium impurity, dehydroxylation of the surface at 1100° results in the formation of a large number of Lewis electron-acceptor centres besides the siloxane groups. The molecules of triethylamine on these centres undergo chemisorption with a heat of adsorption of approximately 50 kcal/mole. Only when all such centres accessible to the N,N,N-triethylamine molecules are filled, does the heat of adsorption sharply decrease to a level corresponding to non-specific adsorption on the siloxane groups of the remaining sample surface. Thus, there are three main levels of interaction energy: the highest, ~ 50 kcal/mole, corresponding to chemisorption of $N(C_2H_5)_3$ molecules on the acceptor centres of the impurity; ~ 20 kcal/mole, a level corresponding to the specific molecular adsorption of $N(C_2H_5)_3$ molecules on silanol groups; and ~ 10 kcal/mole, a level corresponding to the molecular non-specific adsorption of $N(C_2H_5)_3$ molecules by the siloxane groups. Analogous results have been obtained by DAY *et al.*[37] for the heat of adsorption of tetrahydrofuran (a molecule of the B-group) on aerosilogel containing 0.36% of aluminium by wt.). The heat of adsorption of cyclopentane (a molecule of the A-group) at the usual temperature of calorimetric measurements is not affected either by the change of silanol groups into siloxane groups or by the exposure of the aluminium centres[37].

In accordance with this, the chromatograms of B- and D-type molecules on

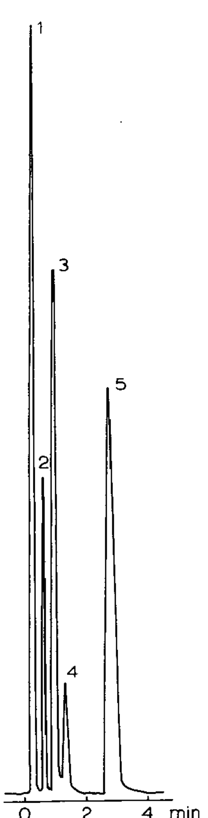


Fig. 9. Chromatogram obtained for Silochrom C-80. 1 = Cyclohexane; 2 = *n*-decane; 3 = aniline; 4 = nitrobenzene; 5 = acetophenone. Column temperature, 200°; column length, 100 cm; sample size, 0.4 μl; carrier gas flow rate, 40 ml/min; flame ionisation detector.

aerosilogels respond with a high degree of sensitivity to the presence of aluminium or boron centres on the silica surface. Thus a separation on columns made of ordinary silica gel containing Al_2O_3 as an impurity only gives chromatograms with symmetrical peaks in the case of hydrocarbons (especially saturated hydrocarbons, *i.e.* molecules of the A-type). Aromatic hydrocarbons, ethers and ketones are only eluted from the column with difficulty, and the corresponding chromatograms are characterised by expanded peaks[15]. On the other hand, such compounds as amines, pyridines, quinoline and other strong organic bases remain on the column *i.e.* they are held by chemisorption on strongly acidic centres distributed on the silica surface[38]. In the case of porous glass adsorbents, the role of such centres is performed by boron atoms[39].

In addition, in accordance with Fig. 8, the HO-groups on pure silica are neither too active energetically nor heterogeneous. From Fig. 9 it can be seen that the hydroxylated surface of chemically pure aerosilogel (Silochrom C-80) does not behave like a chemisorbent towards various molecules of the B- and D-groups, including an amine (aniline), but merely like a specific molecular adsorbent of the second type, which forms hydrogen bonds of different strengths with the molecules of B- and D-groups[40]. The dehydroxylation of a pure silica surface, which decreases its specificity,

only decreases the retention of such molecules and has little effect on the retention of A-group molecules.

The approximate energy values of the specific interaction (in this case the energy of the hydrogen bond) of different molecules belonging to the B-group with the silanol groups have been determined by several workers[35–37]. IR spectroscopic investigation showed that, with respect to the energy of the hydrogen bond, the surface silanol groups are similar to phenol groups[41]. The conjugation of unshared electron pairs in O and N atoms for furan and pyridine molecules, respectively, with the π-electron system of the ring results in a decrease in the specific interaction energy as compared with tetrahydrofuran and triethylamine molecules, whose respective O and N atoms have lone electron pairs[1,35]. For molecules containing several functional groups, the geometrical structure of the molecule is of special significance. In the case of dioxane, the presence of two ether groups isolated from each other by methylene groups does not result in a doubling of the value for the energy of the specific interaction with silanol groups on the silica surface[35]. This is due to the fact that the favourable orientation of one ether group of the chairlike molecule of dioxane precludes the other ether group approaching close enough to the silanol group to form a strong hydrogen bond with the surface. All these factors affect the retention volumes in chromatographic columns filled with Silochroms.

Silica modified by grafted silylalkyl groups. The heat of adsorption on a silica surface which has been modified by reaction with $ClSi(CH_3)_3$ has been investigated by BABKIN *et al.*[42]. It was found that for molecules of relatively large size–benzene, *n*-hexane, and carbon tetrachloride—the initial heat of adsorption measured is considerably less than the corresponding heat of condensation. These results agree with the calculated data for the interaction potential energy, $-\Phi_0$, for the adsorption of benzene and hexane on a silica surface densely packed with methyl groups from the grafted $-Si(CH_3)_3$ groups[43]. Such low values for the heat of adsorption are due to the low concentration of force centres on the outer surface, since the distance of the neighbouring methyl groups from one another is not equal to the C-C bond length (1.5 Å) but is considerably greater and at least equal to the sum of the van der Waal's radii of these groups (4.0 Å). The force field of the silica surface itself, like that of the graphitised carbon black when it was modified by dense monolayers of organic compounds (see below), becomes almost completely shielded. For the same reason, in the case of relatively low concentrations of force centres on the surface, the energy for non-specific adsorption on molecular crystals is smaller than on atomic crystals. Thus the heat of adsorption of *n*-alkanes on phthalocyanine is considerably smaller than on graphite.

A comparison of heats of adsorption and the contribution to their values due to the energy of specific interactions on different adsorbents. Fig. 10 shows the dependence of the heat of adsorption, at a low degree of surface coverage, for *n*-alkanes adsorbed on a number of surfaces described above. From Fig. 10 it can be seen how the heat of adsorption of non-specifically interacting molecules can be varied within a wide range. This means that the retention of these compounds in the chromatographic column can be varied to a much greater extent.

Furthermore, the *relative* role of specific interactions in adsorption on molecular crystals and monolayers containing functional groups can be quite significant, since the contribution of the non-specific interaction energy in this case is small. Thus the

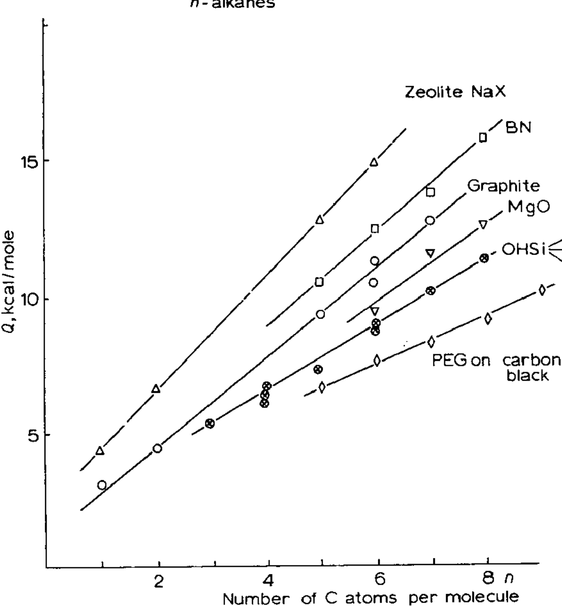


Fig. 10. Dependence of the heat of adsorption of n-alkanes at low degrees of coverage on the number of carbon atoms in the molecule for different adsorbents.

specific interaction with the basal surface of phthalocyanine crystals is strong (see Fig. 4b). It is especially strong for adsorption on metal phthalocyanines. This fact has been utilised for carrying out precise separations[44].

From Fig. 11a it can be seen that the shielding of the surface of graphitised

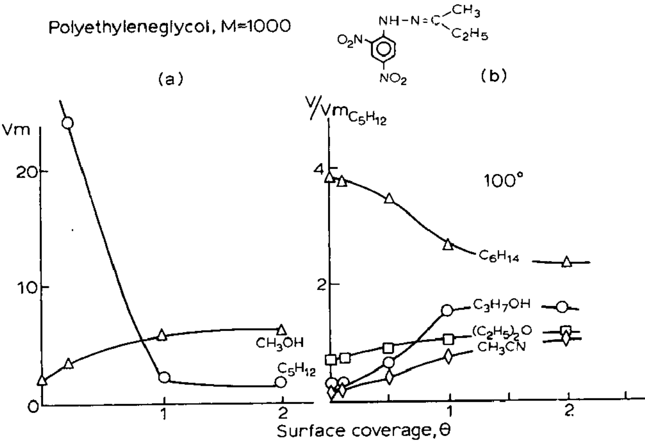


Fig. 11. (a) Dependence of the specific retention volume for n-pentane and methanol on the degree of coverage, θ, of the surface of the graphitised carbon black coated with polyethyleneglycol. (b) Dependence of the specific retention volumes of: n-hexane, n-propanol, diethyl ether, and acetonitrile, relative to the retention volume of n-pentane on the degrees of coverage, θ, of the acetylene black surface coated with the 2,4-dinitrophenylhydrazone of methyl ethyl ketone.

TABLE I

APPROXIMATE VALUES OF THE TOTAL HEAT OF ADSORPTION AT A LOW SURFACE COVERAGE, Q_{total}, AND THE CONTRIBUTION TO THESE VALUES DUE TO SPECIFIC INTERACTIONS WITH THE ADSORBENT, $Q_{specif.}$, FOR DIFFERENT COMPOUNDS (KCAL/MOLE)

The adsorbents are arranged in order of the decreasing $Q_{specif.}$ for benzene adsoption.

Adsorbent	C_6H_6		$(C_2H_5)O$		n-C_3H_7OH	
	Q_{total}	$Q_{specif.}$	Q_{total}	$Q_{specif.}$	Q_{total}	$Q_{specif.}$
$BaSO_4$	12.2	5.0	—	—	—	—
Zeolite NaX	18.0	5.2	21.0	8.6	—	—
Silica gel with hydroxylated surface (degree of coverage $\theta = 0.5$)	10.2	2.8	15.0	7.3	16.0	11.1
Polyacrylonitrile	9.4	2.8	9.0	2.8	9.6	4.8
Polyethyleneglycol (monolayer on carbon black)	9.1	2.5	6.1	0.0	9.4	4.6
2,4-Dinitrophenylhydrazone of methyl ethyl ketone (monolayer on carbon black)	8.3	1.2	8.1	1.5	8.3	2.8
Phthalocyanine (10% by wt. on graphitised carbon black)	7.9	0.7	6.9	0.0	10.4	5.0
Graphitised thermal carbon black	9.2	0.0	8.6	0.0	8.1	<0.5

carbon black, a non-specific adsorbent of the first type, with a dense monolayer of polyethyleneglycol sharply lowers the adsorption of the A-group molecules (n-alkanes) but increases the adsorption of D-group molecules (methanol). By modifying graphitised carbon black with a monolayer of polyethyleneglycol, we can obtain an adsorbent of a third type. In this case we have the electron density concentrated locally at the edge of the ether group; this electron density provides high specific activity with respect to molecules of the D-group and at the same time gives a low background for non-specific interactions[45].

In Fig. 11b, the result of the modification of the surface of the graphitised carbon black with a dense monolayer of 2,4-dinitrophenylhydrazone of methyl ethyl ketone is shown. In this case, different centres appear on the surface, but the surface on the whole acquires the properties of an adsorbent of the third type characterised by the strong retention of alcohols[46].

The change in the contribution to the total heat of adsorption of the energy due to specific interaction and the change in the total heat of adsorption of molecules of different groups on adsorbents of different types are evident from the data summarised in Table I.

Molecular-statistical calculation of Henry constants

Representation of the adsorption isotherm in virial form. In addition to calculating, by the methods of the molecular theory of adsorption, the potential energy of adsorption, which is close to the heat of adsorption, the calculation of the retention volumes as a function of concentration (*i.e.* adsorption isotherms), or at least at zero surface coverage (*i.e.* Henry constants), is of even greater interest. The adsorption equilibrium for many cases can be expressed through the virial form of the equation for the adsorption isotherm:

$$p = \Gamma \exp(C_1 + C_2\Gamma + C_3\Gamma^2 + \dots) \quad (4)$$

where p is the pressure of a given adsorbate in the gas phase; Γ is the Gibbs adsorption per unit surface area of the adsorbent; and C_1, C_2, C_3... are coefficients which depend on the temperature. This equation is obtained by the molecular-statistical method[47] and by substituting into the Gibbs equation the equation for a two-dimensional state in the virial form[1,48]. For a low surface coverage, eqn. 4 expressed as an equation of the Gibbs adsorption isotherm in the virial form changes to:

$$p = K_1'\Gamma + K_2'\Gamma^2 + \ldots \tag{5}$$

where $K_1' = \exp C_1$; $K_2' = C_2 \exp. C_1$. In the molecular-statistical theory of adsorption[4,49] this equation is usually written in reverse in the form of a series of powers of p

$$\Gamma = K_1 p + K_2 p^2 + \ldots \tag{6}$$

Here, K_1 is Henry's constant, which is related to the absolute value (calculated per unit surface area) of the retention volume V_s through the equation (ref.15) :

$$V_s = K_1 RT \tag{7}$$

The remaining constants $K_2, K_3,$... take into account the adsorbate–adsorbate interaction (paired, tripled, etc.) in the adsorbent field.

Molecular-statistical equations for virial coefficients. Eqn. 6 is obtained by the general molecular-statistical method using a large partition function and assuming that the gas far away from the adsorbent surface is an ideal gas[4,49]. The quantitative calculations of coefficients K_1 and K_2 in the case of gas adsorption chromatography on fairly homogeneous surfaces are simpler than in the case of gas–liquid chromatography. This is due to the fact that the force centres of the adsorbent are tightly held. The virial coefficients are expressed through the corresponding statistical partition functions, Z, by following equations (ref. 49):

$$K_1 = \frac{v}{skT}\left(\frac{Z_1}{Z_1^0} - 1\right), \tag{8}$$

$$K_2 = \frac{v^2}{s(kT)^2}\left[\frac{2Z_2 - Z_1^2}{(Z_1^0)^2}\right] \tag{9}$$

Here, Z_1 and Z_1^0 are the partition functions for a single molecule within the volume v when the interaction of the molecule with the adsorbent surface is possible and subsequently when such interaction is absent in the same volume; Z_2 is the partition function within the volume v for two molecules when their paired interaction with one another in the adsorbent field is possible.

Measurements in gas chromatography are usually carried out at fairly high temperatures except in the separation of hydrogen isotopes and isomers[50]. Thus the calculation of the partition functions Z can be made according to classical approximations. By assuming that the partition function which is related to intramolecular motion remains practically unchanged during adsorption (which holds true for non-specific adsorption), Poshkus and Afreimovich[51] obtained the following equation for the Henry constant, K_1, through the corresponding configurational integrals:

$$K_1 = \frac{\int [s_i]^{\frac{1}{2}} \exp(-W/kT)\, [\exp(-\Phi/kT) - 1] \sin\vartheta \mathrm{d}x\mathrm{d}y\mathrm{d}z\mathrm{d}\vartheta\mathrm{d}\varphi\mathrm{d}\psi\mathrm{d}\alpha_1 \ldots \mathrm{d}\alpha_t}{8\pi^2 skT \int [s_i]^{\frac{1}{2}} \exp(-W/kT) \mathrm{d}\alpha_1 \ldots \mathrm{d}\alpha_t} \quad (10)$$

Here, W is the potential energy of the isolated molecule which does not interact with the neighbouring molecules of the adsorbate, this energy being dependent on the angles of the internal rotation of the molecule, $\alpha_1 \ldots \alpha_t$; $-\Phi$ is the potential energy of interaction of the molecule with the adsorbent surface, this energy being the function of the x,y,z coordinates of the centre of the mass, Euler's angles ϑ, φ, ψ describe the orientation of the quasi-rigid core of the molecule in space and $\alpha_1 \ldots \alpha_t$, the internal rotation angles; the function $[s_i]$ is the determinant of the matrix composed of the quadratic equation coefficients for the rotational kinetic energy of the molecule expressed by projection of the angular velocity of rotation of the core and the derivatives of the internal angles of rotation. Thus this equation for K_1 is sufficiently general in form and is not linked to any model of localised or mobile adsorption. However, this equation involves the assumption that the interaction with the adsorbate molecule does not affect the state of the adsorbent which is considered as a field source only, causing deviation of the gas near the surface from its ideal state far away from the surface. For fairly rigid adsorbents, especially in the case of non-specific adsorption, this assumption holds true.

In many cases of practical importance, eqn. 10 for Henry constants can be simplified. For a homogeneous crystal surface, $-\Phi$ is a periodic function of x and y. Often, for example in the case of adsorption on the basal plane of graphite, this periodicity can be neglected by assuming that the regular distribution of the adsorbent density is parallel to the basal plane[3,5,52]. The determinant $[s_i]$ depends on the internal angles of rotation of the linkages i of the molecule. However, if the molecule has a rigid core and if t symmetrical spindles (as, for example, in butane and *n*-pentane molecules) are attached to it, $[s_i]$ becomes constant and eqn. 10 can be abbreviated. If the whole molecule is quasi-rigid, *i.e.* if internal rotations are impossible, $W = 0$. In this frequently occurring case, only the term for the potential energy for the interaction of the isolated adsorbate molecule with the adsorbent remains in the expression for K_1 and:

$$K_1 = \frac{1}{8\pi^2 skT} \int [\exp(-\Phi/kT) - 1] \sin\vartheta \mathrm{d}x\mathrm{d}y\mathrm{d}z\mathrm{d}\vartheta\mathrm{d}\varphi\mathrm{d}\psi \quad (11)$$

Finally, when the molecule is of the one-atom type, the expression for the Henry constant becomes very much simplified and contains only the x,y,z coordinates of the centre of the molecule.

The calculation of the next virial coefficient K_2 is carried out by taking into account the potential energy of the paired adsorbate–adsorbate interaction (this energy is a part of the partition function Z_2 in eqn. 9) in the case of simultaneous interaction of both molecules with the adsorbent (the partition function Z_1 is included in eqn. 9).

The approximation of the additivity of paired adsorbate–adsorbate interaction in the calculation of interaction energy in the equation for K_2 as well as for paired j...i interactions in the calculation of $-\Phi$ (see eqn. 1) is quite satisfactory, since to disregard the interactions of many bodies which strongly depend on their mutual orientation apparently does not lead to large errors.

Eqns. 8, 10, 11 have been used in the work of KISELEV *et al.*[1,15,49,51–55] for the calculation of the Henry constant K_1 (retention volumes V_S for zero sample size) at different temperatures, as well as for the change in the chemical potential, $\Delta\mu$, in the case in which the adsorbate molecule transfers from the gas phase at standard pressure p^0 to the surface with a low value of adsorption Γ (with the assumption that up to this value of Γ the adsorbate–adsorbate interactions can be neglected):

$$\Delta\mu = -RT \ln K_1 + RT \ln (\Gamma/p^0) \tag{12}$$

The calculation of isoteric heat of adsorption Q_1 and the differential molar change in the entropy of the adsorbate, $\Delta\bar{S}$, at low surface coverage requires the calculation of the first derivative with respect to temperature of $\ln K_1$ (and thus also the corresponding configurational integrals) with the assumption that v and s are constants:

$$Q_1 = -RT^2 \frac{\partial \ln K_1}{\partial T} \tag{13}$$

and

$$\Delta\bar{S} = R\left(\ln K_1 + T\frac{\partial \ln K_1}{\partial T} - \ln \frac{\Gamma}{p^0}\right) \tag{14}$$

On the other hand, the calculation of the differential molar change in the adsorbate heat capacity, $\Delta\bar{c}_{v,s}$, requires the calculation of the second derivative of $\ln K_1$ with respect to temperature (and thus the corresponding configurational integrals[54] as well):

$$\Delta\bar{c}_{v,s} = -\frac{\partial Q_1}{\partial T} = R\left(2T\frac{\partial \ln K_1}{\partial T} + T^2 \frac{\partial^2 \ln K_1}{\partial T^2} + 1\right) \tag{15}$$

A comparison of the calculated values of ln K_1 and the experimental values. Such a comparison has been made by KISELEV *et al.*[1,5,49,51–55] for the adsorption of a series of simple and complex molecules on graphitised thermal carbon black and by BRÄUER *et al.*[27b] for the adsorption of some simple molecules on various cationic forms of

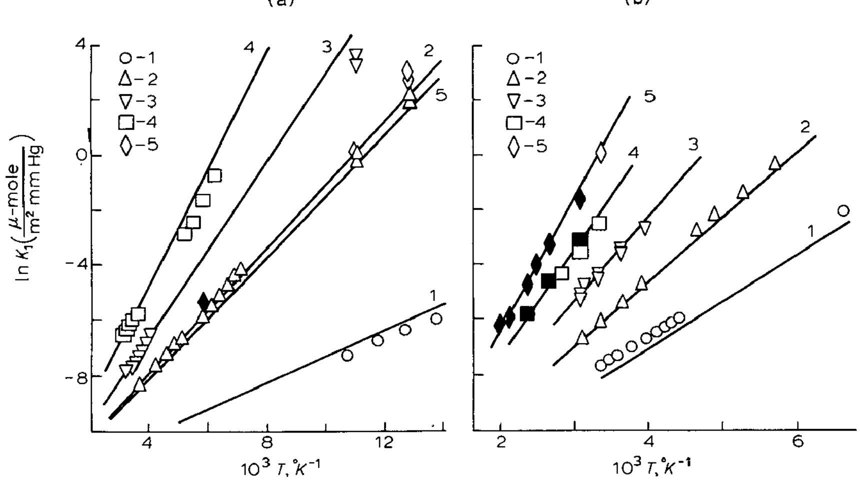


Fig. 12. Dependence of the calculated (lines) and the measured (points) Henry constants as a function of the reciprocal of the absolute temperature on graphitised carbon black. (a) 1 = Neon; 2 = argon; 3 = krypton; 4 = xenon; 5 = nitrogen. (b) For C_1–C_5 *n*-alkanes.

zeolite A. In the latter case, account is taken of the heterogeneous distribution of the molecular field in different directions inside the cavities of a porous crystal.

Fig. 12 shows a comparison of the calculated dependences of $\ln K_1$ on $1/T$ with the experimental data for adsorption on graphitised thermal carbon black. For noble gases and nitrogen, calculations were made by means of eqn. 11. The adsorbate–adsorbate interaction potential $\varphi_{C\ldots i}$ was calculated from eqn. 2, and the summation with respect to the carbon atoms of the semi-infinite lattice of the graphite in the calculation of Φ (z) (see eqn. 1) was carried out by the CROWELL method[3] for a regular distribution in carbon density along the basal planes. The constants C'_{Ci} and C''_{Ci} for these adsorbents were calculated from the KIRKWOOD–MÜLLER theoretical equation[7,8] and an equation analogous to this one[9]. From Fig. 12a, it can be seen that the lines calculated in this manner agree satisfactorily with the experimental data.

In the case of hydrocarbons (n-alkanes from C_1 to C_5), atom–atom potentials $\varphi_{C\ldots C}$ and $\varphi_{C\ldots H}$ and group–atom potentials $\varphi_{C\ldots CH_3}$ and $\varphi_{C\ldots CH_2}$ were used. In the determination of the corresponding C'_{Ci} and C''_{Ci} constants of these potential functions, the calculation of the configurational integrals and the values of $\ln K_1$ for adsorption of the first alkanes considered as quasi-rigid gave results which deviated somewhat from the experimental data (within $\pm$ 10–15%). In order to use these potential functions for all hydrocarbons, a constant correcting factor was introduced for the constants C'_{Ci} and C''_{Ci} (ref. 54), POSHKUS[55] (see also ref. 56) has shown that the effective potential function $\varphi_{C\ldots C}$ obtained for the adsorption of hydrocarbons on graphite is very close to the effective potential function calculated for other cases where there is molecular interaction between carbon atoms. This is indicated by data on the compressibility of graphite[57], the properties of molecular crystals of hydrocarbons[58], and the second virial coefficient of gaseous methane[59]. This means that the effect of interactions between many bodies is insignificant and that the approximation of the additivity of paired interactions is satisfactory.

By using these effective potential functions for atom–atom and group–atom interactions and by taking into account the differences in the adsorption of different rotational isomers (*trans*- and *skew*-forms of n-butane and n-pentane), KISELEV *et al.*[55] calculated $\ln K_1$ as the function of $1/T$ for all C_1–C_5 n-alkanes. From Fig. 12b it can be seen that in the case of the adsorption of complex molecules, the calculated values are close to the experimental values. Analogous results have been obtained for quasi-rigid molecules of benzene, ethylene, and acetylene.

The calculated values for $\Delta\bar{c}_{v,s}$ (ref. 60) can be compared with the calorimetric measurements[61] only for the case of adsorption of benzene on graphitised thermal carbon black; and these values are in good agreement. The value of $\Delta\bar{c}_{v,s}$ in this case amounts to approx. 5 cal/mole·deg; this indicates that in the treatment of gas chromatographic data obtained at wide intervals of column temperature, Q_1 as a function of temperature should be taken into account.

Molecular-statistical and thermodynamic estimation of the separation of components leaving a column and the order of the appearance of these components. This problem is of great interest in gas chromatography, since the order of emergence of the peaks for two given components depends on the nature and the geometry of their molecules, the adsorbent surface and on the temperature.

Usually the order of the $\ln V_s$ values for two components on the same adsorbent corresponds to the order of the values of the heat of adsorption[15]. However,

with a change in temperature a reversal in the order of appearance of the peaks is often observed. The molecular-statistical calculation of the ln V_s values and the heat of adsorption is not sufficiently precise because of the small changes in these values on changing over from adsorption of one molecule to that of another of similar structure. However, the difference in these values can be calculated fairly accurately, since in both cases the calculations involve the same assumptions and approximations.

In the papers[62] for this symposium (see also ref. 63) POSHKUS examines the separation of deutero-substituted molecules in great detail from the point of view of the molecular statistical theory. He also cites the relevant literature on the subject. The quantum-statistical effects related to the intramolecular vibrations rapidly decrease with increase in temperature. Translational and rotational quantum effects increase the adsorption of the heavier molecules among the series of the isotopes investigated, but these effects rapidly disappear with the increase in the mass and moment of inertia of the molecule and increase in temperature. Thus, under conditions of chromatographic measurement, the difference in the potential functions of the interaction of deuterated hydrocarbons with the surface is of great importance. This difference is mainly due to the lesser polarisability of the deuterated linkages of the molecule. KISELEV AND POSHKUS[62,63] give an equation, obtained by quasi-classical approximation, describing the relative difference in the retention volumes for a number of organic substances in the H- and D-substituted forms of the molecules. In the same work, the corresponding expressions for the differences in the heat and entropy of adsorption and the changes in the heat capacity of the adsorbate are given. In accordance with the molecular adsorption theory, the retention volumes and the heats of adsorption on graphitised carbon black[64,65] and carbonised Saran[66] are smaller for D-substituted compounds than those for H-substituted compounds.

The separation of various geometrical isomers and positional isomers on graphitised thermal carbon black and the possibility of using gas adsorption chromatography in the investigation of molecular structure have been reviewed in detail by KISELEV *et al.*[56]. In the paper by KOUZNETSOV AND SHEHERBAKOVA[67] presented at this symposium, new results on the use of gas chromatography, based on non-specific adsorbents having a homogeneous plane surface, for the estimation of the molecular structure of a number of hydrocarbons are reported.

The investigation of the frequently observed inversion of the order of appearance of the components from a column upon a change in temperature is of great interest. KISELEV AND POSHKUS[68] have examined the conditions leading to the intersection of the plots of ln V_s as a function of $1/T$ in the case of low surface coverage. In this case (see eqns. 7, 12–14) we have:

$$\ln K_1 = \ln (V_s/RT) = Q_1/RT + [\Delta\bar{S}/R + \ln(\Gamma/p^0)] \qquad (16)$$

The entropy of the adsorption $\Delta\bar{S} + R\ln(\Gamma/p^0)$ is usually negative[15,48,69,70]. The value of $\Delta\bar{S}/R + \ln(\Gamma/p^0)$ as well as that of $K_1 = (\Gamma/p)_{\Gamma,p\to 0}$ and Q_1 are limited and finite. That is, they are independent of Γ. But all these values K_1, V_s, Q_1, and $\Delta\bar{S}/R + \ln(\Gamma/p^0)$ depend on temperature. With an increase in Q_1, an increase in ln K_1 and in the absolute value of the entropy of adsorption also occur. The dependence of these values on Q_1 is often linear[15]. This question has been examined in theoretical studies by BARRER AND REES[69], BARKER AND EVERETT[48], and KISELEV AND POSHKUS[70].

For adsorption on graphitised thermal carbon black the theoretical slopes of these relations were calculated for the noble gases and n-alkanes[70].

The difference between the $\ln K_1$ values for two adsorption systems at the same temperature, T, and for adsorption of two components on the same adsorbent is in accordance with eqn. 16:

$$\Delta \ln K_1 = \Delta Q_1/RT + \Delta[\Delta\bar{S}/R + \ln(\Gamma/p^0)] \qquad (17)$$

The temperature at which the retention of both components becomes equal, *i.e.* when $\Delta \ln K_1$ becomes zero, and an inversion of the order of appearance of the peaks occurs, is described by the equation:

$$T_{\text{inversion}} = -\frac{\Delta Q_1}{\Delta[\Delta\bar{S} + R\ln(\Gamma/p^0)]} \qquad (18)$$

The difference in the heats of adsorption and the entropies of adsorption are related here to the temperature $T_{\text{inversion}}$. The intersection of the plots of $\ln K_1$ against $1/T$ for two components takes place in the range of temperatures usually encountered in gas chromatographic measurements, if the small differences in the heats of adsorption ΔQ_1 correspond to sufficiently large differences in the entropy of adsorption $\Delta[\Delta\bar{S}+R\ln(\Gamma/p^0)]$. The inversion temperature can be predicted both on the basis of experimental measurements of V_s as a function of T for the corresponding pair of components and by means of molecular-statistical calculations of $\ln K_1$ (or Q_1 and $\Delta\bar{S} + R\ln(\Gamma/p^0)$). Fig. 13 shows the results of such calculation for the adsorption of benzene and cyclohexane on graphitised thermal carbon black. This calculation suggests that the inversion of the order of appearance of the peaks should take place at fairly high temperatures. Unfortunately, however, this cannot be confirmed owing to the fact that experimental data obtained so far are not sufficiently precise.

SOME ANALYTICAL APPLICATIONS OF GAS CHROMATOGRAPHY USING NEW ADSORBENTS

Graphitised thermal and acetylene carbon black

The advantages of graphitised carbon black as a non-specific adsorbent with

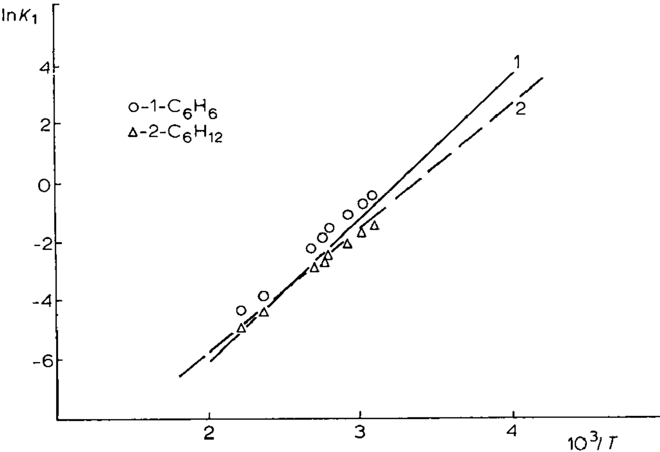


Fig. 13. Calculated (solid and dotted lines) and measured (points) dependence of $\ln K_1$ on $1/T$ for benzene (1) and cyclohexane (2) on graphitised thermal carbon black.

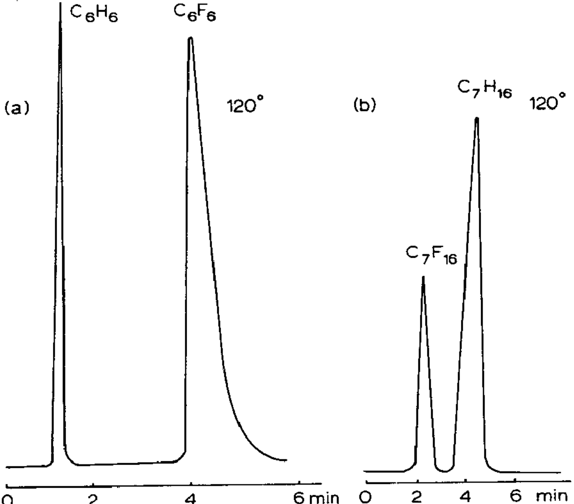


Fig. 14. Separation on graphitised thermal carbon black column: (a) benzene + perfluorobenzene; (b) perfluoroheptane + *n*-heptane. Experiments by I. A. MIGUNOVA and YA. I. YASHIN

the plane faces of the particles of graphite being formed by the basal planes are well known[14,15,56,65,67]. In this work, therefore, we shall only cite examples of new applications of graphitised thermal and acetylene carbon black.

Graphitised thermal carbon black, an adsorbent with a plane surface, is particularly effective in the separation of structural and geometrical isomers. Several examples of separations of isomers are considered by KOUZNETSOV AND SHCHERBAKOVA[67] in their paper for this symposium. The theory for the separation of isotopes has been examined by KISELEV AND POSHKUS[62,63].

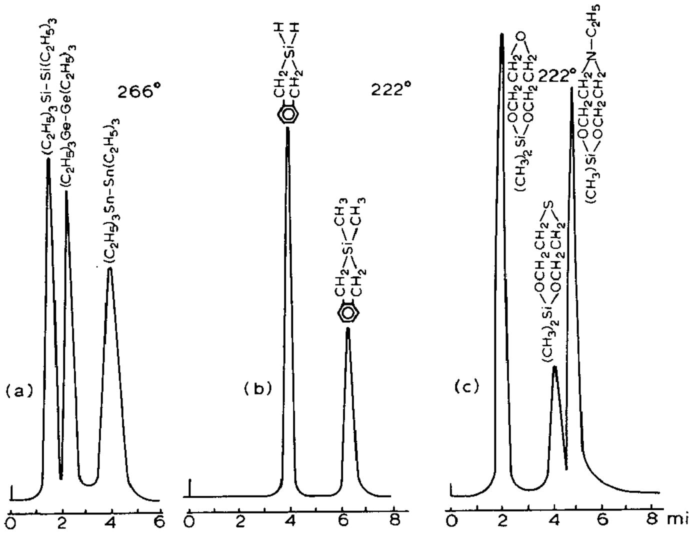


Fig. 15. Separation on graphitised thermal carbon black column: (a) bielemento-organic compounds containing silicon, germanium and tin; (b) organosilicon compounds; (c) heterocyclic compounds.

The separation on graphitised thermal carbon black of the usual and fluorine-saturated aromatic hydrocarbons is of interest. In the case of benzene the substitution of hydrogen by fluorine takes place in the plane of the aromatic ring and therefore does not lead to an increase in the distance between the carbon atoms of the molecule and the basal plane of the graphite. The polarisability of the fluorinated molecule is somewhat higher, and thus the energy of a non-specific interaction with graphitised thermal carbon black in the case of perfluorobenzene is greater. From Fig. 14a it can be seen that in accordance with this, perfluorobenzene leaves the column after benzene. In the case of the fluorination of methyl and methylene groups, their radius increases and the carbon atoms of fluorinated alkanes are at a greater distance from the basal plane of the graphite. Consequently, the energy of a non-specific interaction of fluorinated alkanes and cycloalkanes[71] with the basal plane of the graphite is smaller than that of the unfluorinated alkanes and cycloalkanes. From Fig. 14b it can be seen that *n*-perfluoroheptane leaves the column first, while the *n*-heptane remains longer on the column.

As a chemically inert non-specific adsorbent, graphitised thermal carbon black is effective in the separation of elemento-organic compounds. Fig. 15a shows the separation of bielemento-organic compounds of silicon, germanium, and tin; Fig. 15b shows the separation of organosilicon compounds of the type

$C_6H_4(CH_2)_2SiR_2$ (benzene ring fused with $-CH_2-SiR_2-CH_2-$) where $R = CH_3$ or H; while Fig. 15c shows the separation of heterocyclic organosilicon compounds of the type

$$(CH_3)_2Si\begin{matrix} \diagup OCH_2CH_2 \diagdown \\ \diagdown OCH_2CH_2 \diagup \end{matrix}X$$

where X is $>O$, $>S$, and $>NC_2H_5$ (ref.72). The efficiency of this separation on graphitised thermal carbon black exceeds that obtained in gas chromatographic columns filled with various non-polar and polar liquid phases.

Graphitised thermal carbon black is a fine powder. The granular particles obtained after shaking and passing it through a sieve are irregular in shape and have low mechanical strength. This has a detrimental effect on the hydrodynamic conditions inside the column and decreases its efficiency. KISELEV *et al.*[73] investigated the optimal conditions for reinforcing the carbon black particles by treatment with an adhesive polymer (Apiezon L, 0.01% by wt.). This treatment yielded mechanically strong particles without affecting the properties of graphitised thermal carbon black as a non-specific adsorbent having a homogeneous plane surface. We have named this new material Carbochrom. From Fig. 16 it can be seen that in the separation of C_5–C_{10} mixtures of *n*-alcohols the efficiency of the Carbochrom column is twice that of a column of the same length filled with untreated graphitised thermal carbon black.

It has been found that Carbochrom obtained from graphitised carbon black having a higher specific surface area is effective for the separation of more volatile mixtures. A suitable material for this purpose is acetylene black. During its preparation acetylene black is subjected to thermal treatment at 2500–3000° (ref. 74). Fig. 17a illustrate the dependence of the specific surface area of acetylene black on the amount of polyisobutylene and polyphenylmethylsiloxane used as adhesion modifying

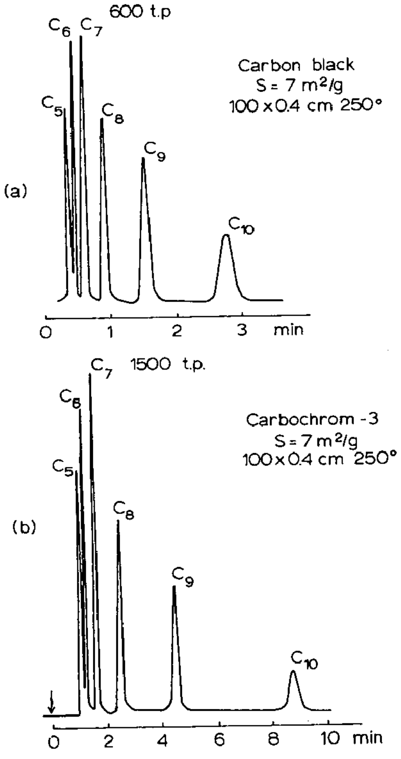


Fig. 16. Separation of C_5–C_{10} *n*-alcohols: (a) on graphitised thermal carbon black without modification with an adhesive material; (b) on Carbochrom (graphitised thermal carbon black + 0.01% by wt. Apiezon L).

agents[73]. As shown in Fig. 17b, the addition of polyisobutylene (0.5% by wt.) considerably increases the efficiency of the acetylene black column.

Silochroms

Silochroms are prepared from aerosilogels subjected to an additional treatment for the purpose of obtaining the desired specific surface area and pore structure. As shown earlier (Figs. 8 and 9), the hydroxylated and dehydroxylated surface of pure silica is only capable of molecular adsorption, even with rather strong organic bases. In Fig. 18, some examples are shown illustrating the separation of compounds of the B-group, *i.e.* those which can specifically interact with the hydroxyl groups of the silica surface[75]. The peaks obtained in chromatographic separations with columns containing pure macroporous silica are fairly symmetrical, and the temperature of the column need not be high. The relatively low energy of non-specific interactions makes its possible to utilise macroporous silochromes for the separation of high-boiling-point substances at relatively low temperatures. Data in Fig. 19 illustrate the separation, at 250°, of a mixture of phthalic acid esters on macroporous silochrome having a specific surface area of 80 m²/g.

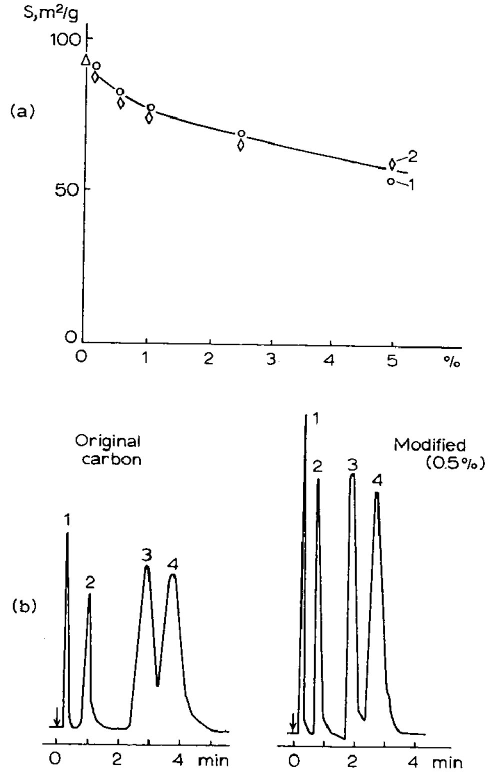


Fig. 17. (a) Dependence of the specific surface area, *s*, of acetylene black on the amount of adhesive material. 1 = Polyisobutylene; 2 = polyphenylmethylsiloxane. (b) Separation of aromatic hydrocarbons: 1 = benzene; 2 = toluene; 3 = cumene; and 4 = *p*-xylene; all on acetylene black and on acetylene black modified by the addition of 0.5% by wt. polyisobutylene.

Barium sulphate

Data in Fig. 6 and Table I show the high specificity of $BaSO_4$ as an adsorbent of the second type. Thus barium and magnesium sulphates are suitable for separating molecules of the B-group, which undergo weak specific interactions with adsorbents of the second type. The selection of adsorbents such as these enhances the weak specific interaction. Fig. 20 shows a chromatogram[21] for some aromatic hydrocarbons separated on barium sulphate. Even in this preliminary experiment with a low-efficiency column, there is complete separation of the *o*-, *m*-, and *p*- mixture of xylenes. This example shows that it is possible to control the adsorption properties, particularly the specificity of the adsorbents, using various inorganic crystalline substances (especially those having a laminar structure). The modification of such adsorbents can often be achieved by the ion-exchange method. In this respect compounds with an organic cation should be effective. TARAMASSO AND VENIALE[76] showed that the separation of aromatic hydrocarbons on a beidellite-type adsorbent treated with long-chain alkyl–ammonium complexes can be highly efficient. Apparently in this case we have an effective combination of the non-specific behaviour of the organic layer and the residual specific behaviour of the mineral itself.

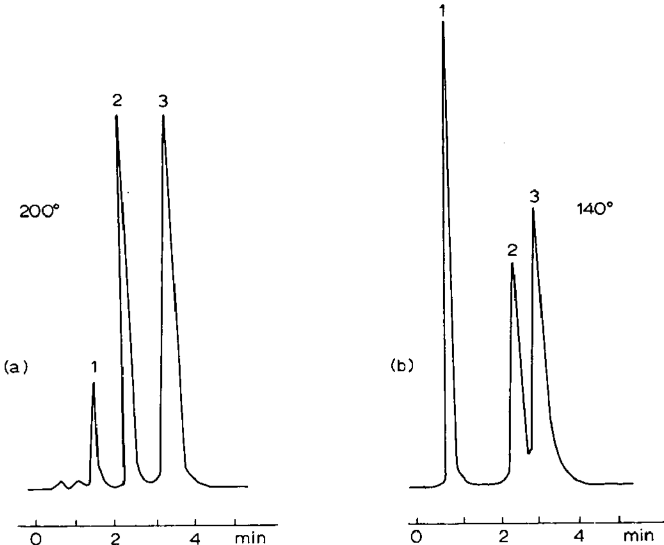


Fig. 18. Chromatograms obtained with Silochrom C-80. (a) Mixture of ketones: 1 = acetone; 2 = methyl ethyl ketone; 3 = diethyl ketone. Column temperature, 200°; sample size, 0.4 μl. (b) Mixture of esters: 1 = ethyl formate; 2 = ethyl acetate; 3 = methyl isobutyrate. Column temperature, 140°; sample size, 0.4 μl.

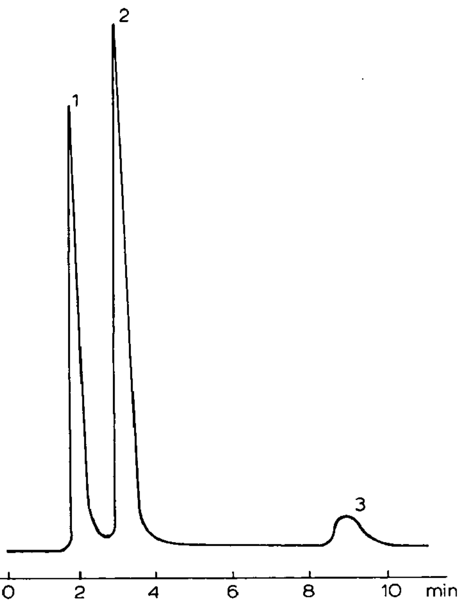


Fig. 19. Chromatograms obtained with Silochrom C-80 of a mixture of phthalic acid esters: 1 = dimethyl phthalate; 2 = diethyl phthalate; 3 = dibutyl phthalate. Column temperature, 270°; sample size, 0.4 μl.

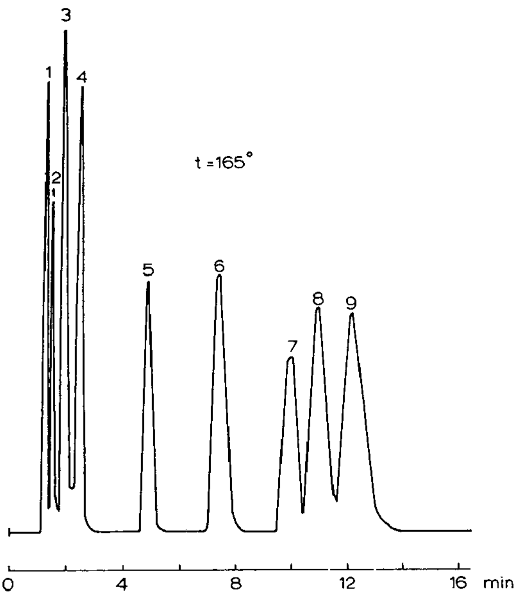


Fig. 20. Chromatograms for mixtures of aromatic hydrocarbons separated on $BaSO_4$. Column temperature, 165°; column length, 400 cm; column diameter, 0.6 cm; flame ionisation detector; carrier gas, nitrogen; carrier gas flow rate, 55 ml/min. 1 = Cyclohexane; 2 = cyclohexene; 3 = 1,3-cyclohexadiene; 4 = benzene; 5 = toluene; 6 = ethylbenzene; 7 = *p*-xylene; 8 = *m*-xylene; 9 = *o*-xylene.

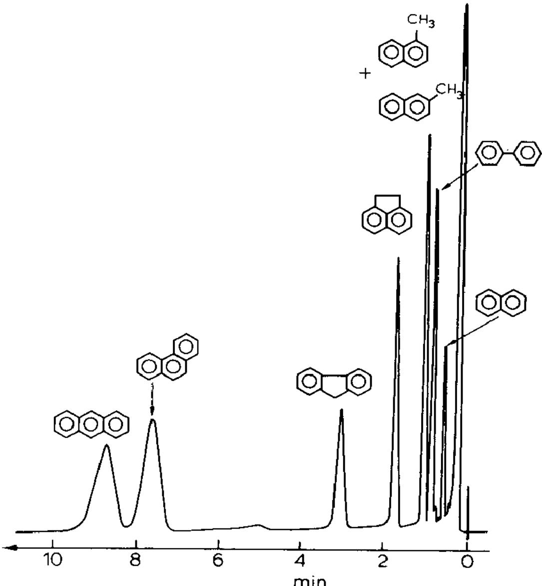


Fig. 21. Chromatogram for the separation of aromatic hydrocarbons on graphitised thermal carbon black containing 1% by wt. copper phthalocyanine. Column temperature, 265°; column length, 1 m; carrier gas, hydrogen; carrier gas flow rate, 28 cm/sec.

Phthalocyanine and metal phthalocyanines

The characteristic adsorption properties of phthalocyanine have been illustrated in Fig. 4. The possibility of introducing metallic complex-forming ions leading to the formation of different phthalocyanines widens the prospect of controlling the specific nature of these adsorbents. These properties have been exploited successfully[44] in excellent separations of complex mixtures. An example of such a separation is shown in Fig. 21.

Adsorbents coated with dense monolayers

The coating of adsorbent supports having a large surface area with dense monolayers of various substances provides a number of advantages[15,45,46,77]. These are:

(1) The shielding of the adsorbent support field with a dense monolayer of molecules or macromolecules (see Fig. 3) leads to a sharp decrease in the concentration of force centres on the coated surface as compared with the uncoated surface; this results in a considerable decrease in the adsorption energy.

(2) The detrimental effects due to heterogeneity of the adsorbent surface are completely or partially overcome.

(3) The degree of specificity of molecular interactions with the adsorbent surface can be controlled effectively by coating the adsorbent with monolayers of compounds containing such functional groups as $-COOH$, $-OH$, $-NH_2$, $-CN$, $>O$, $>CO$, $-C_6H_5$, etc.

(4) The monolayers are much less volatile than the corresponding volume phase, since the monolayers are located in the strong adsorption field of the adsorbent support.

Monolayers can be applied on the adsorbent support surface by adsorption from a solution (a particularly useful method when coating with non-volatile macromolecular polymers[15,45,46,77]) or by adsorption from the carrier gas directly on to the adsorbent in the chromatographic column. The latter method is very convenient with substances that undergo chemisorption from the gas phase on the adsorbent support[38,78a]. KISELEV AND YASHIN[78b] have examined the effect on the order of appearance of the peaks in a separation of C_1–C_4 alcohols on macroporous silica gel coated with various amounts of glycerine, sorbitol and triethanolamine. When the surface is almost completely covered with a dense monolayer, a change occurs in the relative retention volumes of this series of alcohols (see Fig. 11). The functional groups of glycerine, sorbitol, and triethanolamine become partially involved in a specific interaction with the HO-groups on the silanol surface, forming strong hydrogen bonds with them. Because of this, the strong specificity of the adsorbent is obstructed by the monolayer coating. Such modified adsorbents therefore show relatively weak adsorption of alcohols from the gas phase. As a result, the separation of alcohols can be carried out at a relatively low column temperature.

The specific nature of the monolayer surface becomes even less pronounced when the adsorbent support is coated with planar molecules which, like phthalocyanine (see Fig. 3), are located parallel to the surface of the adsorbent support. As a result, the adsorbent exhibits weak specificity. This makes it possible to separate the lower alcohols at yet lower temperatures. Fig. 22 shows a comparison of the chromatograms obtained for C_1–C_4 alcohols on macroporous silica gel with a specific

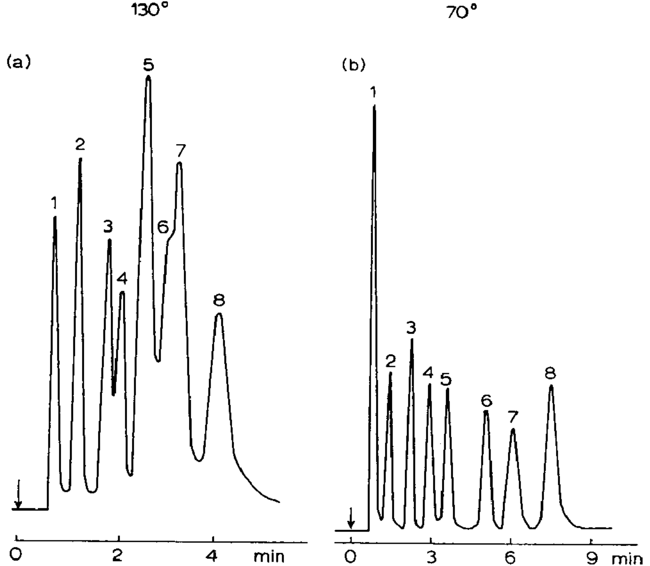


Fig. 22. Separation of C_1–C_4 alcohols on dense monolayers: (a) sorbitol on macroporous silica gel with specific surface area $s = 60\ m^2/g$; (b) 2,4-dinitrophenylhydrazone of methyl ethyl ketone on acetylene carbon black with specific surface area $s = 90\ m^2/g$.

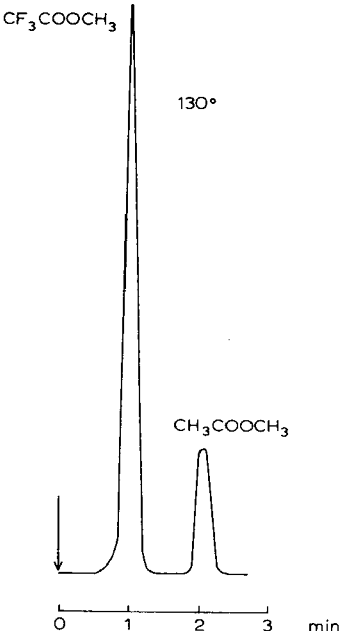


Fig. 23. Separation of the mixture $CH_3COOCH_3 + CF_3COOCH_3$ on Chromosorb-101.

surface area, $s = 60\ m^2/g$, coated with a dense monolayer of sorbitol and on an acetylene carbon black adsorbent ($s = 90\ m^2/g$) coated with a dense layer of the 2,4-dinitrophenylhydrazone derivative of methyl ethyl ketone. In the latter case all eight C_1–C_4 alcohols are well separated at a column temperature of 70°. The hydrazone monolayers on an acetylene carbon black surface do not affect the background of the flame ionisation detector at column temperatures up to 150°.

Porous polymers

Porous styrene–divinylbenzene copolymers such as the Chromosorbs-101 and -102 manufactured by the John Manville Co. and Polysorbs[79] are weakly specific adsorbents of the third type. Some of their adsorption properties with respect to the molecules of groups A, B, and D have been described by GVOZDOVICH *et al.* [80]. The use of porous polymers in preparative chromatography is discussed in the paper by ZELVENSKY AND SAKODYNSKY[81] at this symposium. Owing to their weak adsorption of water, porous polymers find wide applications in the analysis of various mixtures containing water[82,83]. In Fig. 23 an example is given illustrating the separation of the B-group molecules, CH_3COOCH_3 and CF_3COOCH_3, on Chromosorb-101. Owing to the large radius of the fluorine atom, the volume of the CF_3 group is considerably greater than that of the CH_3 group, hence the main non-specific dispersion interaction of the fluorinated compound with Chromosorb-101, in this case the adsorption of B-group molecules on an adsorbent of the third type, is weaker, and CF_3COOCH_3 is the first to leave the column. This case is analogous to the separation of a mixture of perfluoroalkane and *n*-alkane on a non-specific adsorbent (see Fig. 14b). A more detailed analysis of the chromatographic separation of fluorinated compounds is discussed in the paper by GVOZDOVICH AND YASHIN[84] presented at this symposium.

LIQUID-MOLECULAR ADSORPTION CHROMATOGRAPHY

Some characteristics of liquid molecular adsorption chromatography

In 1903 TSWETT discovered chromatography as a liquid adsorption process[85]. One reason for the slow development of liquid chromatography, apart from the fact that TSWETT's discovery at first received little attention, was the absence of sensitive and versatile detectors. In recent years liquid chromatography has become a subject of intense research. This interest is related to the development of new detection methods and to the fact that the shortcomings of gas chromatography had in the meantime become apparent. In its usual form (with a low pressure of weakly adsorbing carrier gas) gas chromatography can only be used in the separation and analysis of relatively volatile and thermally stable compounds. Highly compressed and strongly adsorbing carrier gases can be used to decrease the Henry constants in the case of heavy molecules[86,87]. Actually this type of high pressure gas and fluid chromatography is similar to liquid chromatography, since the molecules of the carrier are concentrated and actively compete with the molecule of the components to be separated for the sites on the adsorbent surface.

In the case of liquid chromatography the separation takes place in the liquid layer immediately adjacent to the surface of the solid adsorbent. The separation proceeds at lower pressures and temperatures (usually at room temperature). Sepa-

ration in liquid molecular adsorption chromatography can be controlled by regulating not only the chemical nature and geometrical structure of the adsorbent surface but also the nature of the adsorbable mobile phase (eluant).

Depending on the diffusion rate of the components in solution and the pore size of the adsorbent, liquid chromatography can approach the conditions of equilibrium adsorption chromatography or molecular sieve chromatography. In this part of the paper we shall consider the first case, where the question of establishing a relationship between liquid adsorption chromatography and the theory of adsorption from solutions[88–90] is of special significance. Unfortunately, however, this question has received very little attention as yet.

Adsorption isotherms from solution, equilibrium constants, and distribution function

Liquid molecular adsorption chromatography, in the case where the system approaches equilibrium, is based on the difference in the equilibrium constants for the solution–adsorbent system. Even where complete separation of the components of a mixture takes place, the elution peak of a given component contains at least two substances: the component from the mixture being analysed and the solvent. A characteristic of adsorption from solution is the mutual displacement of the molecules of the components of the solution on the adsorbent surface. That is, the adsorption of some molecules is inevitably accompanied by the desorption of others. The heat of adsorption of a given component from a liquid solution is determined by the difference in the energy of interaction of its molecules with the molecular fields of the adsorbent and the solution. Thus it is several times lower than the heat of adsorption of the same component from the gas phase. This makes it possible, firstly, to carry out liquid adsorption chromatography at significantly lower temperatures and, secondly, to utilise the changes in the molecular field both of the adsorbent and the eluant. In the case of mixed solvents being used during the elution of a given component of a mixture, adsorption takes place from at least a three-component system: a given component of the mixture being separated and the eluant which consists of the main

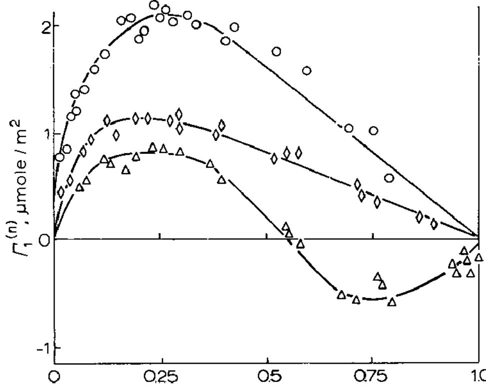


Fig. 24. Gibbs adsorption isotherm $\Gamma_1^{(n)}$ of toluene from solution in n-heptane on: ○ = hydroxylated silica gel; ◊ = oxidised carbon black; △ = non-oxidised carbon black. Mole fractions of the equilibrium bulk solution are on the abscissa.

solvent and a substance added to it. The increase in the concentration of this substance remains constant or is programmed.

The effect of the chemical nature of the adsorbent surface and its modifications in liquid adsorption chromatography is in general similar to that in gas chromatography. However, the competing interaction with the molecules of the solvent should be taken into account here. Fig. 24 shows the effect of decreasing the specificity of the adsorbent on the Gibbs adsorption isotherm $\Gamma_1^{(n)}$ from binary solutions of toluene (component 1) + *n*-heptane[91]. On going over to a non-specific adsorbent of the first type, graphitised carbon black, the magnitude of the Gibbs adsorption of the B-group molecules (toluene) from *n*-alkane solutions (molecules of the A group) sharply decreases. In this case, at sufficiently high concentrations of toluene, its adsorption changes sign. It becomes negative (while the adsorption of *n*-heptane becomes positive), and the isotherm passes through the azeotropic point.

TABLE II

EQUILIBRIUM CONSTANTS FOR A BINARY SOLUTION–ADSORBENT SYSTEM AT ROOM TEMPERATURE

Mixture	*Adsorbent*	*Equilibrium constant*
Benzene + *n*-hexane	Hydroxylated silica	9.1
Toluene + *n*-heptane	Hydroxylated silica	10.4
Benzene + *n*-hexane	Dehydroxylated silica	2.6
Toluene + *n*-heptane	Oxidised carbon black	3.0
Toluene + *n*-heptane	Non-oxidised carbon black	1.5

Table II gives a summary of the values for the equilibrium constants for the adsorption of an aromatic hydrocarbon from a solution in a saturated hydrocarbon on the surfaces of adsorbents in the order of their decreasing specificity[91,92]. These values were calculated with the assumption that there is monomolecular adsorption, constant orientation of the molecules of the solution components near the surface, and equal areas occupied by these molecules on the adsorbent surface[89,91]. With an increase in the degree of dehydroxylation of the silica surface or in the graphitisation of the carbon black, *i.e.* as one goes from specific adsorbents to non-specific, the equilibrium adsorption constant decreases. With the decrease in the equilibrium constant to 1.5 (in the case of adsorption on graphitised carbon black), the adsorption isotherm passes through the azeotropic point.

Fig. 25 shows the effect of the structure of aromatic compounds (benzene, naphthalene, biphenyl, phenanthrene) on the adsorption from solutions in non-specifically adsorbing solvents (*n*-alkanes) on the hydroxylated surface of silica gel. Here the adsorption is expressed in mole fractions of the surface solution $x_1^{(s)}$ calculated from experimental Gibbs adsorption values, $\Gamma_1^{(n)}$. In this case account is taken of the effect of the mutual displacement of the molecules of the aromatic compound and the solvent molecules. This effect is reflected in the ratio of the area occupied by the aromatic molecules to that occupied by the solvent molecules on the adsorbent surface[91].

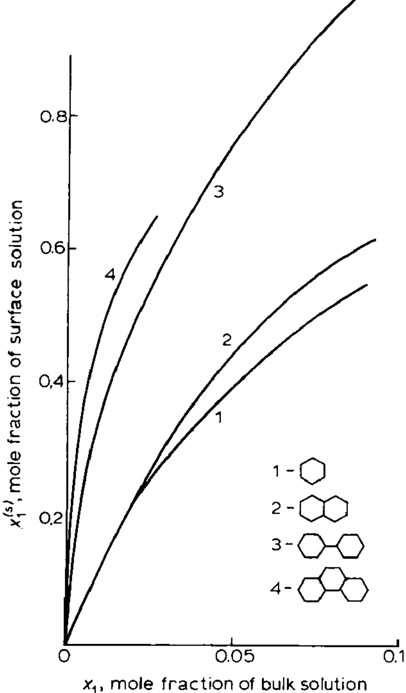


Fig. 25. Adsorption isotherms for benzene, naphthalene, biphenyl and phenanthrene from *n*-alkane solutions on silica gel with a hydroxylated surface. The adsorption values are given in mole fractions of the aromatic hydrocarbons in the surface solution, $x_1^{(s)}$.

In this case, the values for the activity in the bulk solutions are unknown, and the areas occupied by the component molecules are considerably different. Thus the equilibrium constants could not be calculated. But the order of these values in the case of strong specific adsorption of one of the components of the solution (aromatic hydrocarbon) could be expressed by means of the distribution function $f = \frac{x_1^{(s)}}{x_2^{(s)}} \Big/ \frac{x_1}{x_2}$ when $x_1^{(s)} = x_2^{(s)} = 0.5$. Here, $x_1^{(s)}$, $x_2^{(s)}$ and x_1, x_2 are the mole fractions of the components of the surface and bulk binary solutions, respectively. Table III summarises the values of f at $x_1^{(s)} = 0.5$ for the adsorption of a series of aromatic hydrocarbons from solutions in saturated hydrocarbons on a hydroxylated silica surface[92]. The successive order of the f values for polynuclear aromatic hydrocarbons from solutions in saturated hydrocarbons on specific adsorbents coincides with that of the retention times for polynuclear aromatic hydrocarbons in the case of the liquid adsorption chromatography (experiments of FROLOV AND YASHIN[100]) and in the case of fluid gas chromatography at high pressures and with a strongly adsorbable carrier gas[86].

It will be necessary to establish in future work a clear relationship between the equilibrium constants for adsorption from solutions or at least the equilibrium values

TABLE III

DISTRIBUTION COEFFICIENT f IN THE ADSORPTION OF AROMATIC HYDROCARBONS ON A HYDROXYLATED SILICA SURFACE FROM SATURATED SURFACE SOLUTIONS

Mole fraction $x_1^{(s)} = 0.5$; at room temperature.

Aromatic hydrocarbons	*Distribution coefficient f*
Benzene	12
Naphthalene	15
Biphenyl	47
Phenanthrene	90

of the distribution coefficients, f, examined and the corresponding retention characteristics in liquid adsorption chromatography.

The adsorption from tertiary solutions is much more complex. Owing to mutual displacement of different molecules of the component in the surface solution, at least one of the components is negatively adsorbed, as in the case of binary systems. The magnitude of the adsorption can be determined by analysing the composition of the equilibrium solutions by means of gas chromatography[93] or directly by liquid chromatographic analysis on adsorption columns.

The adsorption isotherms in this case are not in the form of curves, but of curved surfaces. They can be conveniently expressed by projection by means of the Gibbs triangle. Isotherms thus obtained for adsorption of dioxane, benzene, and n-hexane from tertiary solutions of these components[93] are shown in Fig. 26. The strong

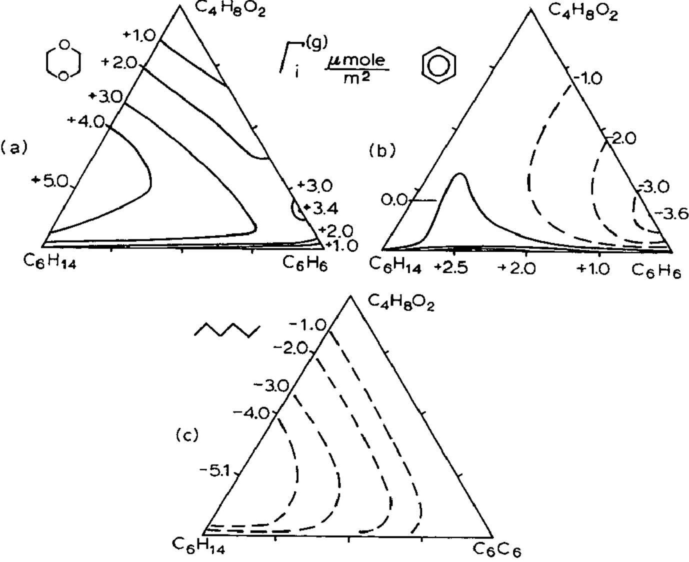


Fig. 26. Gibbs adsorption isotherms (micromoles/m²) of: a = dioxane; b = benzene; c = n-hexane on hydroxylated silica gel from their mixtures. The solid lines are projections of the positive surface adsorption; dotted lines are the projections of the negative surface adsorption.

specific interaction of dioxane molecules with the silanol groups on the surface makes the adsorption of dioxane positive throughout the whole range of concentrations. On the other hand, the adsorption of *n*-hexane is negative throughout. The adsorption of benzene whose molecules can only undergo weak specific interactions with the silanol groups on the adsorbent surface has different signs at different concentrations. The adsorption of benzene is positive at low concentrations of dioxane and negative at high concentrations of dioxane. This example shows the extent to which one can vary the adsorption of the component by changing the nature and concentration of the solvent mixture.

SNYDER[94] has made a comparison of the results obtained from liquid and gas adsorption chromatographic analyses of the same mixtures. In liquid chromatography, as in gas chromatography, the retention volumes on non-specific and weakly specific adsorbents (in particular on various types of carbon black) are practically independent of the nature of the functional groups, but are determined by the configuration and the electron polarisability of the molecules of the components. In separations with a specific adsorbent such as aluminum oxide, the retention volumes in liquid adsorption chromatography sharply increase when the molecules of the components contain polar groups or π-bonds[95] in the case where the solvent belongs to the A-group, *i.e.* where the solvent cannot undergo specific interaction with the adsorbent.

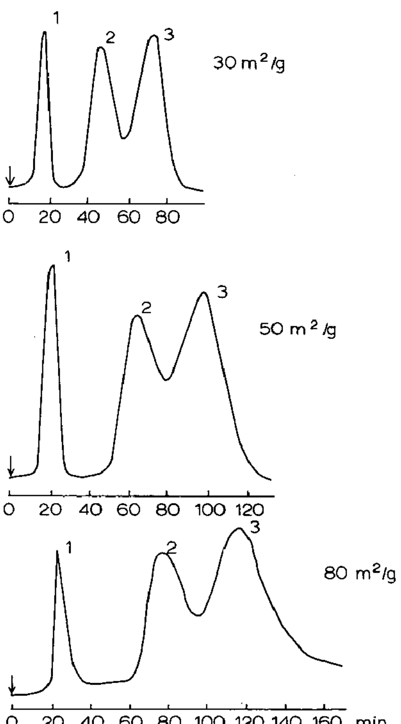


Fig. 27. Chromatogram obtained on aerosilogels of different geometrical structures for the mixture of isomers: 1 = *o*-dihydroxybenzene; 2 = *m*-dihydroxybenzene; 3 = *p*-dihydroxybenzene. Column length, 15 cm; column diameter, 0.6 cm; column temperature 18°; mobile CCl_4 phase flow rate, 0.5 ml/min; specific surface area (from top to bottom): 30, 50, 80 m^2/g.

The effect of the nature and porosity of the adsorbent on liquid chromatography of molecules

SNYDER[96] has studied the effect of various experimental parameters on the efficiency and selectivity of the separation in liquid chromatography. These are sample size, the nature of the adsorbent, the dimensions of the adsorbent particles, the nature of the mobile solvent and the rate of its flow, and the column diameter. The effect of the adsorbent porosity on the performance of a chromatographic column is of great interest; an increase in pore size, corresponding to a decrease in the specific surface area from 830 to 340 m^2/g increases the efficiency (the number of theoretical plates) five times[97]. By selecting the optimal pore size and controlling the porosity relative to the grain depth (*e.g.* by using surface-porous adsorbents such as were originally developed by ZHDANOV *et al.*[98]), one can considerably raise the efficiency of liquid chromatographic columns and carry out separations in shorter periods of time[99].

Data in Fig. 27 illustrate the effect of pore size of aerosilogels in the chromatographic separation of dihydroxybenzene isomers[100]. With the decrease in the surface area of the adsorbent and the increase in the average diameter of the pores, the retention times and peak width decrease. The best results are obtained on columns with a more macroporous aerosilogel.

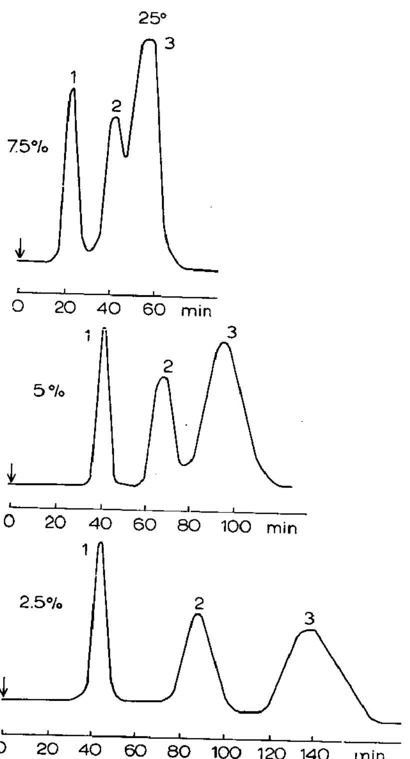


Fig. 28. Chromatograms obtained on aluminium oxide at various concentrations of ethanol (the amount of ethanol is indicated on each chromatogram) in benzene as the main solvent. 1 = *o*-nitrophenol; 2 = *m*-nitrophenol; 3 = *p*-nitrophenol. Column length, 50 cm; column diameter, 0.8 cm; column temperature, 25°; flow rate, 0.44 ml/min.

The effect of changes in the nature of the eluant and in temperature

The effect of changes in the nature of the solvent on the retention volumes and the separation selectivity in liquid adsorption chromatography is illustrated in Fig. 28 (ref. 100). The separation of nitrophenol isomers can be improved upon the addition of ethanol to benzene used as the main solvent. However, at higher concentrations of ethanol, separation becomes less efficient owing to a big decrease in the adsorption of nitrophenol. This makes it possible to control the adsorption of nitrophenol isomers and consequently the retention time. In applying the method of gradient elution, ALM *et al.*[101] and SNYDER[102] increased the concentration of the strongly adsorbing additive in the main solvent as a function of time.

An increase in the column temperature in liquid adsorption chromatography leads to more diverse results than in gas chromatography[103]. This is apparently due to the fact that in adsorption from multi-component solutions the competition for the most favourable sites on the adsorbent surface results in different dependences of the adsorption of the various components on the temperature. From Fig. 29, it can be seen[100] that with an increase in temperature, the retention volumes of *o*- and *p*-nitrophenol on an aluminium oxide column, with benzene as the main solvent containing various amounts of ethanol, do not decrease but even increase slightly. With the increase in temperature the relative role of the specific interactions, in particular the formation of hydrogen bonds, of ethanol with the adsorbent decreases. The retention volumes when large molecules are being separated depend to a lesser degree on temperature. In this case the main contribution to the energy of adsorption is due

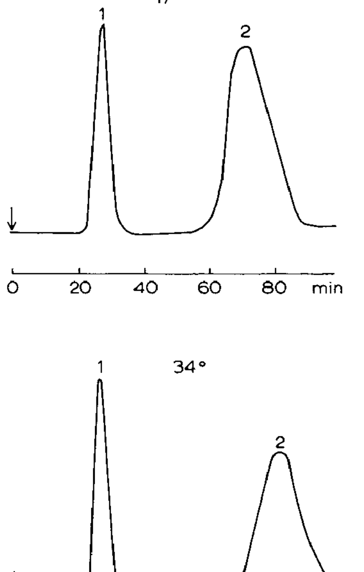


Fig. 29. Chromatograms obtained on an aluminium oxide column: 1 = *o*-nitrophenol; 2 = *p*-nitrophenol. Column temperature indicated on the chromatogram; column length, 50 cm; column diameter, 0.8 cm; mobile phase, 97.5% benzene + 2.5% ethanol; flow rate, 0.44 ml/min.

to dispersion interaction. Apparently at higher temperatures large molecules displace a fraction of the ethanol molecules from the more active sites and become themselves adsorbed more strongly than the ethanol molecules.

Gas chromatography versus liquid chromatography as applied to the separation of the same mixture

In many cases the time for analysis by liquid chromatography may approach that for gas chromatography[104,105]. This is illustrated in Fig. 30, where the data obtained for the same mixture of *o*-, *m*-, and *p*-nitrophenol separated by gas and liquid chromatographic methods[100] are shown. The separation times by these methods

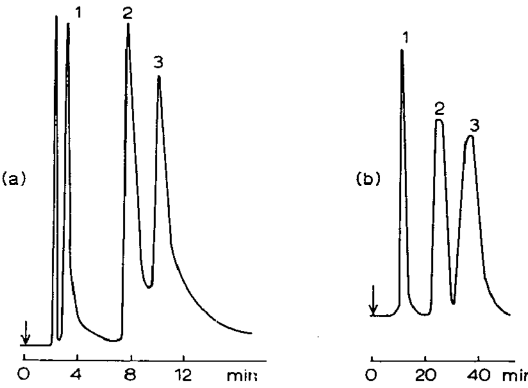


Fig. 30. Chromatograms of the mixture: 1 = *o*-nitrophenol; 2 = *m*-nitrophenol; 3 = *p*-nitrophenol. (a) Obtained by gas chromatography on a column (100 × 0.3 cm) with Chromosorb W + 3% OV-17 at 150°; (b) obtained by liquid chromatography on a column (50 × 0.8 cm) with aluminium oxide at 25°; mobile phase, 95% by wt. benzene + 5% by wt. ethanol; flow rate, 0.44 ml/min.

are comparable, though the liquid chromatographic separation is more complete. From data in Fig. 31 it can be seen that the mixture of diaminodiphenylmethane isomers is completely separated on the liquid adsorption column, whereas on the gas–liquid column the mixture of 4,4'- and 3,3'-diaminodiphenylmethanes is not separated at all[100].

The examples cited show the importance of liquid molecular adsorption chromatography for analytical and preparative use. For the development of this method it is necessary to have more versatile and sensitive detectors and, what is especially important, new and homogeneous adsorbents with controlled geometric and chemical surface structure. It is important to develop further the theory of liquid chromatography on the basis of the adsorption theory for binary and tertiary systems as well as for solutions of a more complex nature under both static and dynamic conditions.

LIQUID MOLECULAR SIEVE CHROMATOGRAPHY BASED ON MACROPOROUS SILICA GEL AND AEROSILOGEL

Separation of synthetic polymers

The molecular sieve effect can be used in liquid molecular adsorption chromato-

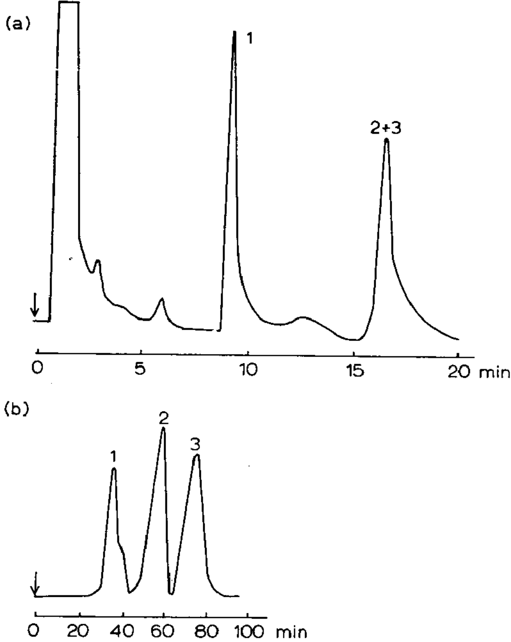


Fig. 31. Chromatograms of (1) 2,2'-diaminodiphenylmethane; (2) 4,4'-diaminodiphenylmethane; (3) 3,3'-diaminodiphenylmethane. (a) Obtained by gas chromatography on a column (100 × 0.3 cm) with Chromosorb W + 3% by wt. OV-17 at 200°; (b) obtained by liquid chromatography on a column (15 × 0.6 cm) of aluminium oxide; mobile phase, 99% by wt. CCl_4 + 1% by wt. C_2H_5OH; column temperature: 18°; flow rate, 0.44 ml/min.

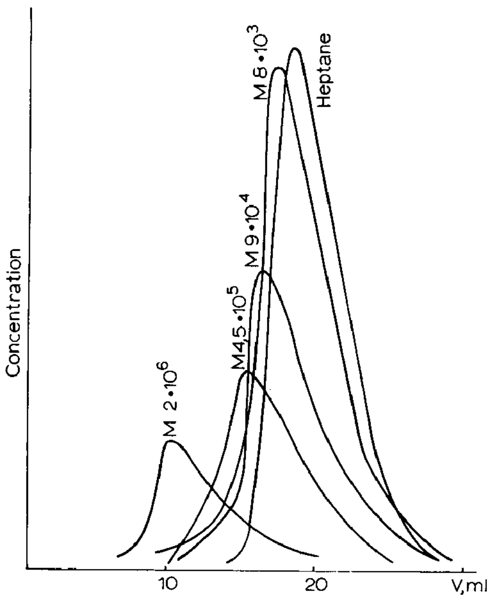


Fig. 32. Chromatograms for toluene solutions of polystyrene on: Silocrom with d ≈ 1500 Å; column size, 40 × 0.6 cm; flow rate, 0.5 ml/min.

graphy as in gas adsorption chromatography. However, the slow diffusion rate prevents separation under equilibrium conditions with respect to the adsorption process. Thus molecular sieve separation, under conditions of liquid chromatography, finds its application not so much on the basis of the differences in adsorption as on the basis of the differences in diffusion rate through the pores of the adsorbent. The ordinary molecular sieve effect is the limiting case of this phenomenon, that is the molecules of a given component of the mixture cannot pass through the pores of the

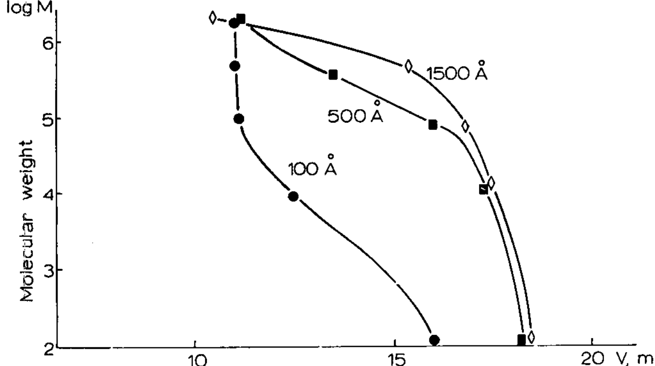


Fig. 33. Dependence of retention volumes for polystyrene as a function of molecular weight obtained on silica gels with effective pore diameters 1500, 500, and 100 Å. Eluant, toluene.

sieve. This effect has found important applications in liquid chromatography of polymers and microbiological substances on macromolecular sieves.

In their usual form macromolecular sieves are cross-linked polymers (*e.g.* Sephadex). Good results have been obtained with cross-linked copolymers of vinyl acetate in separating oligophenylenes and polystyrene into fractions of different molecular weights[106]. However, organic polymers in liquid media often swell to

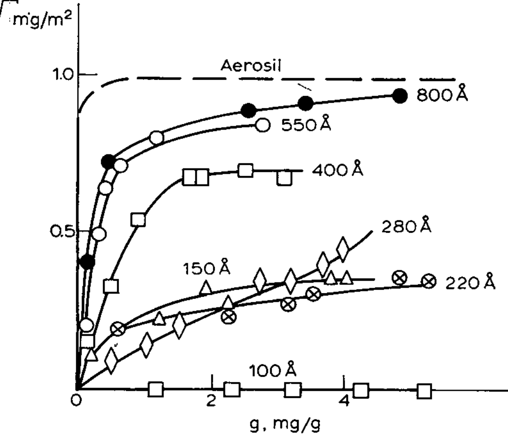


Fig. 34. Adsorption isotherms for polystyrene (mol. wt. ≈ 300 000) from CCl_4 solutions on aerosil (dotted line) and macroporous silica gel with different effective pore diameters as indicated.

various degrees, depending on the solvent system. Therefore in addition to this type of sieve, the macromolecular sieves that are rigid and do not swell are of great interest. Such sieves should possess the maximum possible homogeneous distribution of pores according to their size. They should have a sufficiently large total volume of pores and a definite chemical composition of the surface and should readily undergo regeneration. These problems are now being solved on the basis of macroporous glass[107–109], macroporous silica gels[110,111] and macroporous aerosilogels (Silocroms)[34]. Some results obtained by KISELEV *et al.*[112] are given below.

In Fig. 32, chromatograms are shown of the separation of polystyrene solutions in toluene on an aerosilogel with an effective pore diameter d ≈ 1500 Å. The highest molecular weight fraction of the polymer passes through a column without hindrance. Macromolecules with the lowest molecular weight diffuse into the pores and return to the eluant flow somewhat later. The corresponding dependence of the retention volumes as a function of the molecular weights of the polystyrenes separated on silica gels having pore sizes of approx. 1500, 500, and 100 Å is shown in Fig. 33. In the case of silica gel having the large pore size (d ≈ 1500 Å), the best results were obtained for polystyrene having molecular weights in the range of one million to several hundred thousands. Silica gel with a d ≈ 500 Å gives the best fractionation for samples with molecular weights in the range of several hundred thousands to tens

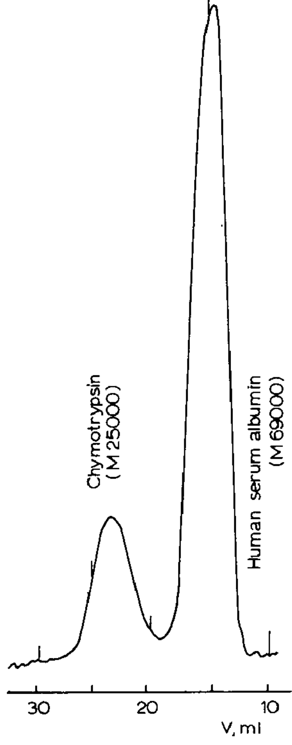


Fig. 35. Chromatograms for human serum albumin (mol. wt. = 69 000) and chymotrypsin (mol. wt. = 25 000) in 0.1 *M* acetic acid on silica gel with d ≈ 280 Å. Column size, 45 × 0.8 cm; flow rate, ≈ 0.5 ml/min.

of thousands. The adsorbent with the small pore size (d ≈ 100 Å) effectively separates only relatively low molecular weight fractions of polystyrene.

However, the residence time of the polystyrene macromolecules in the macropores of the silica gel is related not only to the direct and reverse diffusion of the polystyrene molecules inside the pores but also to their adsorption. In Fig. 34, adsorption isotherms are shown for polystyrene, with a molecular weight ≈ 300,000 in CCl_4 solution, on non-porous aerosil and on a number of macroporous silica gels with gradually decreasing pore size (from 800 to 110 Å), as calculated per unit surface area of these adsorbents[113]. The values of the limiting adsorption of polystyrene on the surface of macroporous silica gels with effective pore size ranging from 500 to 1000 Å practically coincide with each other and are close to the corresponding values for the adsorption on non-porous aerosil (0.9 mg/m^2). This value corresponds approximately to the dense monolayer of polystyrene macromolecules spread over the adsorbent surface. A molecular sieve effect is only apparent with a pore size up to ≈ 200 Å. However, under the dynamic conditions of liquid chromatography, one should take into account the slowness of the diffusion and adsorption processes. The time required for attaining equilibrium in the cases shown in Fig. 34 was 1 h for the aerosil adsorbent, 2 h for macroporous silica gel having pore size of *ca.* 800 Å; no equilibrium was attained after several days for silica gel having a pore size of *ca.* 280 Å. The usual time required for carrying out a separation on a liquid chromatographic column is about 1 h. Thus the adsorption equilibrium for the above-mentioned silica gels will

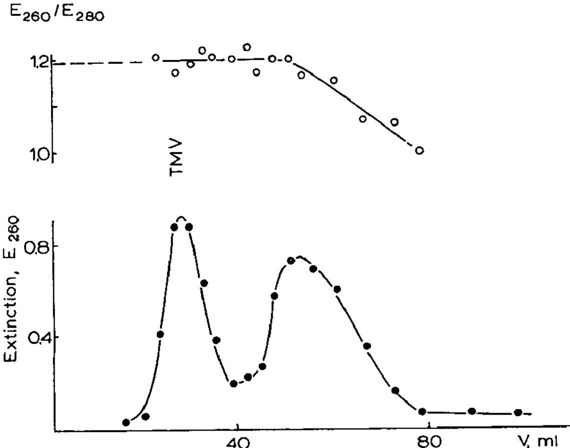


Fig. 36. Separation of tobacco mosaic virus on Silochrom with d ≈ 1500 Å in 0.01 *M* phosphate buffer solution, pH = 7.7. Column size: 60 × 1.0 cm; flow rate: ≈ 0.5 ml/min.

not be achieved. Nevertheless, the adsorption of polystyrene in the macropores does take place to a certain extent. Thus in the case of molecular sieve liquid chromatography, the retention of polystyrene in the column is due entirely to the molecular sieve effect (macromolecules of the largest size cannot enter the pore at all) and to parallel processes of diffusion and adsorption occurring on the most accessible parts of the surface.

Separation of proteins and purification of viruses

Data in Fig. 35 illustrate the separation of proteins on a macroporous silica gel. The pore size of the silica gel is *ca.* 280 Å. Serum albumin (M = 69,000) passes through the column without hindrance, as albumin hardly enters the pores of the silica gel. Chymotrypsin (M = 2500) enters the pores and is thus delayed in the column. It should be noted that in this case the adsorption of the macromolecules takes place both on the grain surface and in the macropores; this is indicated by the irreversible adsorption of the first parts of the protein sample and by the gradual decrease in the working macropore volume.

A pure molecular-sieve effect is of special importance in the fractionation of biopolymers of higher molecular weight such as viruses. Fig. 36 shows the molecular sieve chromatogram of a tobacco mosaic virus extract fractionated on macroporous silica gel with d $\approx$ 500 Å. The particles of the virus (3000 $\times$ 500 Å) do not pass into the pores and leave the column without retention. The ratio of optical densities $E_{260}/E_{280} = 1.2$ shown in the figure is characteristic of this virus. The proteins and pigments contained in the virus sample do enter the pores or become adsorbed.

Thus macroporous silica can be used in liquid molecular sieve chromatography in the fractionation of polymers and other high molecular weight compounds.

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CHROM. 4617

SOME ASPECTS OF THIN-LAYER CHROMATOGRAPHY AND OF ITS APPLICATION IN PHARMACOGNOSY

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SUMMARY

Some applications of TLC in the pharmaceutical research laboratory are given. Separations of steroids from *Solanum paniculatum*, metabolites of some nitroimidazole derivatives and some pharmacognostic investigations are discussed in detail.

INTRODUCTION

I am sure that one of the reasons why thin-layer chromatography (TLC) has gained prompt and widespread acceptance in many laboratories is its remarkable adaptability to the modifications and technical refinements that each laboratory or individual investigator is often forced to make in order to solve certain immediate problems.

From the investigations carried out in our research institute, concerning the metabolism of drugs and their pharmacognosy, I would like to give a few examples where TLC was particularly succesful.

DRUG METABOLISM

In chromatographic studies of synthetic and metabolic derivatives of amino-spirostanes, isolated in the *iso-* form from the roots of *Solanum paniculatum* (Fig. 1), we were able to confirm certain results already partly known from the literature. We found that the introduction of a double bond in the 5- position of a steroid group decreases the polarity of this group and that the 6β- and 7β-hydroxyl derivatives are less polar than the 6α- and 7α-hydroxyl derivatives, respectively. Also, the 3β-azide compounds are less polar than their 3α-azide counterparts (Fig. 2). Although our attempts to separate the *iso-* forms (25α or 25R) of these compounds from the neo-forms (25β or 25S) were unsuccessful, comparative studies of other *iso-* and neo-steroid forms showed that whenever separation is possible, the *iso-* are less polar than the neo- forms. These observations represent a useful criterion for the discrimination of various isomers; they are confirmed in the literature.

Investigations on nitroimidazole derivatives endowed with antiprotozoan properties, and more particularly trichomonacidal activity, have shown that whereas microanalytical results were consistently in agreement with theoretical values, the

1) R=H R'=H
2) R=OH R'=H
3) R=H R'=OH

(25R)-3β-Amino-5α,22α,O-spirostan and 6α-/9α-hydroxyderivatives from *Solanum Paniculatum* roots.

Fig. 1. General structural formula for aminospirostanes isolated from *Solanum paniculatum* roots.

microbiological activity varied from one preparation to another. These products are obtained by reacting the sodium salt of nitroimidazole with ethyl morpholine chloride (see Fig. 3). As a result of TLC, we were able to separate two isomers whose polarity, in a neutral medium, was inverted when basic eluents were subsequently used. Evidently the two compounds had different basicities, and the more strongly basic of

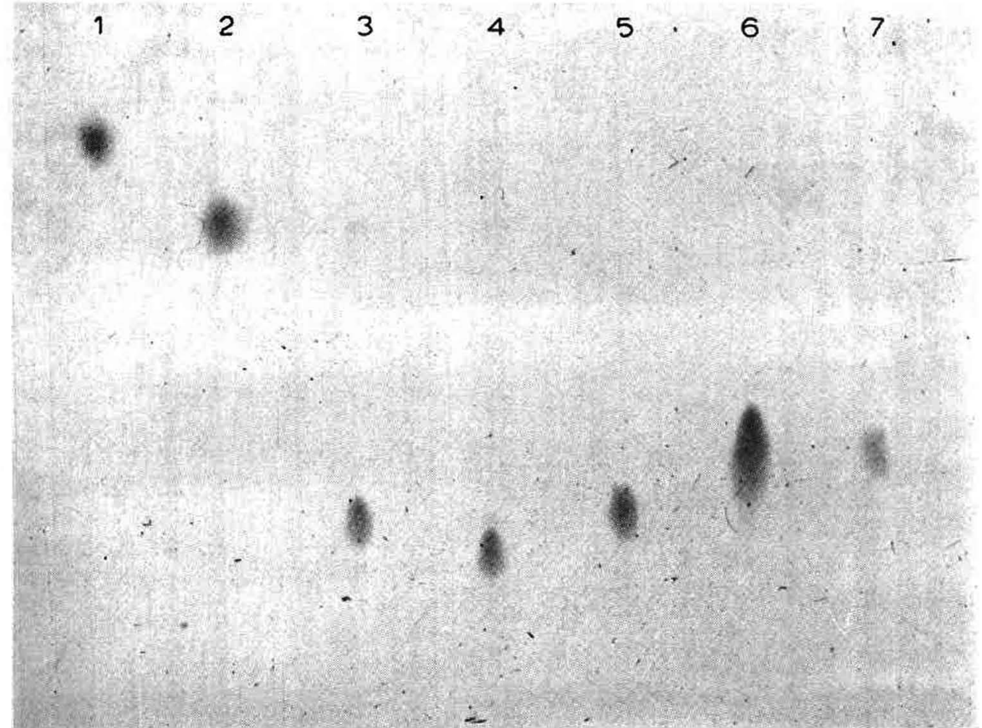


Fig. 2. Thin-layer chromatogram of isomeric steroids. Adsorbent: silica gel. Solvent system: chloroform–methanol–conc. ammonia (100:15:1.5). Detection: vanillin–sulphuric acid reagent. 1 = 3β-amino, Δ^5; 2 = 3β-amino; 3 = 3α-amino, 6α-ol; 4 = 3β-amino, 6α-ol; 5 = 3β-amino, 6β-ol; 6 = 3β-amino, 7β-ol; 7 = 3β-amino, 7α-ol.

Fig. 3. Structural formulae for the 4- and 5-nitro isomers of imidazole-N(β-ethylmorpholine).

the two migrated less in a neutral medium but faster in a basic medium. The following reactions were carried out on the plate itself: reduction with titanium trichloride followed by diazotization with amyl nitrite and coupling with naphthylethylene-

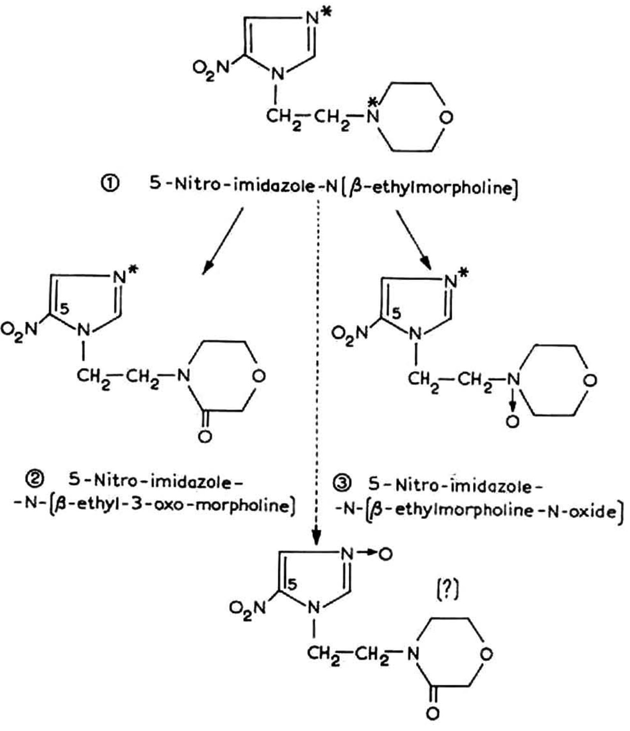


Fig. 4. Structural formulae for the metabolic derivatives of 5-nitro-imidazole-N(β-ethylmorpholine) found in humans.

diamine. This showed that both substances contained a nitro group. Even before we proceeded to separate the mixture on a column and carry out spectrometric analyses, we guessed that we were dealing with 4- and 5-nitro-imidazole-N (β-ethylmorpholine), respectively, and that the isomer with the lower R_F in the basic eluent was the 4-nitro compound, its characteristics being more acidic as a result of the greater electron-withdrawing power of the 4-nitro group.

Following oral administration to humans of 5-nitro-imidazole-N(β-ethylmorpholine), three metabolites were recovered in the urine (Fig. 4); these, in a basic TLC system, were distributed on the plate according to their basicity. (See Fig. 5.)

Isolation and determination of structures 2- and 3-, as shown in Fig. 4, provided confirmation of the polarity/acidity scale, and also were in accordance with the literature data that the human metabolism of nitroimidazole derivatives is prevalently oxidative. The 4th structure has not been definitely determined as yet because of the

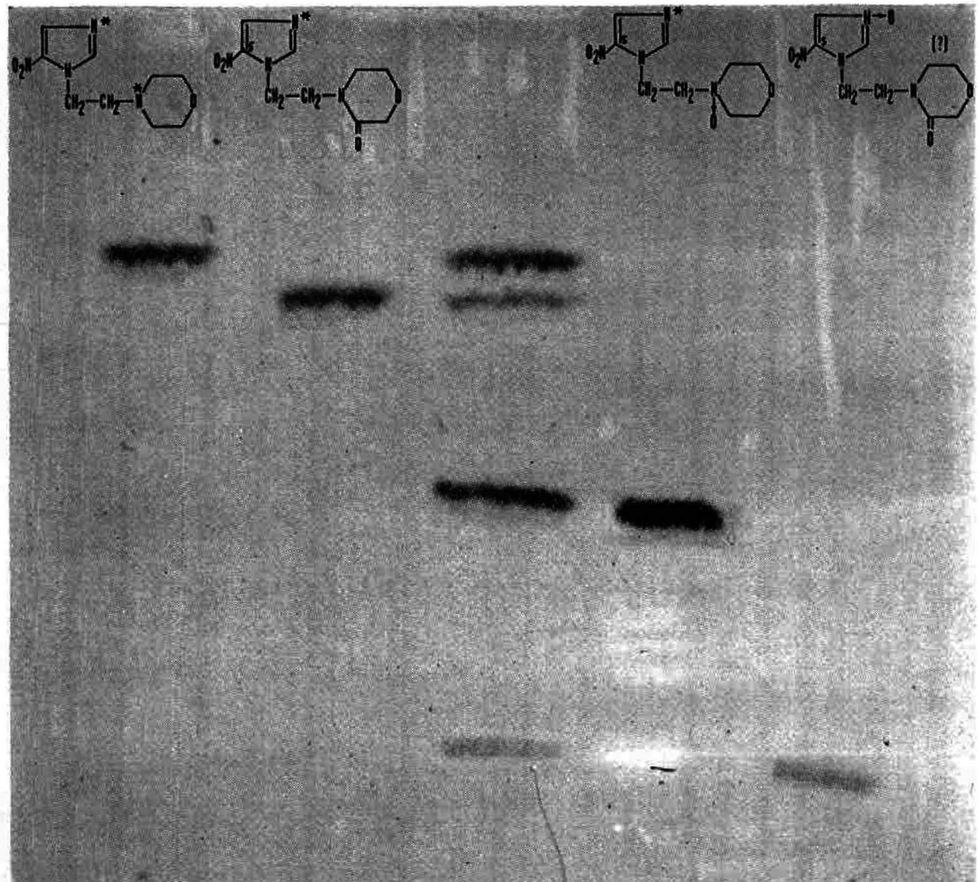


Fig. 5. Chromatogram of the metabolic derivatives of 5-nitro-imidazole-N(β-ethylmorpholine) found in humans. Adsorbent: silica gel. Solvent system: chloroform–methanol–conc. ammonia (70:20:5). Detection: sprayed first with a 50% solution of phenylacetonitrile in acetone and then with a saturated solution of trimethylbenzylammonium hydroxide in methanol, yielding brown spots.

difficulties encountered in its isolation and purification; however, considering that its basicity is practically nil, we are inclined to believe that it is compound 4 in Fig. 4, as the amidic nitrogen is not basic and both the basic nitrogens are substituted.

PHARMACOGNOSY

The many difficulties confronting the pharmaceutical chemist, including the identification of different chemical species in plants where two botanically identical plants may contain different chemical substances, for instance, have promoted the introduction of chromatographic techniques in pharmacognosy. One advantage of TLC is the availability of many more methods of detection than are available with other procedures.

Thus the reaction by which catechins react with aldehydes in the presence of strong acids to yield triphenylmethane dyes was utilized to detect catechinotannins selectively on thin-layer chromatograms. Developed chromatograms were sprayed with a saturated solution of vanillin in 50% phosphoric acid and then heated for a few minutes at 110°. Cherry-red spots appear in the presence of catechino-derivatives; gallic and ellagic acid compounds do not react. The sensitivity is about 0.5 μg (Fig. 7). On the other hand, another reagent, Fast Blue Salt B, reacts with all three types of tannin. In the pharmaceutical industry where the requirements are reflected by that

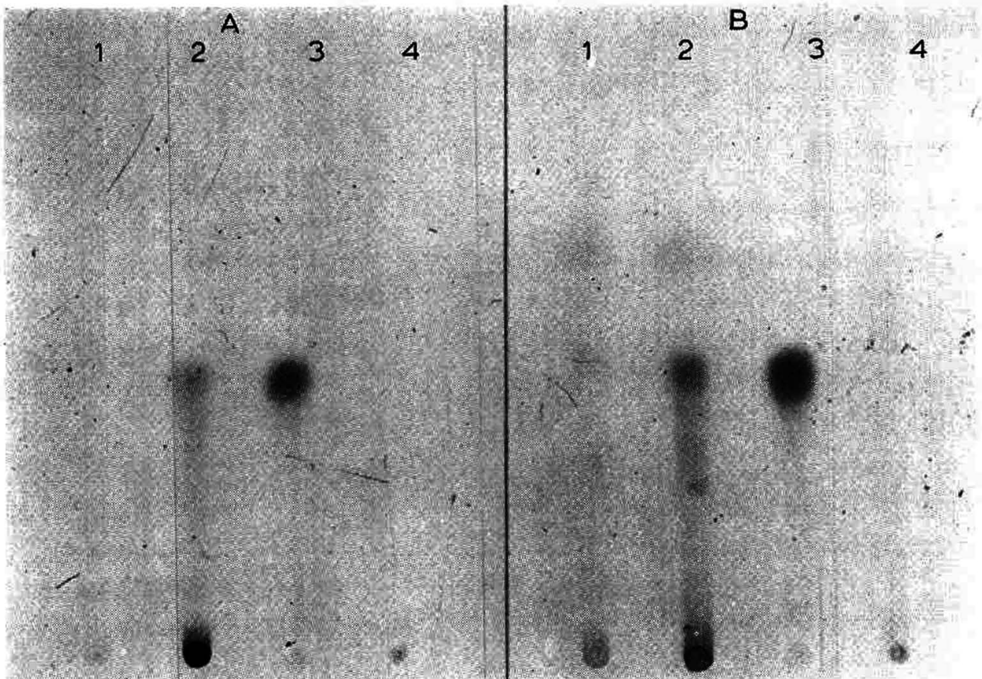
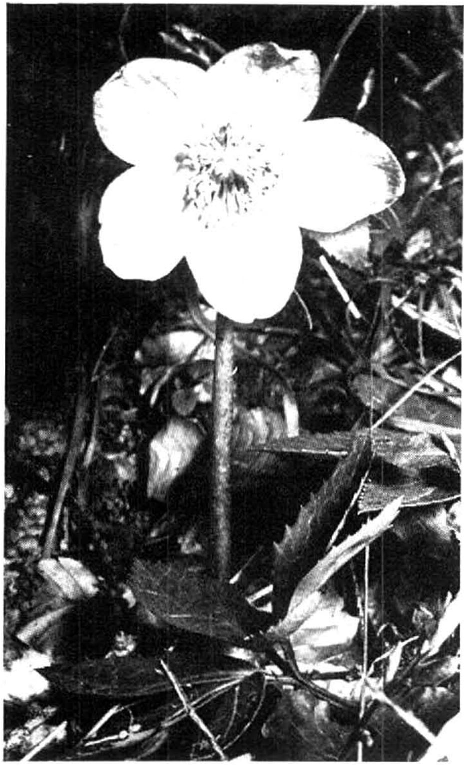
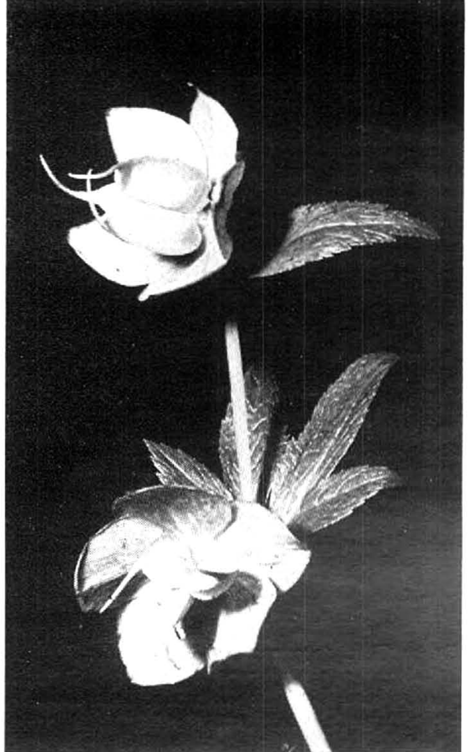


Fig. 6. Chromatogram of catechinotannins using silica gel as adsorbent and chloroform–ethyl acetate–formic acid (5:4:1) as the solvent system. Detection: A = 1% vanillin in sulphuric acid; B = Fast Blue Salt B reagent. 1 = Gallotannin; 2 = *Polygonum bistorta*; 3 = catechin; 4 = ellagic acid.





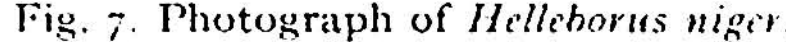


Fig. 7. Photograph of *Helleborus niger*.

Fig. 8. Photograph of *Helleborus viridis*.

of the pharmacopoeia, the pharmacognostic problem has therepeutic and economical implications. The following examples illustrate this point. KARRER's[1] investigations showed that *Helleborus niger* (Fig. 7), gathered in central and northern Europe contains hellebrin, one of the most potent cardiotonics in existence, whereas the similar species, *Helleborus viridis* (Fig. 8), is practically devoid of it. In specimens gathered in Italy, however, we found that the opposite was true (Fig. 9).

Although *Aloe vera L.* (Curaçao aloe) is reputed to be the species containing most aloin, and is therefore used for its extraction, there are specimens of this plant that contain no aloin at all (Fig. 10).

The *Bulbus Scillae* were once divided into a wide variety, which contained cardiotonic scillarens, and a red variety, which contained inactive scilliroside. In fact, many varieties of white squill contain neither scillaren A nor proscillaridin A, and many varieties of red squill contain rather little scilliroside (Fig. 11).

Another example of current interest concerning the chemical composition of different botanical species is that of valerian. The study of the chemistry of this drug seems to have received a new impetus from the discovery of the valepotriates (Fig. 12) which are thought to represent the sedative principles of this plant. Thanks to TLC, we were able to detect (and later to isolate in a column) the same esters from other *Valerianaceae*, such as *Fedia cornucopiae*, a typically Mediterranean

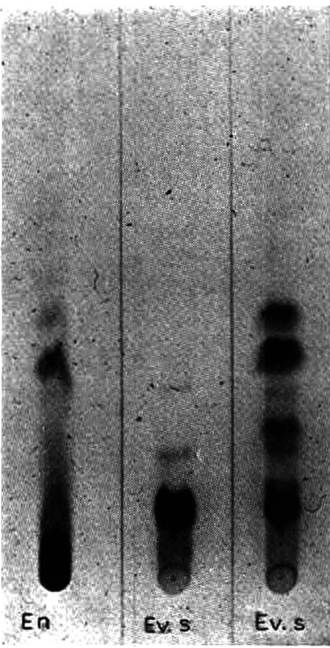


Fig. 9. Chromatogram of hellebrin in *Helleborus niger* and *Helleborus viridis*. Left to right: En = *Helleborus niger* extract; Ev5 = hellebrin; Evs = *Helleborus viridis* extract. Adsorbent: silica gel. Solvent system: chloroform–methanol–conc. ammonia (100:20:2). Detection: vanillin–phosphoric acid reagent.

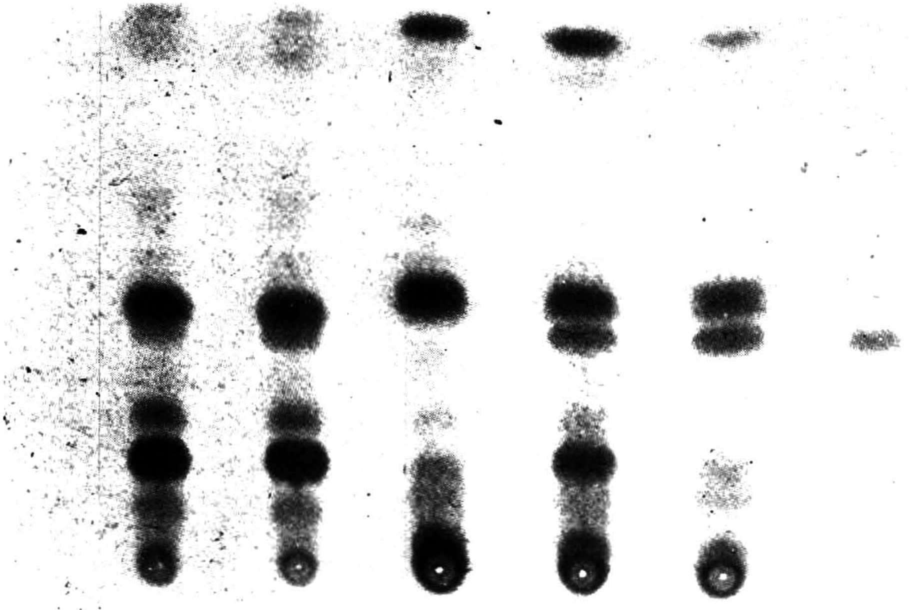


Fig. 10. Chromatogram of species of *Aloe*. From left to right: aloin standard, Indian Aloe, Curaçao Aloe containing aloin, Curaçao without aloin, and Kap Aloe (last two). Adsorbent: silica gel. Solvent system: ethyl acetate–ethanol–water (100:13.5:10). Detection: Fast Blue Salt B reagent.

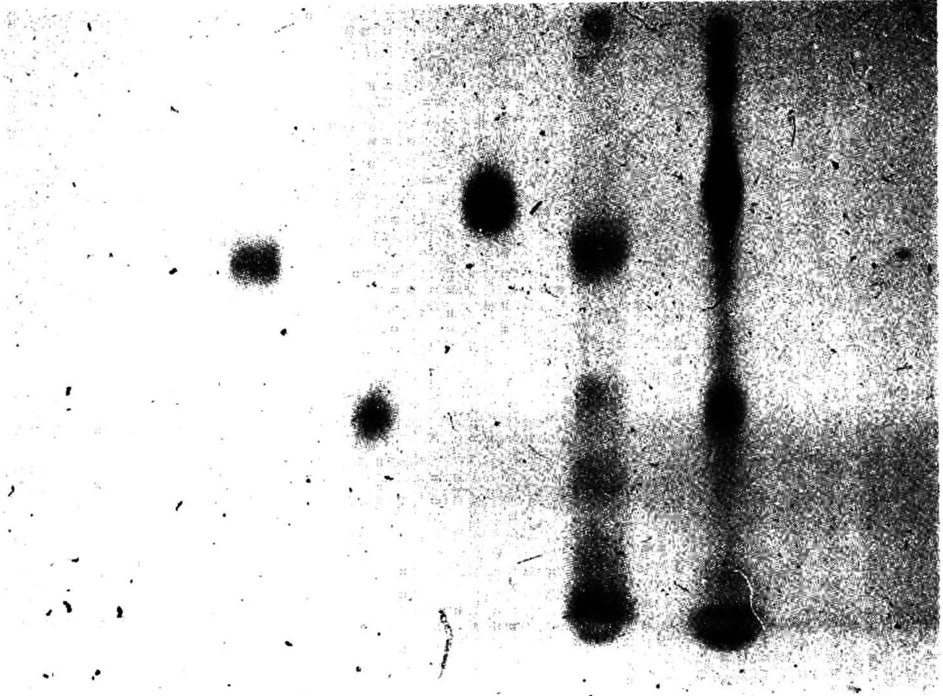


Fig. 11. Chromatogram of varieties of *Scilla*. From left to right: scilliroside, proscillaridin A, scillaren A, and 2 scilla extracts. Adsorbent: silica gel. Solvent system: ethyl acetate–ethanol–water (100:13.5:10). Detection: 25% $SbCl_3$ in chloroform.

A

Valtratum

R_1 or $R_2 = COCH_2CH(CH_3)_2$

R_1 or $R_2 = COCH_2C(CH_3)_2\,OCOCH_3$

B

Acevaltratum

C

Didrovaltratum

Fig. 12. Structural formulae of *Valepotriates*.

plant; on the other hand, many samples of European valerian are devoid of valepotriates (Fig. 13).

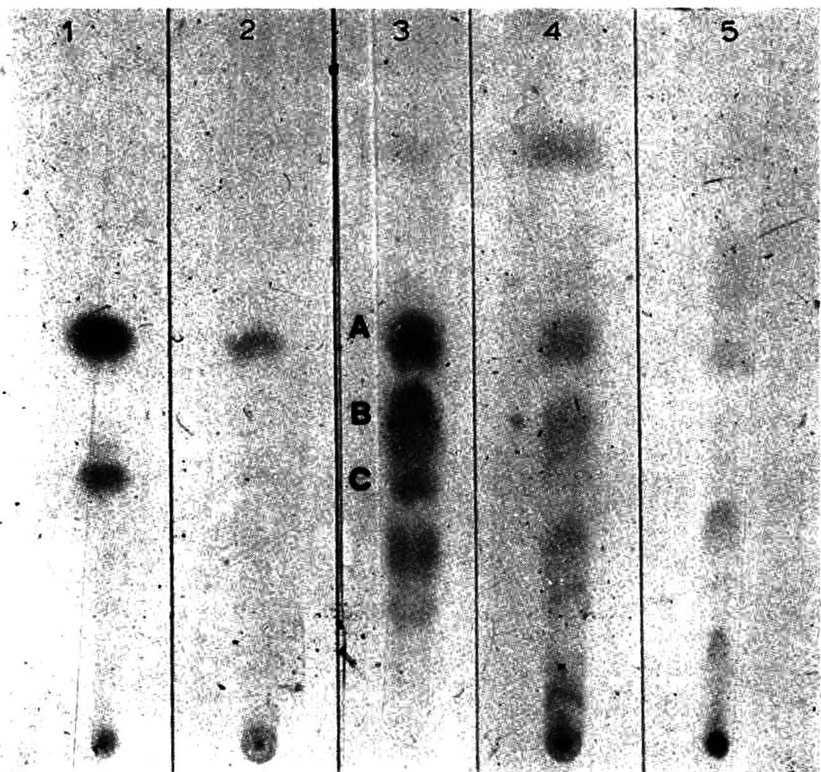


Fig. 13. Chromatogram of valepotriates detected in some *Valerianaceae*. Adsorbent: silica gel. Solvent system: *n*-hexane–ethyl acetate (73:27). Detection: vanillin–sulphuric acid reagent. 1 = *Centranthus ruber* roots; 2 = *Fedia cornucopiae* plant; 3 = valepotriates mixture, A = valtratum, B = didrovaltratum, C = acevaltratum; 4 = *Valeriana wallichii* root-stock (India); 5 = *Valeriana officinalis* roots.

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I wish to thank Dr. E. PESCE, editor of Fitoterapia, for the slides of *Aloe* and *Scilla*, and Mr. C. CONFALONIERI of our Institute, for information.

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CHROM. 4614

THE USE OF HIGH EFFICIENCY PACKED COLUMNS FOR GAS-SOLID CHROMATOGRAPHY

II. THE SEMI-PREPARATIVE SEPARATION OF ISOTOPIC MIXTURES

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SUMMARY

High efficiency packed adsorption columns have been prepared with graphitized carbon black as adsorption medium. The influence of carrier gas, column design, packing characteristics, sample retention and size on column efficiency have been investigated. A HETP value of about 1 mm has been obtained for the 120-m column with methane when 0.5 c.c. s.t.p. of methane is injected.

The separation of CH_4 and CH_3D is shown using different column lengths.

The R^2/t ratio for this separation is of an order of magnitude greater than that found in the literature up to date. The implications of these results for a preparative separation of isotopes are discussed.

INTRODUCTION

Since the introduction of capillary columns by GOLAY in 1958[1], high resolution gas chromatography has been performed mainly with this type of column. However, because of the need of semi-preparative and preparative gas chromatography a good deal of work has been done in order to combine high resolution with high loading capacity.

SCOTT, in his classical work[2], has shown that about thirty thousand theoretical plates can be obtained with "high packed" columns. In a subsequent paper[3], the same author pointed out that resolution, capacity and speed of analysis are linked together in such a way that one can realize the optimum conditions for any two of these parameters, but only by sacrificing the third.

In this paper the first results of our efforts to construct semi-preparative columns for the separation of isotopes are reported; because of the very low separation factor of isotopes resolution must be regarded as the most important parameter. The problem can be summarized as the necessity to make gas chromatographic columns of about

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10^5 theoretical plates, with a reasonable analysis time and capacity. We have chosen CH_4–CH_3D as the test system for our columns because we have considerable experience with it[4] and it is relatively easy to separate this pair.

EXPERIMENTAL

Apparatus

A Carlo Erba ATC/f gas chromatograph equipped with a thermal conductivity detector was slightly modified for high pressure and low temperature work. Two different gas lines were used for the reference arm of the cell and the column inlet in order to avoid high pressure and low flow rate in the former. The carrier gas is purified of any condensable impurity by means of two traps containing molecular sieves, 5 Å; one was at room temperature and the other was maintained at the temperature of liquid nitrogen. The apparatus was also modified by connecting the column, fitted in a Dewar container, to the cell by means of two narrow copper tubings of negligible volume.

Column packing

Graphon, a partially graphitized carbon black, kindly supplied by Dr. W. R. SMITH (Cabot Corp., Billerica, Mass.) was used as packing material. The particle size was 40–60 mesh. Tailing of the peaks was eliminated by treating the adsorbent with a small amount of squalane (1 ‰ w/w). The columns were copper tube, 4 mm I.D., 6 mm O.D., made according to a previously described procedure[5].

Samples were injected by using gas tight and small cross section syringes; no particular difficulties were encountered up to a pressure of 15 kg/cm^2. A more sophisticated inlet system must be used for higher pressures.

RESULTS AND DISCUSSION

Column design and packing characteristics

The choice of column diameter, particle size and amount of squalane are ruled by the necessity of obtaining high efficiency, high permeability, a relatively fast analysis and a reasonable loading capacity. The most important of these parameters is efficiency, as pointed out earlier. The 40–60 mesh particle range assures a satisfactory value of HETP[6] together with a low packing density[7].

The 4 mm I.D. results in negligible dispersion of the chromatographic zone[8], and it lies in a rather flat zone of the plot of permeability *vs.* column diameter[9]. The retention times and the tailing of the peaks decrease sharply as the amount of squalane is increased; with as little as 0.1% peaks are obtained for *n*-butane at 55° and for methane at −78° without tailing.

The capacity ratio value for methane at this temperature is about 4; this permits a short analysis time together with satisfactory exploitation of the resolving power of the column.

The idea of deactivating the adsorbent with a liquid phase, and thus prevent tailing, was first described by EGGERTSEN *et al.*[10]. These authors used a 1.5% concentration to deactivate Pelletex carbon. In our case, however, the amount of squalane needed was about ten times less. This is due to the structure of graphitized carbon

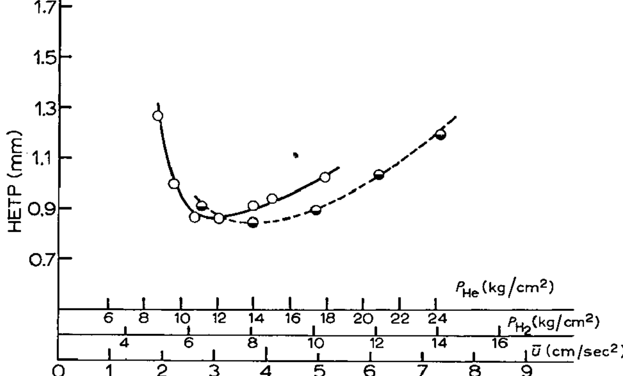

Fig. 1. HETP *vs.* $\bar{u}$ at $-78°$ for CH_4. Column: Graphon + 0.1 % squalane, 4 mm I.D., 6 mm O.D., 60 m long. Carrier gases: ○ helium, ◒ hydrogen.

black, which is essentially a non-porous adsorbent with a few active sites mainly due to some cracks and cravices. The number of molecules of squalane, although much smaller than the monomolecular layer, does not essentially affect the adsorption process, but just block the few active sites.

Effect of the carrier gas

The most important problem was the limitation of the inlet pressure, so that only helium and hydrogen were considered as carrier gases; they also have a better thermal conductivity than other gases. Hydrogen gives the best results as can be clearly seen from the plots in Fig. 1, where the Van Deemter curves for methane at $-78°$ are reported. The triple abscissa serves to show that the same linear gas velocity

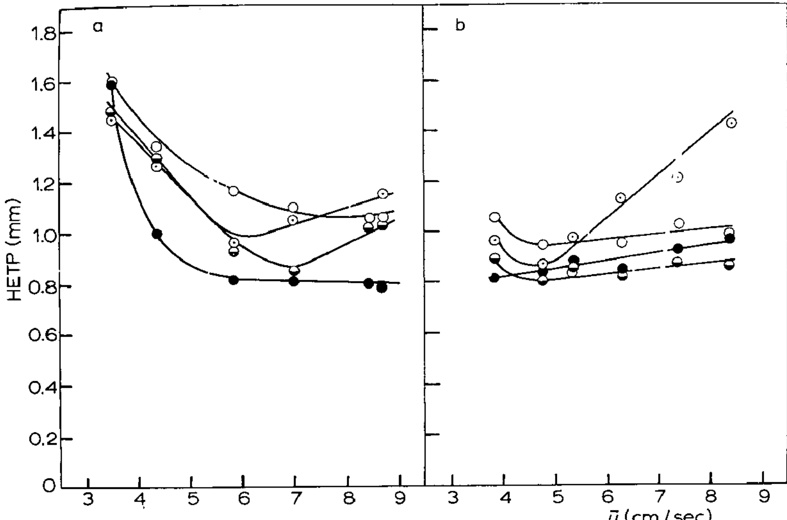

Fig. 2. HETP *vs.* $\bar{u}$ plots for different compounds on different lengths of column. (a) 15 m; (b) 60 m; T = 55°. Samples: ○ C_2H_4; ◒ C_2H_6; ⊙ C_3H_6; ● C_3H_8.

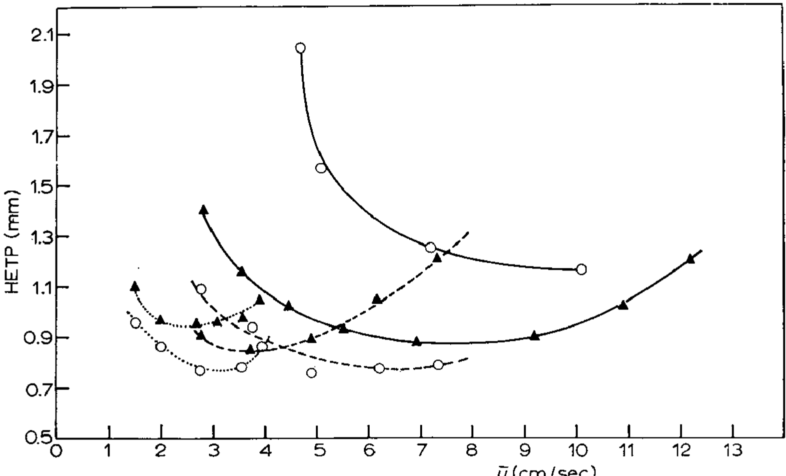

Fig. 3. HETP *vs.* $\bar{u}$ plots for columns of different length at −78°. - - - - - - 120 m; — — — —, 60 m; ———, 15 m. Samples: ○ N_2; ▲ CH_4.

is reached with the two gases using quite different pressures. With hydrogen, the same average linear gas velocity is reached at a much lower pressure and $\bar{u}_{min}$ is shifted towards the higher velocities, improving the speed of analysis.

Effect of sample retention, column length and sample size on HETP.

Fig. 2 shows the Van Deemter curves for compounds of different retention at 55° relative on columns of different length. It should be noted that the constant C is larger for the unsaturated compounds than for the analogous saturated compounds.

The unsaturated hydrocarbons do in fact tail slightly, while perfectly symmetrical peaks are obtained for the alkanes. The same minimum HEPT is obtained on the two columns.

Fig. 3, in which the same plots are reported for methane and nitrogen at −78° for three columns of 15, 60, 120 m, respectively, is more interesting. The well retained peaks (methane) show about the same minimum HETP, while a noticeable decrease of HETP minimum with column length is observed for the more volatile material (nitrogen). The apparent decrease of HETP is however illusory because of the effect of the "dead time" on it.

Furthermore, the longer the column, the lower is the $\bar{u}_{min}$ value. This is explained by the fact that the diffusion effect decreases with the gas pressure so that for longer columns the B term of the Van Deemter curve becomes less important. The same effect has been observed by Halász *et al.*[11] who compared packed capillary columns of an order of magnitude shorter.

Fig. 4 shows the increase of n, the number of theoretical plates, and N, the number of effective theoretical plates[12], with increase in column length. The use of N is suggested by the low value (~ 4) of the capacity ratio for methane at −78° on our columns. Points are taken in the region of $\bar{u}_{min}$ in every case. A slight curvature is observed for long column lengths; this effect is probably due to the high inlet pressure, which causes the flow rate to be very high in the last part of the column.

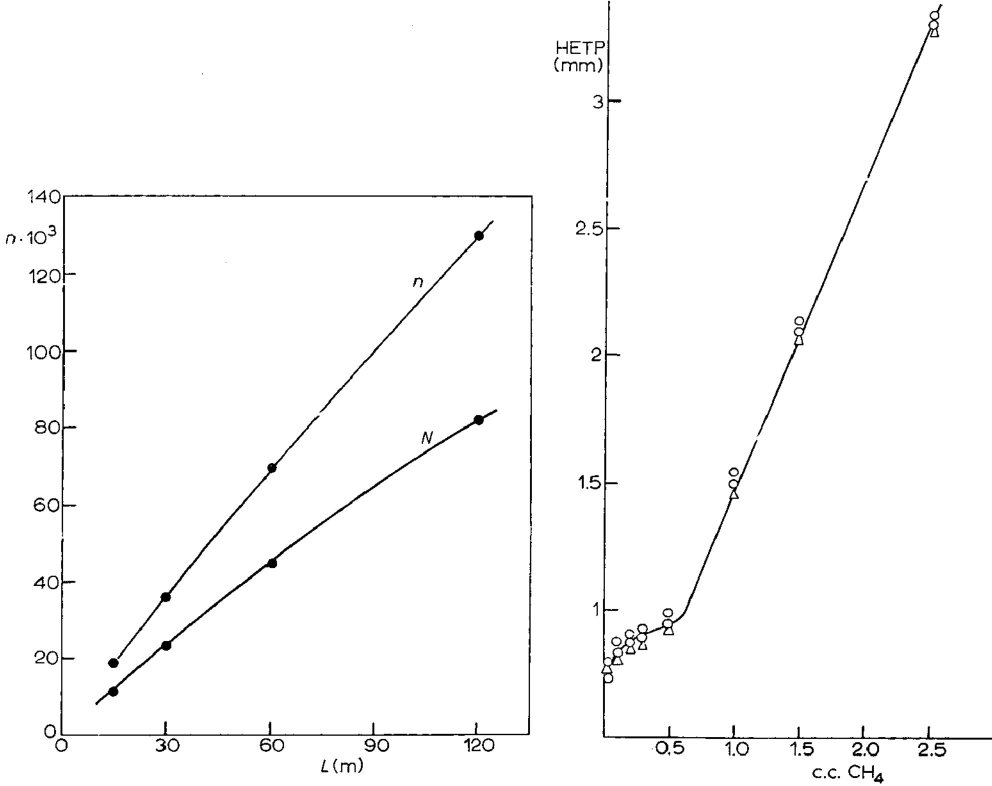

Fig. 4. Number of theoretical plates *(n)* and of effective theoretical plates *(N)* as a function of column length. Sample 0.5 c.c. CH_4; T = $-78°$.

Fig. 5. HETP as a function of sample size. Different dots refer to different columns, 15 m long each.

Fig. 5 shows the effect of sample size on HETP for three different columns packed with the same material. Efficiency is comparable with that of analytical columns up to 1 c.c. A value of HETP higher than 2 results in an unsatisfactory separation for the system CH_4–CH_3D even on the 120 m column. The most significant separations of CH_4–CH_3D, obtained on columns of different length at $-78°$, are reported in Fig. 6. The separation shown in (a) is remarkable for the short elution time and its resolution. It is useful for comparison with analogous separations obtained under different conditions, but it is of little interest for preparative purposes.

The separations obtained in (b) and (c) for the pair CH_4–CH_3D are for the 60 m and the 120 m columns. Chromatogram (c) can be compared with one previously reported[13] which was obtained at the temperature of liquid nitrogen with a column packed with powdered etched glass. The resolution is about the same but in the present case the analysis time has been reduced to about one half.

It should be noted that at $-78°$ the "abnormal" isotope effect takes place but this is much lower as an absolute value than the "normal" one which occurs at $-196°$, so that the same resolution is only obtained with a much longer column.

A comparison of the efficiency of a given gas chromatographic separation can

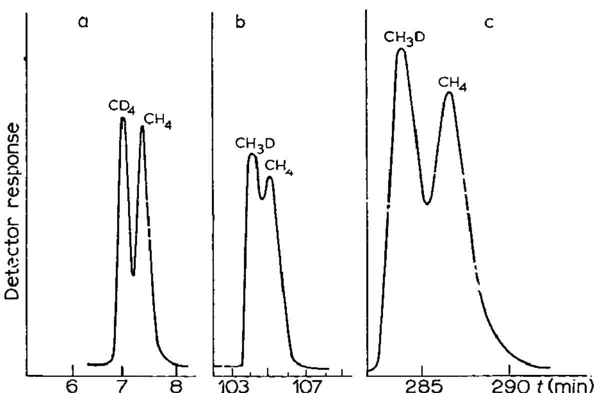

Fig. 6. Separation of isotopic systems on columns of different length. (a) CH_4–CD_4 on 15 m column; (b) CH_4–CH_3D on 60 m column; (c) CH_4–CH_3D on 120 m column. T = −78°. $\bar{u}_{min}$ in every case. Samples: 1 c.c. of mixture.

be made in terms of the factor R^2/t (ref. 14), where R is the resolution expressed in the usual way and t the analysis time. The results obtained by several authors are compared in Table I. The R^2/t value is much higher in the present work and this compensates the use of so long a column for the separation of CH_4–CH_3D.

Such results, which are promising for preparative applications, have been possible because of the peculiar characteristics of graphitized carbon black, the use of which allows one to make use of the selectivity of adsorption gas chromatography and the linearity of the gas–liquid chromatography isotherm. Furthermore, it is possible to adjust the retention volumes by simply changing the amount of liquid phase used.

TABLE I

MEAN R^2/t VALUES ON DIFFERENT ADSORPTION MEDIA FOR THE SYSTEM CH_4–CD_4 (PACKED COLUMNS)

Author	*Ref.*	*T (°C)*	*Adsorption medium*	*Column length (m)*	*R*	*$R^2/t \times 10^4$ (sec^{-1})*
Gant and Yang	15	−3.5	High activity charcoal	15	1.0	0.9
Rowland *et al.*	16	20	Molecular sieves 5 Å	(recycling)	0.9	0.4
Bruner *et al.*	13	−196	Etched glass	8	1.3	0.5
Czubryt *et al.*	17	−45	Porapak Q	30	1.3	5.7
Possanzini *et al.*	18	−78	Porapak Q	4	0.9	2.3
This work		−78	Modified graphitized carbon black	15	0.75	15.0

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CHROM. 4598

MOLECULAR-STATISTICAL CALCULATION OF THE THERMODYNAMIC CHARACTERISTICS FOR SEPARATION OF DEUTERATED MOLECULES BY ADSORPTION

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SUMMARY

Calculations have been made of the ratio of the retention volumes, the differences in the isosteric heats and differential entropies of adsorption for the pairs of the isotopic-substituted molecules CH_4–CD_4, C_2H_6–C_2D_6, C_2H_4–C_2D_4, C_6H_6–C_6D_6 and C_6H_{12}–C_6D_{12} on graphitised carbon blacks. The differences in the potential functions of the interaction between the isotopic molecules and the adsorbent surface and the differences in the quantum effects for the vibrations of the isotopic molecules mass centres normal to the surface have been taken into account. The results of the calculations are compared with experimental data found in the literature. The differences in the quantum effects for the vibrations of the mass centres of the hydrocarbon isotopic molecules normal to the surface are small and only appreciable for the lightest molecules. The isotopic effect on adsorption of the hydrocarbon molecules on graphitised carbon blacks is mainly caused by the difference in the potential functions of the interactions of isotopic molecules with the surface. The zero point energy effect can also contribute to the isotopic effect for hydrocarbon molecules.

INTRODUCTION

The adsorption properties of isotopic molecules slightly differ[1–25]. Depending on the mass and the structure of the molecule, the nature of the surface and the temperature, deuterated molecules may be adsorbed both more strongly[1–16,19–22] or more weakly[16–18,21,23–25] than the hydrogenated form.

The isotope effect on the adsorption is apparently caused by three following factors: (1) the differences in the potential functions Φ of the interaction between the isotopic molecules and the adsorbent surface (the quantum mechanical effect); (2) the differences of the quantum effects in adsorption for the external (translational and rotational) degrees of freedom of isotopic molecules, due to the difference of their masses and moments of inertia; and (3) the different change of intramolecular vibrational energy of the isotopic molecules on adsorption, due to the difference of the

masses of the vibrating atoms (the zero-point energy effect). As a first approximation these effects can be regarded as independent.

The potential functions Φ for the deuterated and the hydrogenated forms of hydrocarbon molecules must always apparently differ somewhat. In the case of adsorption on a non-polar surface this effect causes a decrease in the adsorption of the deuterated molecules, and in the case of heavier molecules, this is apparently the main effect[26,27].

The translational and rotational quantum effects always increase the adsorption of deuterated molecules by comparison with the adsorption of the hydrogenated form. These effects, however, decrease rapidly when the mass and moment of inertia of the molecule and the temperature increase, but they are considerable for the lightest molecules at low temperatures. In the case of hydrogen isotopes the quantum effects for the external degrees of freedom are the main ones[10,28,31].

Depending on the sign of the change of the force constants of intramolecular vibrations in the adsorption, the zero-point energy effect can cause either an increase or a decrease in the adsorption of deuterated molecules. With an increase of the temperature, this effect decreases considerably more slowly than the quantum effects for the external degrees of freedom. The contribution of this effect increases with an increase in the number of substituted atoms. Therefore the zero-point energy effect may be significant for hydrocarbon molecules with a great number of the substituted atoms[23,32,33].

Of these three effects only the quantum effects for the external degrees of freedom have been investigated to any extent[10,28–31]. The quantum mechanical and the zero-point energy effects have been investigated much less extensively[23,26,27,32].

We have calculated the potential functions Φ of the interaction between a number of the isotopic molecules (CH_4–CD_4; C_2H_6–C_2D_6; C_2H_4–C_2D_4; C_6H_6–C_6D_6; C_6H_{12}–C_6D_{12}) and the basal face of graphite on the basis of the physico-chemical properties of the isotopic molecules and the graphite lattice. The ratio of the retention volumes, differences of the isosteric heats and differential entropies of adsorption for these systems of isotopic molecules at zero coverages were calculated from the calculated potential functions.

In a previous paper[27], the groups of atoms were assumed to be the force centres of the molecules. In this paper the atoms are assumed to be the force centres of the molecules. This approximation allows for an apparently better representation of the spatial structure of the molecules under consideration.

STATISTICAL EXPRESSIONS

The ratio of the retention volumes V_s for a pair of isotopic molecules at zero coverage and at a fixed temperature T and surface area A is given by the following general expression[34]:

$$V_s^{\mathrm{H}}/V_s^{\mathrm{D}} = (Q_1^{\mathrm{H}}/Q_1^{0\mathrm{H}} - 1)/(Q_1^{\mathrm{D}}/Q_1^{0\mathrm{D}} - 1), \quad (1)$$

where Q_1 and Q_1^0 are the partition functions for one molecule in a volume V in the presence and in the absence of the adsorbent, respectively. The upper indices H and D refer to the hydrogenated and deuterated forms of the molecule.

In the classical approximation for the external degrees of freedom of a rigid molecule, assuming that the energy of intramolecular vibrations and the rest of energy are separable,

$$Q_1/Q_1{}^0 - 1 \approx (1/8\pi^2 V)\,(Q_{vib}/Q^0{}_{vib}) \int [\exp(-\Phi/kT)-1]\sin\delta \mathrm{d}x\mathrm{d}y\mathrm{d}z\mathrm{d}\delta\mathrm{d}\varphi\mathrm{d}\psi, \quad (2)$$

where Φ is the potential energy of the interaction between the molecule and the surface of the solid as a function of the Cartesian coordinates x, y and z of the molecule mass-centre and the Euler angles δ, φ, ψ, which determine the orientation of the molecule to the surface, Q_{vib} and $Q^0{}_{vib}$ are the vibrational partition functions of the adsorbed molecule and the molecule in the gas phase. Putting (2) into (1) and assuming $Q_{vib} = Q^0{}_{vib}$, we obtain

$$V_s{}^{H}/V_s{}^{D} = S_1{}^{H}/S_1{}^{D}, \quad (3)$$

where the configurational integrals S_1 are given by the expression:

$$S_1 = \int [\exp(-\Phi/kT)-1]\sin\delta \mathrm{d}x\mathrm{d}y\mathrm{d}z\mathrm{d}\delta\mathrm{d}\varphi\mathrm{d}\psi. \quad (4)$$

If the potential function Φ does not depend on the translational coordinates of the molecule mass-centre parallel to the surface (coordinates x and y) and on the rotational coordinates in the plane parallel to the surface (angle φ), which is approximately correct, for instance, in the case of adsorption on the basal face of graphite, and if we assume that the vibrations of the molecule mass-centre normal to the surface are harmonic, then

$$\Phi = \Phi_0 + (\Phi''_z/2)\,(z-z_0)^2, \quad (5)$$

where $\Phi_0\,(\delta, \psi)$ and $\Phi''_z\,(\delta, \psi)$ are the values of Φ and the second derivative of Φ with respect to the coordinate z at the equilibrium distance $z_0\,(\delta, \psi)$ of the molecule mass-centre at fixed angles δ and ψ, and

$$S_1/2\pi A = \int (2\pi kT/\Phi''_z)^{\frac{1}{2}} \exp(-\Phi_0/kT)\sin\delta \mathrm{d}\delta\mathrm{d}\psi. \quad (6)$$

If the system deviates from the classical one, but not significantly, and the quantum corrections can be calculated by the Pitzer and Gwinn approximation[35], then

$$V_s{}^{H}/V_s{}^{D} = S_1{}^{H}\, v^{**H}/S_1{}^{D}\, v^{**D}, \quad (7)$$

where

$$v^{**} = \prod_r \frac{hv_r}{kT}\,\frac{\exp(-hv_r/2kT)}{1-\exp(-hv_r/kT)} \quad (8)$$

Here v_r is the frequency of the harmonic oscillations of the molecule near the potential minimum for the r-th degree of freedom. The Pitzer and Gwinn approximation however, assumes that the degrees of freedom for which we calculate the quantum corrections are harmonic oscillations.

POTENTIAL FUNCTIONS Φ

In the case of the interaction between a non-polar molecule and the surface of a non-polar solid, *e.g.* graphitised carbon black, the main attractive forces are dispersion forces. In this case the potential functions Φ of the interaction between different molecules and the same adsorbent are mainly determined by the chemical and spatial structure of the molecule, by the electromagnetic properties (polarisability α, diamagnetic susceptibility χ) and by the van der Waals radius r_0 of the molecular force centres.

The average length of the C–D bond is shorter than the average length of the C–H bond by 0.004–0.005 Å[36–40], and the polarisability of deuterated molecules is 0.5–1.5% less than the polarisability of the hydrogenated form[37,41–43]. The van der Waals radii r_0 of the H and D atoms in molecules are apparently equal[26,44–46]. Therefore the difference in the potential functions Φ of the isotopic molecules is mainly caused by the difference in the lengths of the C–H and C–D bonds and by the difference in the electromagnetic properties of the force centres of the isotopic molecules. In calculating Φ both these factors are taken into consideration. Atoms are assumed as the force centres of the molecules. It is assumed that Φ is equal to the sum of the potential functions φ of the interaction between the atoms of the molecule and the C atoms of the graphite lattice, $\Phi = \Sigma\Sigma\varphi$. For the interaction between the C and H atoms of the molecules and the C atoms of the graphite lattice we use the semi-emperical potential functions[34,47]:

$$\varphi_{\mathrm{C}\ldots\mathrm{C}} = -331\,r^{-6} - 513\,r^{-8} + 4.52\cdot 10^{4}\exp(-3.57\,r), \tag{9}$$

$$\varphi_{\mathrm{H}\ldots\mathrm{C}} = -119\,r^{-6} - 227\,r^{-8} + 0.86\cdot 10^{4}\exp(-3.57\,r), \tag{10}$$

where the distance r between the atoms is in Å and φ is in kcal/mole.

According to the Kirkwood-Müller formula the ratio of the dipole–dipole constants C_1 of the dispersion interaction between the D and H atoms of the molecule and the C atoms of the graphite lattice is

$$\frac{C_1^{\mathrm{D}}}{C_1^{\mathrm{H}}} = \gamma = \frac{\alpha_{\mathrm{D}}}{\alpha_{\mathrm{H}}}\left(1 + \frac{\alpha_{\mathrm{H}}/\chi_{\mathrm{H}} - \alpha_{\mathrm{D}}/\chi_{\mathrm{D}}}{\alpha_{\mathrm{D}}/\chi_{\mathrm{D}} + \alpha_{\mathrm{C}}/\chi_{\mathrm{C}}}\right), \tag{11}$$

where α_{H}, α_{D}, α_{C} are polarisabilities and χ_{H}, χ_{D}, χ_{C} are diamagnetic susceptibilities of the H, D and C atoms. If we assume that the ratio $C_2^{\mathrm{D}}/C_2^{\mathrm{H}}$ of the constants of a dipole–quadrupole interaction is equal to the ratio $C_1^{\mathrm{D}}/C_1^{\mathrm{H}}$ and the rest of parameters (the separation of the atoms at the potential minimum and the constant in the exponential) of the potentials $\varphi_{\mathrm{H}\ldots\mathrm{C}}$ and $\varphi_{\mathrm{D}\ldots\mathrm{C}}$ are the same, then

$$\varphi_{\mathrm{D}\cdots\mathrm{C}} = \gamma\varphi_{\mathrm{H}\cdots\mathrm{C}}. \tag{12}$$

Assuming that the decrease in the polarisability α of the hydrocarbon molecules on the substitution of a D atom for an H atom is due to the smaller polarisibility of the D atoms, then on the basis of the experimental results for the systems CH_4–CD_4[37], C_6H_{12}–C_6D_{12}[41,42] and C_6H_6–C_6D_6[41,42] we obtain that $\alpha_{\mathrm{D}}/\alpha_{\mathrm{H}} \approx 0.98$. The diamagnetic susceptibility χ of deuterated molecules apparently has not been measured. It is assumed that the isotopic substitution does not change α/χ (ref. 26) or χ (ref. 48). Using eqn. 11, we obtain $\gamma = 0.98$ and $\gamma = 0.99$, respectively.

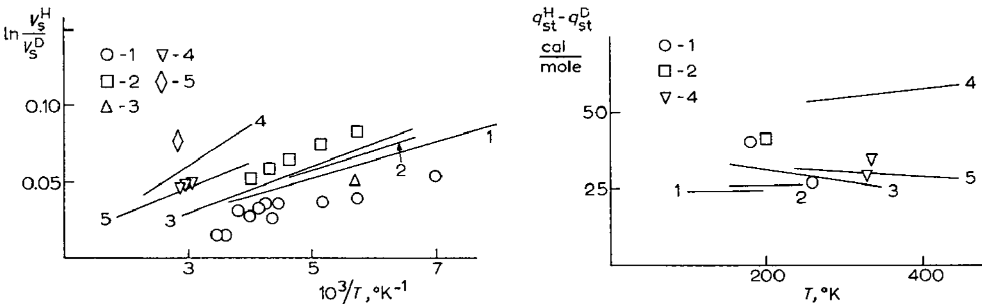

Fig. 1. Calculated (curves) and experimental (points) values of ln $V_s{}^H/V_s{}^D$ for the systems: CH_4–CD_4 (1); C_2H_6–C_2D_6 (2); C_2H_4–C_2D_4 (3); C_6H_6–C_6D_6 (4); and C_6H_{12}–C_6D_{12} (5) as a function of $1/T$.

Fig. 2. Calculated (curves) and experimental (points) values of $q_{st}{}^H - q_{st}{}^D$ for the systems: CH_4–CD_4 (1), C_2H_6–C_2D_6 (2), C_2H_4–C_2D_4 (3), C_6H_6–C_6D_6 (4) and C_6H_{12}–C_6D_{12} (5).

In our calculations γ is assumed to be equal to 0.985. The position of the force centre of the D atom, compared with the position of the force centre of the H atom, is assumed to be displaced in the direction of the C atom by 0.004 Å in the CD_4 molecule and by 0.005 Å in the other deuterated molecules.

THERMODYNAMIC QUANTITIES

The calculated values of ln $V_s{}^H/V_s{}^D$ and $q_{st}{}^H - q_{st}{}^D$ are compared with the corresponding experimental data[16,24,25] in Figs. 1 and 2. The calculated curves are fairly close to the experimental data. In contradiction of the experimental data, however, the calculated values of ln $V_s{}^H/V_s{}^D$ for the system C_2H_4–C_2D_4 are greater than those for the system C_2H_6–C_2D_6; and also the calculated values of ln $V_s{}^H/V_s{}^D$ for the system C_6H_6–C_6D_6 are greater than those for the system C_6H_{12}–C_6D_{12}. This divergence of calculated values and experimental data can be explained in various ways, for example, it may be caused by neglecting the zero-point energy effect. The contribution of the zero-point energy effect for the systems C_2H_6–C_2D_6 and C_6H_{12}–C_6D_{12} can be greater than for the systems C_2H_4–C_2D_4 and C_6H_6–C_6D_6 because of the greater number

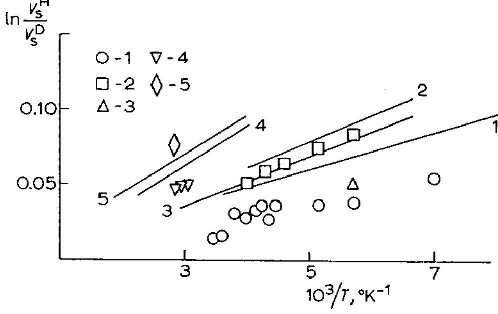

Fig. 3. Calculated (curves) and experimental (points) values of ln $V_s{}^H/V_s{}^D$ as a function of $1/T$. The calculated values are obtained assuming that the position of the force centres of the D and H atoms coincide. The designations are the same as in Fig. 1.

of the substituted H atoms in C_2D_6 than in C_2D_4, and in C_6D_{12} than in C_6D_6, as well as the difference of the spatial structure of these molecules. This divergence can also be caused by the errors in the estimation of the differences in the positions of the force centres of the H and D atoms. The variation in the value of this difference hardly changes the results of the calculation for the plane molecules C_2H_4–C_2D_4 and C_6H_6–C_6D_6, but has a comparatively strong effect on the results for the rest of the molecules. If we assume, that the positions of the force centres of D atom and the H atom coincide, then the calculated curves $\ln V_s^H/V_s^D$ *vs.* $(1/T)$ (Fig. 3) should have the same sequence as the experimental data. Moreover, the calculated values are very sensitive to the value of the coefficient γ: a change in the coefficient γ of 0.005 causes a change in the values of $\ln V_s^H/V_s^D$ and $q_{st}^H - q_{st}^D$ of 30–50%. Therefore, the above discrepancy between the calculated values and the experimental data can also be caused by small changes in the coefficient γ for these systems.

The translational quantum effect for the isotope substituted hydrocarbon molecules is small: for the system CH_4–CD_4 at 100°K the quantum corrections are equal to -0.016 for $\ln V_s^H/V_s^D$ and -6 cal/mole for $q_{st}^H - q_{st}^D$. The quantum corrections for the rotational degrees of freedom must be added to this effect. However, the calculation of the last effect for the polyatomic molecules is very difficult. Allowance for this effect would lower the calculated values for CH_4–CD_4 slightly. For heavier molecules, not at very low temperatures, the quantum statistical effects for the external degrees of freedom are not significant and may be neglected.

The calculated values of $(\Delta\bar{S}_a^H - \Delta\bar{S}_a^D)/R$ are 0.01–0.03.

Thus, the results of the calculations show that the isotopic effect on the adsorption of hydrocarbon molecules on graphitised carbon blacks is mainly caused by the difference of the potential functions Φ of the interaction of isotopic molecules with the surface. The comparison of the calculated values with the experimental data shows that the zero-point energy effect apparently contributes significantly to the isotopic effect for the hydrocarbon molecules. The quantum effects for the external degrees of freedom are only significant in the case of the lightest molecules.

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CHROM. 4599

SOME NEW CHROMATOGRAPHIC TECHNIQUES

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SUMMARY

Six new methods for chromatography without carrier gas are discussed. They allow an increase in precision, sensitivity, and speed of analysis. Examples are given of the use of these methods for the analysis of mixtures of permanent gases, enrichment of microconcentrations, and preparation of high purity gases. A discussion of the efficiency of gas chromatographic columns with a new sorbent having small and uniform channels is presented.

The special features inherent in elution analysis, in particular the necessity of precise sampling and sensitive detection, substantially restrict the possibilities of improving some of its characteristics. This is especially true with respect to the determination of trace quantities of impurities and the possibility of developing continuous analysis.

In the present article a brief description of some new techniques will be given, opening up some additional potentialities in these fields. The first group referred to is chromatography without carrier gas (CWCG). In some cases the absence of a carrier gas sharply reduces the broadening of the zones, often resulting in concentration of the components, and minimizes the requirements imposed on the detecting and sampling devices.

Four versions of CWCG will be considered. In the first (elution CWCG) the test mixture is passed continuously through the column. A change in some parameter at the inlet end of the column (injection of sample of any composition, short duration variations in temperature or flow rate, etc.) leads to the formation of a series of zones of a changed composition moving along the sorbent bed with their individual velocities. The molar fraction of the i-th component (N_i) is related to the specific retention volume (Z_j) and the Henry coefficient (Γ_i) by the equation

$$N_i = \frac{\Pi\,(Z_j - \Gamma_i)}{\Pi\,(\Gamma_k - \Gamma_i)} \qquad (1)$$

Thus the composition of the mixture may be determined by measuring the retention volumes. In this case the detector plays the role of a null instrument. Using as an example the case of binary mixtures it has been shown[2] that the precision of the method is substantially higher than that of elution analysis, the error being about 0.3%.

TABLE I

RESULTS OF THE ANALYSIS OF A C_4H_{10}–C_3H_8–C_2H_6–CH_4 MIXTURE ON β,β'-IMINODIPROPIONITRILE

No. of mixture	*Components*	*Taken (%)*	*Found (%)*	δ^a *(%)*
I	Butane	35.0	35.6	+ 1.7
	Propane	30.0	31.8	− 6.0
	Ethane	16.0	15.5	− 3.1
	Methane	19.0	17.1	−10.0
II	Butane	25.0	24.6	− 1.6
	Propane	15.0	15.2	+ 1.3
	Ethane	20.0	20.0	0
	Methane	40.0	40.2	+ 0.5
III	Butane	40.0	38.8	− 3.0
	Propane	10.0	9.5	− 5.0
	Ethane	12.9	13.4	+ 5.6
	Methane	37.1	38.3	+ 3.2
IV	Butane	20.0	21.5	+ 7.5
	Propane	28.0	26.8	− 4.3
	Ethane	20.0	20.0	0
	Methane	32.0	31.7	− 0.9
V	Butane	20.0	21.0	+ 5.0
	Propane	20.0	19.8	− 1.0
	Ethane	35.0	35.6	+ 1.7
	Methane	25.0	23.6	− 5.6

[a] $\delta = 3.3\%$.

In Table I, the results are given for a determination of the composition of a four-component system of C_1–C_4 paraffins on β,β'-iminodipropionitrile. This stationary phase was chosen in order to use eqn. 1, which is valid only for linear isotherms. A nonpolar solvent–hydrocarbon system gives a specific deviation from linearity, causing some complications in the calculation[3]. The large magnitude of errors is due to some inaccuracy in determining the composition of the initial mixture. The elution CWCG method may be extended to more complex systems.

In differential chromatography without carrier gas (DCWCG)[4] the mixture to be analyzed is introduced into a column previously filled with a reference mixture (or *vice versa*). The heights of the steps produced are directly related to the values of differences in the concentrations of each component of these mixtures.

Fig. 1 represents a chromatogram of a three-component mixture (methane, nitrogen, argon), and in Table II the results are given of an analysis of a five-component mixture (CH_4, N_2, Ar, O_2, H_2) on NaX molecular sieves ($L = 70$ cm).

The differences in concentrations of the mixtures compared have been measured with an accuracy of 15 to 20%, the error in determining the concentrations themselves not exceeding 0.3–0.7%.

Thus it will be seen that the precision of DCWCG is superior to that of elution analysis. It should be noted that DCWCG permits the analysis of mixtures containing pairs of inseparable components. In the case considered here it is the argon–oxygen pair.

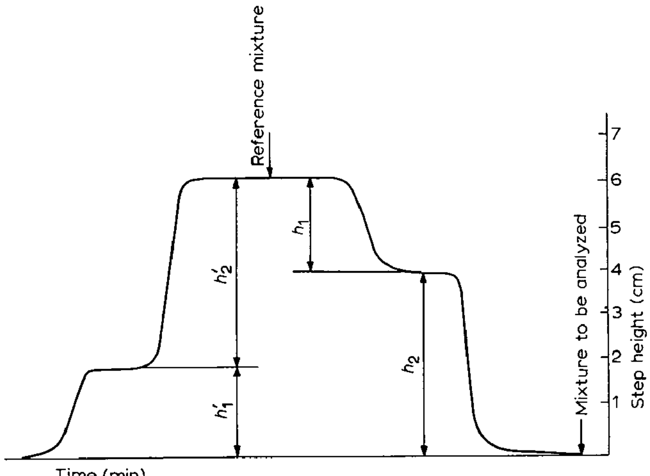

Fig. 1. Chromatogram of a three-component mixture (CH_4, N_2, Ar) obtained by the differential chromatography method.

TABLE II

RESULTS OF THE ANALYSIS OF A FIVE-COMPONENT MIXTURE BY DCWCG

(N_{CH_4} = 0.291; N_{N_2} = 0.20; N_{Ar} = 0.24; N_{O_2} = 0.23; N_i = 0.0100).

ΔN_i	ΔN_i *taken*	ΔN_i *found*	ΔN_i *taken* − ΔN_i *found*	% *error* ΔN_{H_2}	% *error* N_i
ΔN_{CH_4}	+1.70	+1.70	0	0	0
ΔN_{N_2}	−0.50	−0.41	−0.09	18.0	0.44
ΔN_{Ar}	−0.70	−0.54	−0.16	22.8	0.69
ΔN_{O_2}	−0.58	−0.66	+0.08	13.8	0.34
ΔN_{H_2}	−0.11	−0.09	−0.02	15.0	0.45

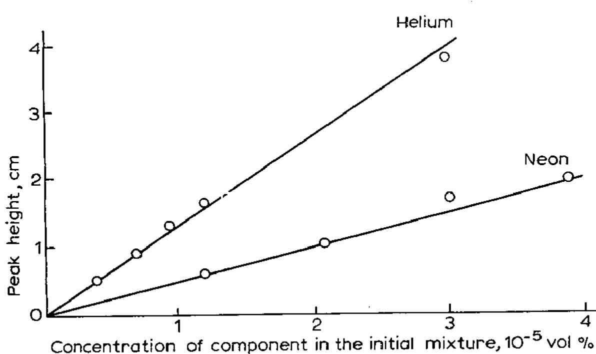

Fig. 2. Calibration curves for the determination of the He and Ne content in air.

In the determination of trace amounts of poorly adsorbed components it is advisable to carry out a preliminary chromatographic enrichment by filling an evacuated column with the mixture to be analyzed[5]. A small portion of the mixture enriched in the poorly adsorbed components is then transferred to the separation column where elution analysis is carried out. Either the test gas or some other gas is used as carrier. The principle of this method has been used as the basis of the Luch chromatograph designed and constructed in collaboration with the Dzerzinsk Affiliated Branch of the Experimental Construction Bureau of Automatics.

Fig. 2 shows some calibration curves for the determination of the He and Ne content in air. The sensitivity of the method is 10^{-6} to 10^{-5} vol. %. This chromatograph has been successfully applied to the determination of traces of hydrogen in argon.

In order to enrich well-adsorbed impurities it is necessary to pass a "pushing gas", which is adsorbed more strongly than any of the components of the mixture, through the bed saturated with the test mixture.

In Fig. 3 a calibration curve is shown for the determination of traces of benzene in water or in aqueous solutions. In this particular case water vapour plays the part of "pushing gas" in the chromatographic process of enrichment of trace amounts of benzene (column packed with MSM silica gel; length of column, 180 cm; temperature, 110°), and the role of carrier gas in the elution analysis (column packed with BAU charcoal, a hydrophobic sorbent; length of column, 80 cm; temperature, 110°). By increasing the volume of the sample of water (or aqueous solution) taken for analysis the sensitivity of determination may be increased to $5 \cdot 10^{-7}$ wt. %.

Finally, a combination of CWCG with the heat-dynamic method can be used successfully for preparative purposes. The mixture to be separated is continuously

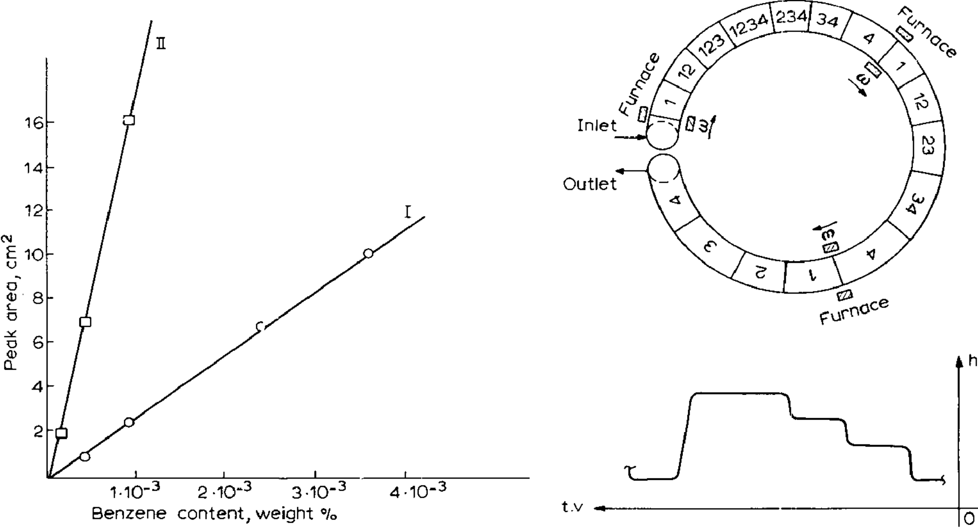

Fig. 3. Calibration curve for the determination of benzene impurities in water and in aqueous solutions.

Fig. 4. Layout of apparatus and chromatogram obtained (the initial mixture contained N_2, C_2H_6, C_3H_8, and C_4H_{10}).

passed through a column having the shape of an unclosed ring. A furnace (or several furnaces) moves continuously along the sorbent bed and pushes out the zones of pure components moving one after the other.

Fig. 4 shows a flow diagram of the device and a chromatogram of the gases emerging from the column. (The initial mixture contained nitrogen, ethane, propane, and butane.)

Belonging to the same group of techniques as the heat-dynamic method without carrier gas is a chromatographic method where fixed concentrations (FC) are used; this was recently developed by the authors in collaboration with GELMAN AND SCHWARTZMAN. If an eluent with a Henry coefficient Γ_{el} and a sufficiently large quantity of a mixture containing n components with Henry coefficients Γ_1, Γ_2, ... Γ_n ($\Gamma_1 > \Gamma_2 > \Gamma_3 > .... > \Gamma_n > \Gamma_{el}$) is passed through a bed of sorbent, the pushing gas is supplied at a velocity a, and a short furnace begins to move along the solvent bed at a velocity, w, then under some definite conditions a stationary regime of movement of the binary zones can arise in the column, as shown in Fig. 5. The material

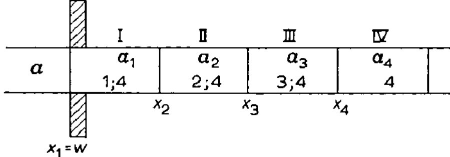

Fig. 5. Diagram of the movement of the zones. Stationary regime (FC method). a = linear velocity of the mixture within a zone, x = velocity of zone boundaries, w = velocity of the furnace.

balance equation of the components permits the derivation of a relationship between the molar fraction (N_i) of a component and the velocity of the furnace (w), flow rate (a) of the "pushing gas" and the adsorption characteristics:

$$N_i = \frac{\Gamma_i - \frac{a}{w}}{\Gamma_i - \Gamma_{el}} \tag{2}$$

where Γ_{el} and Γ_i are the Henry coefficients of the "pushing gas" and of the component, respectively. Eqn. 2 has been verified on a He–Ar binary system (activated carbon, length of column = 125 cm, length of furnace = 10 cm, temperature of furnace = 150°). In Fig. 6 two chromatograms are shown, obtained with samples of pure argon and of a mixture containing 20% argon and 80% helium. It will be seen that the height of

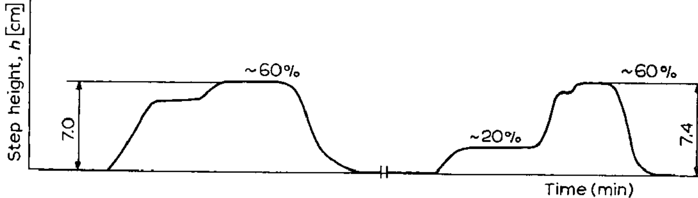

Fig. 6. Chromatogram obtained in the analysis of samples of pure argon and of a mixture of 20% argon and 80% helium.

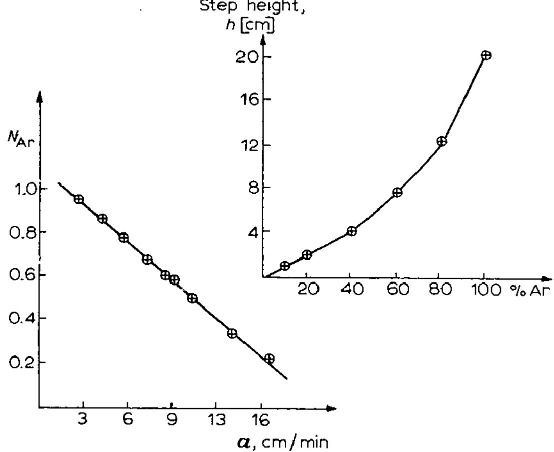

Fig. 7. Linear dependence of the argon molar fraction on the "pushing gas" flow rate and calibration curve of the detector.

the step formed does not depend on the composition of the sample. Fig. 7 shows an experimentally obtained relationship between N_{Ar} and α, which is linear in accordance with eqn. 2.

Five possible applications of the new method are suggested.

Since the value of the FC does not depend on the initial concentration in the sample, the FC method may be used to determine impurities, adsorbed better than the main gas. The advantages of the method are not restricted to the effect of a concentration increase, since the concentration in the zone produced is constant and known; the initial concentration of the impurity may be determined directly from the width of the step. The detector functions as a null instrument. As an example the determination of CO_2 in air is examined. The analyses were made on a column (length = 130 cm, diameter = 0.5 cm) packed with MSM silica gel; air was passed continuously through the column. At a flow rate of air of 20 c.c. per min, a step was produced the height of which corresponded to 0.25% CO_2; it emerged within 2.8 min. A calculation based on material balance showed the content of CO_2 in the air sample analyzed to be about 0.015 vol. %.

In this way the possibility is opened up for the analysis of complex mixtures

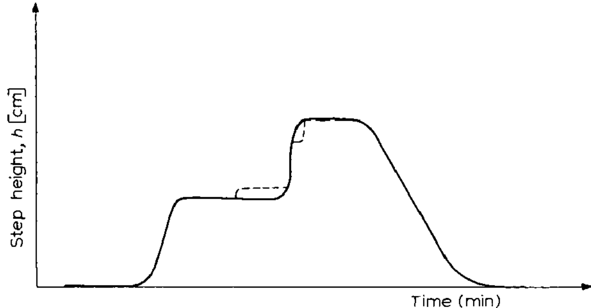

Fig. 8. Chromatogram of the N_2–CH_4 mixture obtained by the FC method.

while retaining an independent calibration since the concentration of each component is determined from the width of one step only. Using the width rather than the height of the step as a parameter offers several advantages (less strict requirements imposed on the detector, decreased effect of fluctuations in the parameters, etc.).

Fig. 8 illustrates this point, where a chromatogram of a nitrogen–methane mixture is shown obtained on a column 130 cm long, 0.5 cm in diameter, packed with activated carbon. Helium was passed through the column. The steps on the chromatogram correspond to helium–nitrogen and helium–methane binary zones.

Since a complex mixture in an FC regime is separated into binary mixtures, each containing only one component of the mixture to be separated, the FC method may be applied to preparative chromatography.

As a fourth application, the determination of the thermodynamic characteristics of a sorbate–sorbent system can be considered. By varying only the "pushing gas" flow rate, characteristic equilibria may be obtained throughout the whole concentration range, using only one sample containing an unknown concentration of the component of interest.

Finally, the method may be employed to prepare a binary mixture of a definite concentration; for this purpose it is sufficient, according to eqn. 2, to fix a value of $\eta' = a/w$.

The methods described above are based on the rejection of the principle of elution chromatography. Within the limits of elution chromatography, however, there are important problems associated with the radical improvement of the characteristics of the methods. These are in the first place concerned with increasing the separation efficiency. A significant decrease in HETP requires a modification of the granular sorbents commonly used. To solve the problem it is not only the diffusion path, *i.e.* the section of the channels along which the carrier gas is moving, that should be decreased, but the uniformity of their sizes and distribution has to be secured as well. Two new approaches for solving the problem are presented*.

Decreasing the grain size of commonly used sorbents is limited by the phenomenon of agglomeration of the particles, with the result that resistance is sharply increased and the mass transfer characteristics are impaired. The agglomeration is produced by the adhesion of particles and associated with the presence of roughness of the surface. That is why in the first version of the method developed it is suggested

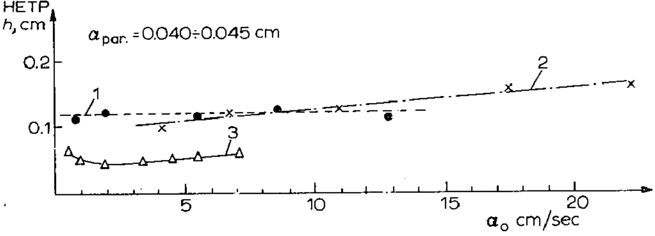

Fig. 9. HETP–a relationship for a filter (1) and for packed columns of different diameter (2, 3) with stainless-steel powder, particle diameter = 0.040–0.045 cm. 1: filter, column length = 16 cm, diameter of column = 1.6 cm, 0.42 w/w % hexadecane. 2: packed column, length 20 cm, diameter 0.65 cm, 0.712 w/w % hexadecane. 3: packed column, silanized, length 7 cm, diameter 3.9 cm, 0.335 w/w % hexadecane.

* The work has been carried out in collaboration with S. M. YANOVSKI and O. N. ALKSNIS.

that very small metallic balls which can be made absolutely smooth are used as a support for a common granular bed. In the second version it is proposed that a rigid structure of the filter type which may be supported on sintered and pressed metallic balls be used. Fig. 9 shows the HETP–α relationship for a filter (Fig. 9, curve 1) and for packed columns of different diameters (curves 2, 3). An examination of curves shows that in addition to high efficiency the new proposed packings result in a very insignificant dependence of HETP on flow rate (α) and column diameter. These facts suggest that such columns may be used in high speed (express) and preparative chromatography.

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INVESTIGATION OF CHARGE-TRANSFER INTERACTIONS BETWEEN CARBON TETRABROMIDE AND AROMATIC DONORS BY GAS CHROMATOGRAPHY*

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SUMMARY

A gas chromatographic method has been employed to study the charge-transfer interactions between alkylated benzenes and molten CBr_4. Resultant activity coefficients have been analyzed in terms of the constituent intermolecular forces. Experimental results indicate a weak, but genuine charge-transfer interaction between aromatic solute and CBr_4. The charge-transfer interaction is temperature dependent, decreasing rapidly in magnitude with increasing temperature. An increasing complexation effect is noted for the addition of electron-repelling groups on the benzene nucleus.

INTRODUCTION

Investigations of charge-transfer complexation by GLC

The utilization of aromatic charge-transfer complexing in GLC was initiated in order to effect difficult aromatic isomer separations. As early as 1955, an aromatic addition complex of picric acid–fluorene was used to separate aromatic compounds[1]. In the ensuing years, many studies invoked the concept of π-electron donation of the solute to the solvent[2–6], but few could unequivocally establish that such interaction was taking place[7].

Perhaps the most extensive studies of aromatic charge-transfer complexing in gas chromatography are those of LANGER, PURNELL, and coworkers. Initially, charge-transfer complexing was inferred from unusual selectivities shown for aromatic compounds and the elution of *m*-xylene before *p*-xylene, an order not predicted on vapor pressure considerations alone[8,9]. Additional studies[10–13] with tetrahalophthalates as well as other aromatic selective liquid phases interpreted chromatographic elution parameters in terms of excess thermodynamic solution functions. Unfortunately,

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these substrates are complex in their electronic effects on solutes, so that the interpretation of excess functions is, at best, difficult. Further studies of this type employing 1,3,5-trinitrobenzene[14] and di-*n*-nonyl tetrachlorophthalate[15] as column substrates have invoked spectrophotometrically determined association constants to help explain trends in retention data.

Additional evidence indicating the value of GC in elucidating complexing phenomena has been provided by CVETANOVIC and co-workers, who studied the interaction of olefinic solutes with 1,3,5-trinitrobenzene[16]. Similarly, charge-transfer interactions have also been postulated from Rohrschneider plots[17,18], and from Kovats retention indices in a study employing molten quaternary ammonium salts as solvents[19].

Charge-transfer interaction between CBr_4 and aromatic donors

Phase studies were perhaps the first positive indication that molecular complexes could form between alkylated benzenes and CBr_4. KAPUSTINSKII AND DRAKIN[20] have examined the phase diagram of CBr_4 with benzene and found a maximum corresponding to a compound of the formula CBr_4–C_6H_6. STRIETER AND TEMPLETON[21] examined the solid adduct formed between CBr_4 and *p*-xylene by X-ray crystallography and found that their data were consistent with an orthorhombic unit cell[22] in which each planar aromatic ring is flanked on both sides by a bromine atom. The authors attributed formation of the solid adduct (m.p. 53° C) to a packing arrangement which cannot be achieved by the acceptor with other xylenes. Thus the selectivity of CBr_4 for the *p*-xylene in separation techniques[14] is spatial in nature and depends little upon the effect of the alkyl substituents in the charge-transfer interaction. This idea has been verified independently by GOATES *et al.*[15], in a study of compounds possessing similar interactions.

HOOPER[23] has studied the pure electrical quadrapole resonance frequencies of the CBr_4–*p*-xylene complex at liquid nitrogen temperatures and concluded that little, if any, charge-transfer interaction is present in the ground state. His conclusions are based on the absence of a frequency lowering for the halogen which if present would indicate a change in the electric field gradient associated with the nucleus. Nevertheless, photochemical irradiation of polyhalogenated methanes, including CBr_4, in hydrocarbon glasses reveals the production of color centers in the matrix[24]. It has been suggested that these centers are actually trapped electrons, produced from charge-transfer interactions.

Spectroscopic techniques have also been used to accumulate considerable evidence for complexing between CBr_4 and aromatic compounds. DOERR AND BUTTGEREIT[25] postulated 1:1 adducts for CBr_4 with hexamethylbenzene and mesitylene based upon the appearance of a new band in the UV spectra of these compounds. More extensive UV spectral results were obtained by TRAMER[26], who investigated the complexes formed between CBr_4 and the following donors: benzene, toluene, *p*-xylene, mesitylene, and α-chloronaphthalene. Polarization spectra of single crystal samples of the CBr_4–*p*-xylene complex were also studied. UV shifts increased as the benzene ring became more alkylated, an effect which was ascribed to charge-transfer transitions. TRAMER asserted that these were weak donor–acceptor complexes, analogous in crystal structure to the corresponding I_2 and Br_2 complexes, but much less stable.

DEMAINE[27] has pointed out the need for non-spectrophotometric studies of complex formation to verify spectrophotometric results, particularly when the asso-

ciative interactions are non-color producing London and dipole–dipole interactions. HAYMANN[28] in a treatise has further questioned the reliability of spectrophotometric methods in comparison with partition methods for determining equilibrium constants of complexes. He asserts that spectrophotometric methods are affected by the formation of termolecular complexes, which do not affect the partition method provided that measurements are carried out in dilute solution.

Gas–liquid partition chromatography offers a method of studying complexing in dilute solution, *i.e.* infinitely dilute solution. Although association constants cannot always be determined by GC, analysis of solution parameters determined by GC can be extremely helpful in ascertaining complexation. Further, as noted by one author[29], GC may indeed be the preferred method of studying weak charge-transfer complexes in solution.

EXPERIMENTAL

Preparation of the columns

CBr_4 (Eastman Distillation Products Industries) having a melting point of 90–91° C was employed for this work. The support upon which the CBr_4 was distributed was Chromosorb G, a product of the Johns-Manville Corporation having a mesh range of 45/60. Coating solutions of CBr_4 were prepared by dissolving CBr_4 with gentle heating in Baker Grade (Reagent) benzene. The method of PARCHER AND URONE[30] was used to distribute the CBr_4 over the Chromosorb. At the conclusion of the fluid drying the coated support was removed carefully and weighed in a stoppered bottle. The amounts of CBr_4 deposited on the support for two different coatings were 19.02% w/w and 29.25% w/w, respectively.

The volatility of CBr_4 necessitated the use of a presaturator to replenish the solvent lost via entrainment in the carrier gas. Presaturator design and use have been adequately described in the literature[31,32]. The presaturator constructed for use in this study consisted of a copper tube, 21.1 cm in length × 1/4 in. O.D., packed with support and solvent in identical proportions to that present in the analytical column.

Instrumental parameters

The necessity of applying a presaturator column voided the use of the normal injection port provided with the commercial chromatograph, hence an injection port was constructed. It consisted of a 1/4 in. O.D., copper Swagelock tee which was wound with resistance wire connected to a rheostat. Asbestos fiber mat was used as insulation to prevent heat loss. The temperature of the injection port was monitored by an iron–constantan thermocouple embedded beneath the insulating fiber.

The injection port was placed in a steel can enclosure and insulated with glass wool. The steel can was fitted flush with an asbestos that covered the oven of the gas chromatograph. A small hole was drilled into the board, concentric with the injection septum mounted in the Swagelock tee. This arrangement when placed in the column oven produced little effect on the SCR controller of the commercial instrument.

The flow pattern is illustrated in Fig. 1. Helium was employed as a carrier gas. In order to minimize gas phase non-ideality in the analytical column, the ratio of p_i/p_o was kept at approximately 1.15. The Moore flow controller supplied with the gas chromatograph, soap bubble flow meter for measuring outlet flow rates, and U-

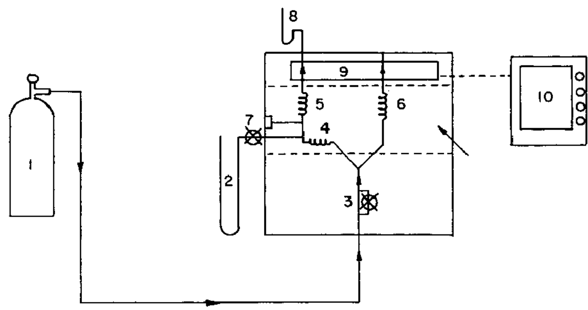

Fig. 1. Experimental flow schematic. 1 = Carrier gas supply; 2 = manometer; 3 = flow controller; 4 = presaturator; 5 = analytical column; 6 = dummy column; 7 = injection port; 8 = flow meter; 9 = thermal conductivity cell; 10 = recorder. Dotted lines indicate thermostatted chamber.

tube manometer for reading inlet pressure were all thermostatted to $\pm 0.5°$ C.

The commercial gas chromatograph employed with the above described modifications was a Varian Aerograph Model 202. The detection unit was a dual thermal conductivity cell employing WX filaments. Differential displays of the elution profiles were recorded by a Sargent Model SRL recorder. Chart paper was kept and measured in a room controlled to $\pm 0.5°$ C in order to prevent expansion or contraction of cellulose fibers.

Temperature measurement

Temperature measurement was accomplished with the apparatus schematically illustrated in Fig. 2. The potentiometer employed was a Leeds & Northrup Model K with a range of 5 V to 1 mV. A D'Arsonval-type galvanometer, Leeds & Northrup Model No. 2430, was employed as a null detector. Its CDRX was 2100 Ω, internal

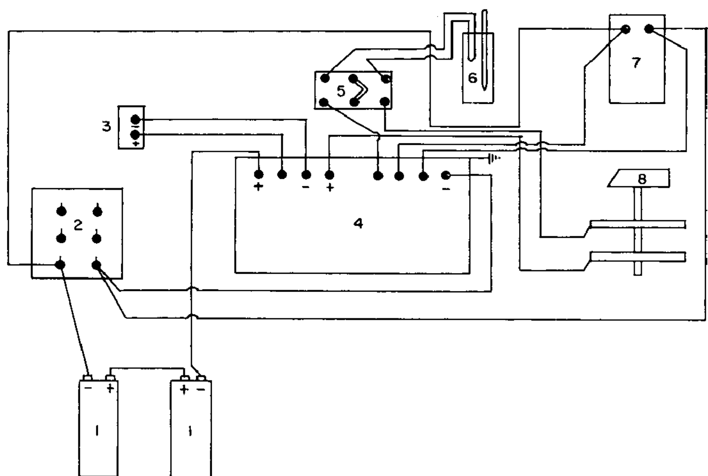

Fig. 2. Electrical measurement schematic. 1 = Dry cells; 2 = resistance box; 3 = standard cell; 4 = potentiometer; 5 = switch; 6 = ice point reference; 7 = galvanometer; 8 = rotary switch.

TABLE I

INSTRUMENTAL PARAMETERS REQUIRED FOR THE CALCULATION OF RETENTION DATA

Property	*93.7 °C*	*104.5 °C*	*105.3 °C*	*113.9 °C*	*123.6 °C*
F (ml/min)	26.2	27.0	31.5	32.0	24.7
T (°K)	366.8	377.7	378.5	387.1	396.8
T_a (°K)	296.2	292.6	296.8	296.8	292.6
p_w (mm Hg)	21.0	19.4	21.8	21.8	19.4
$p_a = p_o$ (mm Hg)	734.0	734.0	737.8	737.8	734.0
F_c (ml/min)	31.5	34.0	39.0	40.5	32.6
p_i (mm Hg)	841.0	841.0	832.0	887.2	841.0
j (unitless)	0.930	0.930	0.939	0.795	0.930
w (g)	6.5209	6.5209	6.1095	6.1095	6.5209

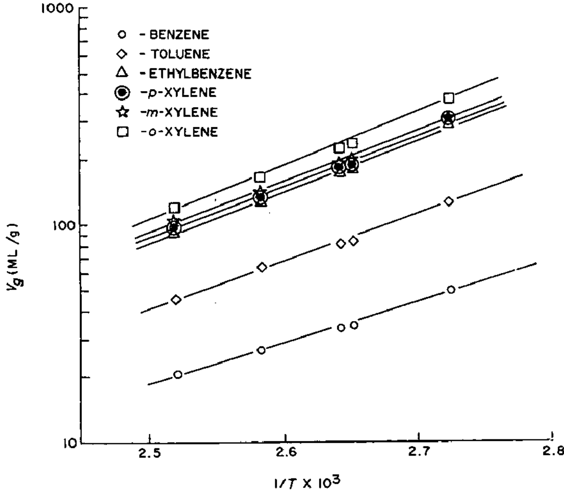

Fig. 3. Specific retention volume *vs.* reciprocal temperature.

TABLE II

SPECIFIC RETENTION VOLUMES *VS.* TEMPERATURE FOR AROMATIC DONORS IN CBr_4

All figures are in units of ml/g.

Compound	*93.7 °C*	*104.5 °C*	*105.3 °C*	*113.9 °C*	*123.6 °C*
Benzene	48.5	33.8	33.2	26.2	20.6
Toluene	123.5	80.6	79.1	62.4	44.9
Ethylbenzene	279.4	172.1	168.3	123.4	91.1
m-Xylene	305.5	188.9	184.5	136.7	101.5
p-Xylene	299.0	183.4	178.9	131.9	97.4
o-Xylene	365.1	224.3	219.0	158.6	118.9

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resistance 25 Ω, giving it a period of 2.7 sec. The d.c. source for this circuit consisted of two $1\frac{1}{2}$-V dry cells. A reference potential was provided by an Epley Student Cell having a rating of 1.0183 V. Variable resistance was provided by a Leeds & Northrup decade box ranging from 0.1–10^3 ohms. A rotary switch with six insulated iron–constantan thermocouples was used to monitor temperature.

Experimental procedure

Solutes were obtained from two sources: the benzene, toluene, and ethylbenzene were Phillips 99 mole % grade while the xylene isomers were obtained courtesy of Sinclair Petrochemicals, Inc. All solutes were used without further purification. The dependence of the solute retention on sample size was checked by varying the injection sample size. Variance of the sample size for a tenfold range (1.0–0.1 μl) produced no change in retention volume or peak symmetry.

The temperature of the injection block was kept at 230° C while the compartment housing the T.C. bridge registered 204° C on the pyrometer gauge. Cell currents were consistently 133 mA. Outlet flow rates ranged from 25–40 ml/min. Temperature fluctuations of the oven bath were minimized to $\pm$0.1° C at 93.7° C and to $\pm$0.15° C at 113.9° C. The latter figures were obtained by a time *versus* temperature fluctuation study of various thermocouple probes.

Analysis of each solute was done in triplicate employing 0.1-μl sample sizes. Although some of the retention times were extremely long at this relatively slow flow rate, the accuracy gained in being able to reproduce retention times gave further credence that equilibrium conditions were prevailing in the column. Retention times were converted to specific retention volumes, V_g, using the following equation:

$$V_g = jF_c 273(t_R - t_A)/w_L T \quad (1)$$

where

j = gas compressibility factor
F_c = corrected flow rate
t_R = retention time of solute
t_A = retention time of unsorbed solute
w_L = solvent weight
T = column temperature

The numerical quantities required for these calculations are listed in Table I. Specific retention volumes have been tabulated in Table II.

It is perhaps pertinent to note the variance of V_g with column temperature. This is depicted in Fig. 3. The ability to reproduce V_g independent of solvent weight is clearly shown in the close agreement of V_g at 104.5° C and 105.3° C. Thus, activity coefficients at infinite dilution, γ^∞, can be calculated with confidence and used to interpret solution processes.

RESULTS AND DISCUSSION

Specific retention volumes were used to calculate activity coefficients at infinite dilution by using eqn. 2

$$V_g = 273R/p^0 M_L \gamma^\infty \quad (2)$$

where

p^0 = saturated vapor pressure of solute at column temperature
M_L = molecular weight of solvent
R = 6.3×10^4 ml-mm/mole-°K

Vapor pressure values were computed using the well-known Antoine equations of the form

$$\log_{10} p^0 \text{ (in mm Hg)} = A - (B/t + C) \quad (3)$$

where t is the temperature of the column in °C and A, B, C are the Antoine constants for the solute[33]. The derived activity coefficients at infinite dilution are listed in Table III.

TABLE III

ACTIVITY COEFFICIENT AT INFINITE DILUTION *vs.* TEMPERATURE FOR AROMATIC DONORS IN CBr_4
All figures are unitless.

Compound	*93.7 °C*	*104.5 °C*	*105.3 °C*	*113.9 °C*	*123.6 °C*
Benzene	0.939	0.994	0.994	1.007	1.015
Toluene	0.915	0.998	0.995	0.984	1.053
Ethylbenzene	0.894	0.998	0.996	1.035	1.052
m-Xylene	0.902	0.998	0.997	1.020	1.027
p-Xylene	0.894	1.000	1.000	1.031	1.045
o-Xylene	0.892	0.986	0.985	1.027	1.036

Activity coefficients can yield much information on the intermolecular forces that are present in solution. Further, the activity coefficient also reflects the superimposition of size effects in the liquid state. From basic thermodynamics, the definition of the excess partial molar free energy, $\Delta\bar{G}_e^0$, is

$$\Delta\bar{G}_e^0 = RT \ln \gamma^\infty = \Delta\bar{H}_e^0 - T\Delta\bar{S}_e^0 \quad (4)$$

from which it can be shown that

$$\delta\Delta\bar{G}_e^0/\delta T = -\Delta\bar{S}_e^0 \quad (5)$$

and

$$\delta(\Delta\bar{G}_e^0/T)/\delta(1/T) = \Delta\bar{H}_e^0 \quad (6)$$

where $\Delta\bar{H}_e^0$ and $\Delta\bar{S}_e^0$ are the excess partial molar enthalpy and entropy of solution, respectively. Eqn. 4 may be rewritten in the form

$$\ln \gamma^\infty = \Delta\bar{H}_e^0/RT - \Delta\bar{S}_e^0/R \quad (7)$$

which suggests that the activity coefficient may be regarded as a composite function, or as expressed by ASHWORTH AND EVERETT[34]

$$\ln \gamma^\infty = \ln \gamma^s + \ln \gamma^e \quad (8)$$

where $\ln \gamma^e$ and $\ln \gamma^s$ are the thermal and the athermal contribution to the activity coefficient.

The sign and magnitude of the partial molar excess enthalpy of solution can be extremely difficult to interpret, especially if either the solute or the solvent are structurally complex. PURNELL AND LANGER[12,35] have recognized that $\ln \gamma^e$ is a composite of heats, $\Delta \bar{H}_{e\alpha}{}^0$, $\Delta \bar{H}_{e\beta}{}^0$, etc., some of which may be favorable to solution (negative) and others positive, forcing the molecules of solute out of the liquid phase. Thus, the fact that $\Delta \bar{H}_e{}^0$ is not negative in sign does not imply that complexing forces are not operative in solution. Further, large positive enthalpic contributions may mask out energetically weak complexing interactions which give rise to negative deviations from Raoult's Law.

Inspection of the activity coefficient values in Table III reveals a trend at 93.7° C supporting increasing charge-transfer interaction with alkylation of the benzene ring. This trend completely disappears at higher temperatures. Therefore, it seems strange that the apparent drop-off in complexing ability is abrupt and not temperature dependent.

Various association parameters can be obtained by comparing the elution data (*e.g.* V_g) of solutes capable of undergoing charge-transfer complexing on an inert solvent *versus* their V_g values on a charge-transfer complexing solvent. By use of eqn. 9

$$V_g = 273R/\gamma' p^0 M_L \tag{9}$$

MARTIRE AND RIEDL[36] have been able to calculate the equilibrium constant of hydrogen-bond formation as well as the corresponding enthalpies of formation. Here γ' is the apparent (measured) activity coefficient which is defined as

$$\gamma' = \gamma_u(1 - c) \tag{10}$$

where γ_u is the activity coefficient at infinite dilution, and c is the fraction of solute molecules complexed. To measure γ_u requires specific retention volumes determined on an analogous non-complexing stationary phase. For CBr_4, the non-complexing analog would be CH_4, which at the temperatures employed in this study would be extremely difficult to use as a liquid phase.

An alternative procedure would be to assume that γ_u is approximately the same for a number of structurally similar solutes. Thus, following the procedure adopted by LANGER *et al.*[9], one could assign a value of unity to $(1 - c)$ and measure relative complexing tendency to a particular solute. If this is done for the data in Table III at 93.7° C and the assignment of $(1 - c) = 1$ is for benzene, then c increases as alkylation of the benzene ring increases. However, following this procedure for solutes at higher temperatures gives anomalous results.

It may well be that molecular size differences account for the results at higher temperatures. Recalling eqns. 7 and 8, it can be shown that

$$\ln \gamma^s = -\Delta \bar{S}_e{}^0/R \tag{11}$$

Using the Flory–Huggins approach, at infinite dilution

$$\gamma^s = (1/m)\, e^{1-(1/m)} \tag{12}$$

where $m = v_2/v_1$ and v_2 and v_1 are the molar volumes of solvent and solute, respec-

tively, the contribution to γ^∞ from γ^s is negligible in the case for our solutes ($\ln \gamma^s =$ 1). This is not surprising since the molar volumes of the solute and solvent in this experiment are nearly equal. Hence the activity coefficient at infinite dilution is directly related to the thermal portion of the activity coefficient or

$$\gamma^\infty = \gamma^e \tag{13}$$

The general form of $\ln \gamma^e$ is given by eqn. 14

$$\ln \gamma^e = k\Delta E \tag{14}$$

where ΔE is the summation of molar interchange energies. Thus $\Delta \bar{H}_e{}^0$ can be regarded as a sum of pairwise potential energies of interaction between solute and solvent molecules, or in general

$$\ln \gamma^e = k(E_{11} + E_{22} - 2E_{12}) \tag{15}$$

As BROWN[37] has pointed out, E_{12} is predominantly a measure of electron donor–acceptor interactions between solute and solvent. Thus, if one could obtain a general equation for the calculation of γ^e, the strength of E_{12} could be ascertained.

In 1961, MARTIRE[38] derived the following equation from the Van Arkel modification of Hildebrand's regular solution theory

$$\ln \gamma^e = (v_1\varphi_2{}^2/RT)\ [(\delta_1 - \delta_2)^2 + (\omega_1 - \omega_2)^2 - K] \tag{16}$$

where

φ_2 = volume fraction of solvent in solution
δ_2 = solubility parameter of the solvent
δ_1 = solubility parameter of the solute
ω_2 = orientation parameter of the solvent
ω_1 = orientation parameter of the solute
K = residue force parameter

Although originally proposed for the prediction of activity coefficients, MARTIRE's equation allows the estimation of molecular forces present in solution. Further, the above equation shows remarkable ability to predict accurate activity coefficients at infinite dilution and has been employed with success by several workers[39,40].

A regular solution is one involving no entropy change when a small amount of solute is transferred to it from an ideal solution of the same composition. Initially, regular solution theory was applied only to molecules displaying dispersion force interaction in solution. To account for other forces, such as orientation fields, the Hildebrand–Scatchard expression for the activity coefficient, eqn. 17, must be modified.

$$\ln \gamma^\infty = (v_1/RT)\varphi_2{}^2\ (\delta_1 - \delta_2)^2 \tag{17}$$

By adding terms characteristic of the positive and negative heats, activity coefficients less than unity become possible to predict. Despite the many assumptions in the regular solution theory, it has worked remarkably well for correlating solution behavior. In gas chromatography alone, the regular solution theory has been applied to predict activity coefficients[41–43], correlate retention data[44,45], and in the selection of liquid phases[35,46].

Inspection of eqn. 16 reveals some very interesting details. If γ^∞ is known and the contribution of dispersion forces, $(\delta_1 - \delta_2)^2$, and orientation forces, $(\omega_1 - \omega_2)^2$, can be estimated, then it should be possible to calculate K, the residue force parameter. If CBr_4 is undergoing charge-transfer interaction with the benzene ring, then K should reflect these interactions, since charge-transfer interactions are known to give negative deviations from Raoult's Law[47]. Further, in this specific complexing case, K should reflect the electron donor–acceptor interaction solely since it has already been shown that size effects do not contribute to $\ln \gamma^\infty < 1$.

The quantity $(\delta_1 - \delta_2)$ requires knowledge of the variance of δ with temperature. The solubility parameter, δ, is a measure of the cohesive energy density of the molecular species under consideration. Since δ is defined as

$$\delta = (\Delta E^{vap}/v)^{\frac{1}{2}} \tag{18}$$

the variation of the solubility parameter with temperature is related to the density dependence of v, the molar volume. Employing eqn. 19 (ref. 48)

$$\mathrm{d} \ln \delta / \mathrm{d} \ln v = -1.25 \tag{19}$$

and integrating

$$\ln \delta_{T_2} - \ln \delta_{T_1} = -1.25 \ln (v_{T_2}/v_{T_1}) \tag{20}$$

yields the desired relationship between δ and T. Solubility parameters, δ_T and v_T were selected from HILDEBRAND AND SCOTT[49]. The computation of δ_{T_2} for CBr_4 was made employing the Clausius–Clapeyron equation

$$\mathrm{d} \ln p^0/\mathrm{d}T = \Delta H^{vap}_{app}/RT^2 \tag{21}$$

using P-V-T data[50]. Calculation of δ_2 was then facilitated by employing eqn. 22

$$\delta \cong [(\Delta H^{vap}_{app} - RT)\, Z/v]^{\frac{1}{2}} \tag{22}$$

Values of δ are listed in Table IV, while the square of the difference in the solubility parameters, $(\delta_1 - \delta_2)^2$, are tabulated in Table V.

The orientation parameter, ω, is estimated from the expression for the average energy of a point dipole in a liquid, eqn. 23, to be

$$e = 2\mu^4/3r^6kT \tag{23}$$

TABLE IV

δ *vs.* TEMPERATURE

All figures are in units of cal. $^{0.5}$/cc.$^{1.5}$.

Compound	*93.7 °C*	*104.5 °C*	*105.3 °C*	*113.9 °C*	*123.6 °C*
Benzene	8.22	7.98	7.97	7.90	7.78
Toluene	8.09	7.90	7.92	7.82	7.63
Ethylbenzene	8.01	7.87	7.87	7.75	7.65
m-Xylene	7.97	7.91	7.91	7.83	7.78
p-Xylene	7.99	7.86	7.85	7.74	7.65
o-Xylene	8.23	7.98	7.98	7.90	7.77

TABLE V

$(\delta_1 - \delta_2)^2$ *vs.* TEMPERATURE

All figures are in units of cal./cc.

Compound	*93.7 °C*	*104.5 °C*	*105.3 °C*	*113.9 °C*	*123.6 °C*
Benzene	0.030	0.078	0.078	0.123	0.212
Toluene	0.032	0.130	0.109	0.185	0.372
Ethylbenzene	0.068	0.152	0.144	0.250	0.348
m-Xylene	0.090	0.123	0.116	0.176	0.212
p-Xylene	0.078	0.160	0.160	0.260	0.348
o-Xylene	0.002	0.078	0.073	0.160	0.221

where

r = distance between dipole centers

μ = the dipole moment of the molecule

The activity coefficient at infinite dilution must be unitless, so provided that ω^2 must have units of energy/volume, the average orientation energy must be divided by the volume for unit consistency. Hence

$$\omega = \mu^2(2/3vr^6kT)^{\frac{1}{2}} \tag{24}$$

making

$$(\omega_1 - \omega_2)^2 = \frac{2N^4\,[\mu_1^2/(v_1)^{\frac{1}{2}} - \mu_2^2/(v_2)^{\frac{1}{2}}]}{3RT\,r^6\,(4.18 \times 10^7)^2} \tag{25}$$

where N is Avogadro's number and k is the Boltzmann constant.

Considering the spherical symmetry of CBr_4, the Clusius–Weigand model employed by MARTIRE is equally applicable here. This model assumes a spherical molecule in the force field of six other molecules. The value predicted by the model for a body-centered lattice is

$$v_2 = (3)^{9/4}r^3/(2)^{7/2} \tag{26}$$

or

$$[\{v_2 \times (2)^{7/2}/(3)^{9/4}\}^{1/3}]^6 = r^6 \tag{27}$$

which yields

$$r^6 = (v_2^2/1.047) \tag{28}$$

Thus, the final expression for $(\omega_1 - \omega_2)^2$ is

$$(\omega_1 - \omega_2)^2 = \frac{3.40 \times 10^7}{v_2^2\,RT}\left(\frac{\mu_1^2}{\sqrt{v_1}} - \frac{\mu_2^2}{\sqrt{v_2}}\right)^2 \tag{29}$$

For the solute–solvent system in this study, contributions to $\ln \gamma^\infty$ from $(\omega_1 - \omega_2)^2$ are small, and in many cases zero. The μ for CBr_4, benzene, and *p*-xylene is zero in deference to their spherical symmetry, while the μ for toluene (0.4 D), ethylbenzene (0.36 D), *m*-xylene (0.32 D), and *o*-xylene (0.52 D) are very small[51]. Nonetheless, $(\omega_1 - \omega_2)^2$ has been calculated and is presented in Table VI.

K values are listed in Table VII. Numerically, the value of K increases as the

TABLE VI

$(\omega_1 - \omega_2)^2$ *vs.* TEMPERATURE
All figures are in units of cal./cc.

Compound	*93.7 °C*	*104.5 °C*	*105.3 °C*	*113.9 °C*	*123.6 °C*
Benzene	0	0	0	0	0
Toluene	0.055	0.053	0.053	0.051	0.049
Ethylbenzene	0.033	0.029	0.029	0.039	0.037
m-Xylene	0.032	0.031	0.031	0.031	0.035
p-Xylene	0	0	0	0	0
o-Xylene	0.087	0.084	0.084	0.082	0.079

benzene ring becomes alkylated. Thus, increasing the electron density of the ring contributes significantly to the donor–acceptor interaction. Table VII also reflects the dependence of charge-transfer interaction on temperature. For practically all solutes employed in this study, there is a seven- to eightfold decrease in charge-transfer interaction in going from 93.7° C to 123.6° C. The similarity of K values for ethylbenzene and the xylenes follows random trends observed in other charge-transfer studies[52]. Apparently, there is no special selectivity for *p*-xylene in molten CBr_4. This lends further substantiation to arguments that the formation of a CBr_4–*p*-xylene addition compound is a result of packing geometry and not particularly strong charge-transfer interaction.

TABLE VII

K VALUES *vs.* TEMPERATURE
All figures are in units of cal./cc.

Compound	*93.7 °C*	*104.5 °C*	*105.3 °C*	*113.9 °C*	*123.6 °C*
Benzene	0.489	0.120	0.120	0.067	0.068
Toluene	0.589	0.144	0.144	0.080	0.083
Ethylbenzene	0.683	0.165	0.165	0.092	0.095
m-Xylene	0.686	0.164	0.164	0.091	0.092
p-Xylene	0.688	0.166	0.163	0.093	0.095
o-Xylene	0.651	0.159	0.159	0.089	0.092

How strong are these charge-transfer forces in liquid CBr_4? A measure of this can be found by comparing K for the CBr_4–benzene complex with the K for a well-known charge-transfer complex, for example I_2–benzene. If one takes the difference in solubility parameters for I_2 and benzene at 25°C, $(\delta_1 - \delta_2)^2 = 24.5$ cal./cc, and assuming $(\omega_1 - \omega_2)^2 = 0$, then $K > 24.5$ cal./cc for this system to exhibit a negative deviation from Raoult's Law. Thus, the CBr_4–benzene charge-transfer interaction is indeed a weak one at elevated temperatures.

CONCLUSION

There is apparently a weak, but genuine charge-transfer interaction between aromatic solute and CBr_4 under the conditions employed in this set of experiments.

This is in agreement with the earlier mentioned phase studies and spectral data. The charge-transfer interaction is temperature dependent, decreasing rapidly in magnitude with increasing temperature. K values determined by the latter method show an increasing complexation trend with the addition of electron-repelling groups on the benzene nucleus. The apparent selectivity of CBr_4 for *p*-xylene so evident in the solid state is absent at these temperatures. Thus, factors other than charge-transfer interaction are responsible for the *p*-xylene–CBr_4 addition complex.

The method presented here for determining charge-transfer complexing is, in theory, applicable to other types of intermolecular forces that cause negative deviations from Raoult's Law, *i.e.* hydrogen bonding. Several requirements will have to be observed, however:

(1) Size effects must be accounted for. This is made possible by subtracting out the γ^s contribution to γ^∞ via eqn. 8.

(2) Accurate δ and ω parameters must be obtained.

(3) K must reflect a dominant force which gives rise to negative departures from solution ideality.

Point (1) is readily obtainable through experiment or statistical–mechanical formulations. Point (2) should present little problems for well characterized molecules. Point (3) of course can only be verified by continuous application to a wide variety of solute–solvent systems. Present work indicates its applicability may be more universal than previously thought. In theory, the above results are internally consistent with the results obtained for electron donor–acceptor interaction using the method advocated by ROHRSCHNEIDER[53] and the extraction postulates of PRAUSNITZ and coworkers[54].

In summary, the above method for ascertaining complexing may be particularly useful when Class A, Class D, and comparative Class B methods, as defined by PURNELL[55], are not applicable. This especially would be the case for volatile solvents where there is a lack of an "inert solvent" for experimentally determining the dispersion contribution to the activity coefficient.

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CHROM. 4657

A STUDY OF THE POLARITY OF SOME STATIONARY PHASES USED IN GAS-LIQUID CHROMATOGRAPHY

ESTERS OF PHTHALIC ACID AND THEIR ISOMERS

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SUMMARY

The relationship between the structure of the stationary phases and their gas chromatographic polarities is examined for a group of phthalic esters.

The dependence of the polarity on the structure of the phase is demonstrated by 25 esters of benzenedicarboxylic acids, their isomers and other similar compounds. It is found that lengthening the chain of the esterifying alcohol in the phthalic esters decreases the polarity of the phase.

The lowest polarities are found in the case of di-*n*-decylphthalate, di-*n*-hexylisophthalate and di-*n*-hexylterephthalate, and the highest in the case of di-γ-phenylpropylphthalate, diallylphthalate and di-*n*-butylphthalyl-bis-glycolate.

It is thought that the data obtained in this work might be of use in practical gas-liquid chromatography as a basis for the selection of a liquid phase from the group consisting of the phthalic esters.

INTRODUCTION

We have previously reported[1-3] the influence of the structure of phthalic acid esters on the activity coefficients of some paraffins, cyclohexanic and aromatic hydrocarbons. The activity coefficient, however, is a complex quantity depending on the properties of the molecules of the solute and the solvent. One of the most interesting properties of the stationary phases used in gas-liquid chromatography (GLC) is undoubtedly their polarity.

The present study is an attempt to ascertain the influence of the structure of the esters mentioned above on their gas chromatographic (GC) polarity. It is the authors' opinion that, apart from the theoretical interest, such an investigation would also be useful from the point of view of practical GLC.

Some years ago ROHRSCHNEIDER[4] suggested an empirical method for the determination of relative GC polarity of a stationary phase. This method, in spite of some weak points, is now the only method widely used in the field of GLC.

The polarity of the liquid phase, according to ROHRSCHNEIDER, could be calculated from the expression:

$$P = 100 - 100 \frac{q_1 - q_x}{q_1 - q_2} \quad (1)$$

where q_1 is the logarithm of the ratio of the specific retention volumes of butadiene: butane measured on a β, β'-oxydipropionitrile column at 70°, q_2 is the logarithm of the ratio measured on a squalane column and q_x is that measured on the column with the liquid phase under investigation.

CHOVIN AND LEBBE[5], with the purpose of making the method more general, suggested a better standard pair, benzene/cyclohexane. These compounds are easily obtainable in every laboratory and with them it is possible to measure the polarity at higher temperatures than with the butadiene/butane pair.

EXPERIMENTAL

The specific retention volumes of benzene and cyclohexane on the liquid phases under investigation were measured with a "Fractovap" model B apparatus, produced by Carlo Erba, Italy.

Pure dry nitrogen was used as carrier gas at an outlet flow rate of 120 ml/min.

The stainless steel column was 2 m long and had an internal diameter of 6 mm. It was packed with ground unglazed tile (particle diameter 0.2–0.4 mm) and the stationary phase amounted to 15.3% (w/w) of the inert support.

Benzene and cyclohexane were used as the standard volatile compounds and β,β'-oxydipropionitrile and squalane were the standard stationary phases.

The polarity of the phases under investigation was measured at 120°. The specific retention volumes of the standard compounds on β,β'-oxydipropionitrile and squalane are shown in Table I. The data in Table I are the arithmetic means from five measurements of every retention volume.

TABLE I

SPECIFIC RETENTION VOLUMES OF BENZENE AND CYCLOHEXANE ON β,β'-OXYDIPROPIONITRILE AND SQUALANE IN ml

No.	*Standard compound*	*Squalane*	*β,β'-Oxydipropionitrile*
1	Benzene	29.3	19.6
2	Cyclohexane	33.3	3.6

All the esters under investigation are shown in Table II. With exception of the dinonylphthalate (BDH Ltd.), all of them were synthesized by the authors as described earlier[1–3].

RESULTS AND DISCUSSION

Table II gives the values of the specific retention volumes of benzene and cyclohexane and the corresponding liquid phase polarity according to eqn. 1. On the

TABLE II

SPECIFIC RETENTION VOLUMES OF BENZENE AND CYCLOHEXANE AND POLARITY OF THE STATIONARY PHASES UNDER INVESTIGATION

No.	*Stationary phase*	*Specific retention volume*		*Polarity*
		Benzene	*Cyclohexane*	
1	Di-*n*-propylphthalate	18.0	35.2	44
2	Di-*n*-butylphthalate	19.2	35.2	40
3	Di-*n*-hexylphthalate	20.2	34.2	36
4	Di-*n*-octylphthalate	21.3	32.5	30
5	Di-*n*-decylphthalate	22.4	31.1	25
6	Diphenylphthalate	9.5	19.8	47
7	Dibenzylphthalate	9.9	21.3	49
8	Di-β-phenylethylphthalate	10.7	23.6	51
9	Di-γ-phenylpropylphthalate	10.9	25.2	53
10	Diisopropylphthalate	18.0	35.3	44
11	Diallylphthalate	14.4	32.8	52
12	Di-*sec.*-butylphthalate	18.7	35.2	42
13	Diisobutylphthalate	17.4	33.3	42
14	Dinonylphthalate	19.8	29.7	29
15	Di-*n*-hexylisophthalate	23.9	32.2	23
16	Di-*n*-hexylterephthalate	24.1	31.3	21
17	Dicyclohexylphthalate	15.4	27.4	38
18	Dibornylphthalate	9.1	18.8	47
19	Catecholdibutyrate	16.6	31.8	43
20	Catecholdibenzoate	11.5	21.8	42
21	Di-*n*-hexyl-4-nitrophthalate	17.0	32.4	42
22	Di-*n*-butyltetrachlorophthalate	17.9	33.7	42
23	Di-*n*-butylphthalyl-bis-glycolate	11.9	27.0	52
24	Di-*n*-hexyladipate	27.2	42.1	31
25	Di-*n*-hexylsuberate	27.5	41.4	30

basis of the data in the table some conclusions are possible about the influence of the structure of the phases on their GC polarity.

For instance, it is evident that the increase of the number of $-CH_2-$ groups in the alcoholic alkyl chains of the di-*n*-alkyl- and di-ω-phenylalkylphthalates leads to a linear change in the polarity. The difference between the polarity of the di-*n*-propyl- and di-*n*-decylphthalate is 19 units. If the phases are di-*n*-alkylphthalates (Fig. 1),

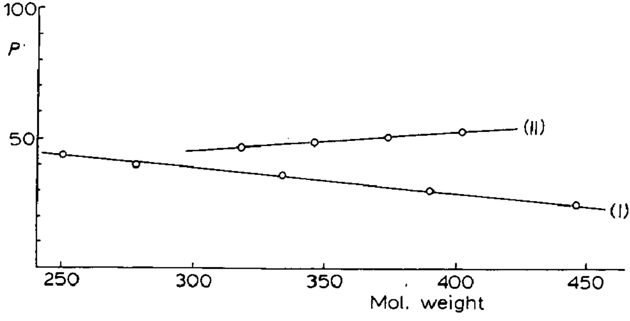

Fig. 1. Plot of the polarity of the phases under investigation *vs.* the molecular weight of the di-*n*-alkylphthalates (I) and of the di-ω-phenylalkylphthalates (II).

an increase in molecular weight causes a decrease in polarity, while in the case of di-ω-phenylalkylphthalates increasing molecular weight increases the polarity.

The nature of the carbon chain in the alcohol alkyl group has only a slight influence on the polarity. This can be seen by comparing the data for the esters of di-*n*-propyl- and diisopropylphthalate, and di-*n*-butyl-, di-*sec.*-butyl and diisobutylphthalate. Also if one compares the value for the dinonylphthalate (No. 14, Table II), which really is di-3,5,5-trimethylhexylphthalate, it corresponds to the polarity of the normal dinonyl ester, as it fits the relationship shown in Fig. 1.

Comparison of the data for the di-*n*-propylphthalate and diallylphthalate suggests that the presence of double bonds in the side chains of the ester leads to an appreciable increase in the polarity.

Comparison of the data for di-*n*-hexylphthalate with those for di-*n*-hexylisophthalate and di-*n*-hexylterephthalate shows that the polarity is highest for the *ortho* and lowest for the *meta* and *para* isomers. The difference between the last two is only 2 units according to ROHRSCHNEIDER's system. These data are in accordance with the values of the dipole moments for esters with similar structure. For example the dipole moments of the dimethyl esters of the isomeric phthalic acids are as follows: *ortho*, 2.75 D; *meta*, 2.46 D; and *para*, 2.40 D[6].

The difference in the polarity of the *m*- and *p*-isomers compared with that of the *o*-isomers is probably due to the steric arrangement of the carboxylic acid groups on the benzene ring: adjacent in the *o*-isomers and on opposite sides in the *p*-isomers.

If the properties of the di-*n*-hexyl-, dicyclohexyl- and diphenylphthalate are compared, it is found that the polarity increases slightly in the presence of a saturated ring structure. When the ring is aromatic, however, this increase is more definite. For example the difference between the polarities of the di-*n*-hexyl- and diphenylphthalate is 11 units.

On the other hand, it is interesting that substitution of the aromatic ring of the phthalic acid with a straight carbon chain with the same number of carbon atoms (total or linear between the ester groups) increases the polarity by about 10 units. (See the esters di-*n*-hexylterephthalate, di-*n*-hexyladipate and di-*n*-hexylsuberate.)

Dibornylphthalate is almost equal to the diphenylphthalate from the viewpoint of its polarity. It can be assumed that the aromatic rings of the latter together with the perpendicular π-orbitals are similar in their steric volume and influence to the bornyl groups.

Catecholdibutyrate does not much differ from its isomer di-*n*-propylphthalate. However with the other isomeric pair—catecholdibenzoate and diphenylphthalate—the difference in the polarities is 5 units.

The introduction of a nitro group in the phthalic ring (*cf.* di-*n*-hexylphthalate and di-*n*-hexyl-4-nitrophthalate) leads to an increase in the polarity of 6 units.

The influence of chlorine as a substituent in this respect is weaker. For instance, the presence of 4 chlorine atoms in di-*n*-butyltetrachlorophthalate increases the polarity only by 2 units with respect to the di-*n*-butylphthalate.

Di-*n*-butylphthalyl-bis-glycolate has a polarity of 12 units higher than that of the di-*n*-butylphthalate. It is thus possible to conclude that an increase in the number of carboxylic groups in the side chains is an effective way of obtaining phases with a high polarity.

The data presented in Table II show that the group of phthalic esters tested as

stationary phases have a low or medium polarity value. Di-*n*-decylphthalate, di-*n*-hexylisophthalate and di-*n*-hexylterephthalate have the lowest GC polarity, and di-γ-phenylpropylphthalate, diallylphthalate and di-*n*-butylphthalyl-bis-glycolate the highest.

The interchange of one phthalic ester for use as a stationary phase with another is possible only on the basis of their polarities.

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CHROM. 4676

REDISTRIBUTION REACTIONS OF TRIS(N-ALKYL)HEXAMETHYL-CYCLOTRISILAZANES

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SUMMARY

Some preliminary investigations into the interchanges involving different cyclotrisilazanes have been carried out. This reaction was found to proceed very easily at moderate temperatures, especially in a polar solvent. Dimethyldichlorosilane was used as the starting material in the preparation of all the heterocycles, synthesis in all cases being relatively straightforward.

INTRODUCTION

MOEDRITZER AND VAN WAZER[1,2] reported the reaction between hexamethylcyclotrisilthian and nonamethylcyclotrisilazane to form two new cyclosilthiazanes. In view of the growing interest in the chemistry of silicon heterocycles containing sulphur and nitrogen atoms in the ring, it was decided to investigate a range of compounds of this type, to see if similar redistributions occurred in other mixed heterocycle systems. It was found that interchange of N-alkyl groups occurred very readily at moderate temperatures, no catalyst being required for the reaction. For each pair of cyclotrisilazanes that were heated together, two new mixed N-alkylcyclotrisilazanes were formed. The reaction appears to be essentially of the same class as that reported for borazoles by MCCLOSKEY *et al.*[3].

NOMENCLATURE

In the present work, several cyclotrisilazanes were used, and the shorthand notation employed is as follows:

$$N^0 = \mathrm{Si{-}N(H){-}Si},\quad N^1 = \mathrm{Si{-}N(Me){-}Si},\quad N^2 = \mathrm{Si{-}N(Et){-}Si},\quad N^3 = \mathrm{Si{-}N(Pr){-}Si}$$

Each cyclotrisilazane can then be written as $N^xN^yN^z$. Thus $N^1N^1N^1$ is nonamethylcyclotrisilazane.

EXPERIMENTAL

Preparation of cyclotrisilazanes

Tris(N-methyl)- and tris(N-ethyl)hexamethylcyclotrisilazanes were prepared as described by ABEL AND BUSH[4]. Hexamethylcyclotrisilazane and octamethylcyclotetrasilazane were prepared by the method of OSTHOFF AND KANTÖR[5].

Preparation of cyclotrisilazanes containing large N-alkyl groups is known to be difficult or in some cases almost impossible by synthetic routes known to date. For example, BREED AND ELLIOTT[6] were unable to prepare the compound $(Me_2SiNBu)_3$, and could recover only 27% of the original bis(N-butyl)dimethylsilane. However, these authors made no reference to any attempt to prepare the tris(N-propyl) compound. In the present work, this compound was synthesized, but could not be isolated in a pure condition.

179 g *n*-propylamine were dissolved in 250 ml dry hexane and cooled in ice. To the well stirred solution was added a solution of 84 g dimethyldichlorosilane in 200 ml *n*-hexane, over a period of 40 min. The reaction mixture was allowed to come to room temperature, and stirred for a further 4 h. The *n*-propylamine hydrochloride was filtered off from the reaction product, *n*-hexane removed by distillation and the residue fractionated to give 81 g bis(*n*-propylamino)dimethylsilane, b.p. 53–54° (12 mm). Microanalysis gave %C = 55.31, %H = 12.83, %N = 15.94 ($C_8H_{22}N_2Si$ requires %C = 55.2, %H = 12.65, %N = 16.1).

77 g of this product was refluxed with 0.1 g ammonium sulphate for 22 h. After this time gas chromatographic analysis indicated the formation of a high-boiling product, but most of the silylamine still remained. An additional small quantity of ammonium sulphate was added, and refluxing continued for a further 16 h.

From the fractionation of the reaction product were obtained 35 g unchanged bis(*n*-propylamino)dimethylsilane, and 9 g of a brown oil, b.p. 56° (20 mm). Gas chromatographic analysis* of the oil showed the presence of a high boiling compound thought to be the desired tris[N-(*n*-propyl)]hexamethylcyclotrisilazane. The oil also contained appreciable quantities of components of low retention volume, and was therefore stripped of these by vacuum sublimation. Microanalysis of the stripped product gave %C = 44.1, %H = 9.48, %N = 10.29 ($C_{15}H_{39}N_3Si_3$ requires %C = 52.1, %H = 11.3, %N = 12.2). Subsequent gas chromatographic analysis indicated that more of the low-boiling impurities had formed. It is possible that some sort of ring-chain equilibrium was occurring, although in such equilibria the chain compound is normally present in very small quantities. The redistribution reactions and gas chromatographic retention data described in this paper leave little doubt that the major component in the oil was, in fact, tris[(N-(*n*-propyl)]hexamethylcyclotrisilazane. The compound also gave an expected distribution of products when heated with hexamethylcyclotrisilthian. Nevertheless, isolation of this compound would obviously be highly desirable.

Redistribution reactions

A series of reactions was run in which $N^1N^1N^1$ and $N^2N^2N^2$ were heated together

* A Pye F104 gas chromatograph, equipped with dual flame ionisation detectors, columns of 6 ft. × 1/4 in. O.D., containing a stationary phase of 12% w/w Silicone Gum SE-30 on Chromosorb W, at a flow rate of 60 ml/min, at 180°.

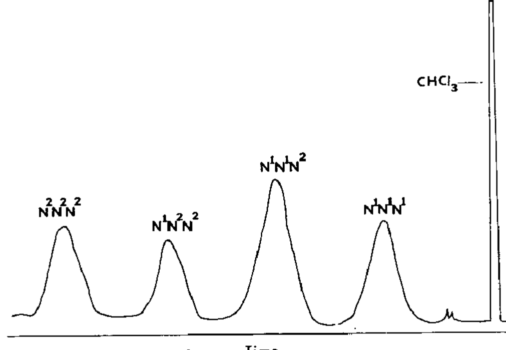

Fig. 1. Chromatogram of the equilibrated mixture from the $N^1N^1N^1$–$N^2N^2N^2$ system in chloroform.

with several solvents at 150° in sealed glass tubes. It was found that two new compounds were formed, with retention volumes intermediate between those of the starting materials (Fig. 1). Neither of these new compounds was formed when the cyclotrisilazanes were heated individually, and their identities were tentatively assigned as $N^1N^1N^2$ [N-ethyl-bis(N-methyl)hexamethylcyclotrisilazane], and $N^1N^2N^2$ [bis(N-ethyl)-N-methylhexamethylcyclotrisilazane], *i.e.*

$$N^1N^1N^1 + N^2N^2N^2 \rightleftharpoons N^1N^1N^2 + N^1N^2N^2$$

It was found that formation of the new compounds was enhanced when chloroform was used as solvent, whereas benzene and hexane tended to inhibit the reaction (Fig. 2). The reaction also occurred when no solvent was used, and in less than 4 h in chloroform. The reaction also proceeded at room temperature (24°) in chloroform, equilibrium being reached in about three weeks. Analysis of the reaction mixture

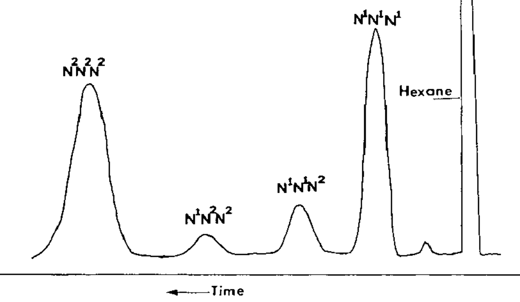

Fig. 2. Chromatogram of the equilibrated mixture from the $N^1N^1N^1$–$N^2N^2N^2$s ystem after two days at 140° in *n*-hexane.

by a gas chromatograph coupled to a selective nitrogen detector (flowing conductivity cell) of the type described by COULSON[7] indicated that each component contained approximately equal quantities of nitrogen.

The proton resonance spectrum of the initial $N^1N^1N^1$–$N^2N^2N^2$ mixture contained five resonances with the following assignments[4] (Table I).

TABLE I

ASSIGNMENT OF PROTON RESONANCE IN A MIXTURE OF $(Me_2SiNMe)_3$ AND $(Me_2SiNEt)_3$

τ-value	*Multiplicity*	*Proton environment*	*Molecule of origin*
9.96	Singlet	$Si{<}^{Me}_{Me}$	$N^1N^1N^1$
9.89	Singlet	$Si{<}^{Me}_{Me}$	$N^2N^2N^2$
9.00	Triplet	C–Me	$N^2N^2N^2$
7.57	Singlet	N–Me	$N^1N^1N^1$
7.18	Quartet	$N–CH_2–C$	$N^2N^2N^2$

The compounds $N^1N^1N^2$ and $N^1N^2N^2$ can each be expected to give rise to six resonances, five of which are also given by the $N^1N^1N^1$–$N^2N^2N^2$ mixture. The only new resonance is that due to silyl methyl groups in the environment $Me_2Si(NMe)NEt$, and is common to both molecules. This resonance can be expected to have a value intermediate between those for $Me_2Si(NMe)_2$ and $Me_2Si(NEt)_2$. The proton resonance spectrum of the equilibrated mixture (Fig. 3) showed a new resonance at 9.92τ, intermediate between the original silylmethyl signals. No other new resonances were produced. The 9.92τ signal from a $N^1N^1N^1$–$N^2N^2N^2$ mixture, which had been equi-

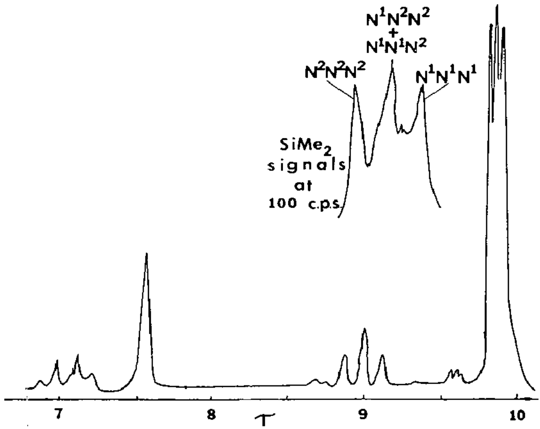

Fig. 3. Proton magnetic resonance spectrum of the equilibrated mixture from the $N^1N^1N^1$–$N^2N^2N^2$ system.

librated in chloroform at room temperature for several weeks, increased in intensity when the mixture was heated at 150° for 6 h. The change in intensity corresponded to the formation of greater quantities of the components assigned as $N^1N^1N^2$ and $N^1N^2N^2$. The compounds $N^1N^1N^2$ and $N^1N^2N^2$ should have two silylmethyl proton nuclear magnetic resonances in the ratio of 2:1, because of the environment Me_2Si-(NMe)NEt.

The fractions collected by trapping the effluent from the analytical gas chromatographic column suspected of being $N^1N^1N^2$ and $N^1N^2N^2$ showed in their resultant PMR spectra that these peaks were in the ratio of 2:1. Attempts were made to prepare further samples by large column preparative gas chromatography, however, the efficiency of the separation was too low to allow isolation of pure components.

Using the mixture containing $N^3N^3N^3$ two more systems, *viz.* $N^1N^1N^1$–$N^3N^3N^3$ and $N^2N^2N^2$–$N^3N^3N^3$, were studied. In each case two new compounds were formed with retention volumes intermediate between those for the starting materials. None of the new compounds was formed when the $N^3N^3N^3$-containing mixture was heated on its own. The suggested equilibria in the two systems are:

These systems also contained numerous components of low retention volume, which originated from the original $N^3N^3N^3$ mixture. In both systems the new components were formed more readily when chloroform was present than when hexane was used as a solvent.

The compounds $N^1N^1N^1$ and $N^1N^1N^2$ form a homologous series, the unit of difference being a methylene group. A plot of log corrected retention volume (or retention time) *versus* carbon number for these compounds should therefore be a straight line, if there are no complicating factors in their chromatography. A plot of log corrected retention time *versus* N-alkyl carbon number was made for the three systems studied, assuming the identities given above for the newly formed compounds. This resulted in a straight line (Fig. 4) which tended to confirm these identities. It is of interest to note that the most asymmetric cyclotrisilazanes, $N^1N^1N^3$ and $N^1N^3N^3$, both had slightly higher retention times than the more symmetrical compounds with the same carbon number. This chromatographic behaviour parallels that observed in these laboratories for asymmetric tetraalkylsilanes[8,9].

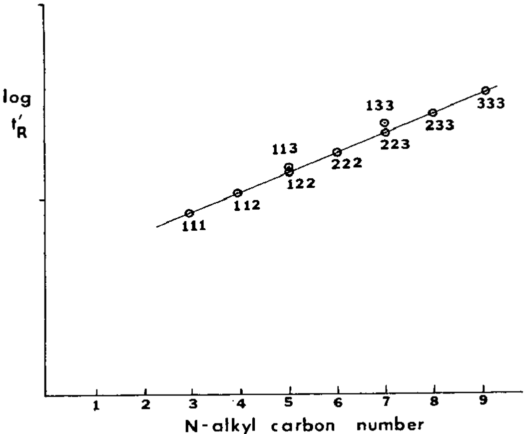

Fig. 4. Plot of log t_R' *versus* N-alkyl carbon number for cyclotrisilazanes.

DISCUSSION

This type of interchange must involve fission either of nitrogen–silicon or nitrogen–carbon bonds. Fission of nitrogen–silicon linkages, involving ring opening, could be expected to give rise to higher membered rings and possible chain products. This is not in accordance with the chemistry of the cyclotrisilazanes, which are known to be thermally stable up to 300° (ref. 10), and no decomposition products of this nature could be detected. An indication that nitrogen–carbon bond fission may be involved was given by the fact that no interchange at all could be obtained in the $N^0N^0N^0$–$N^1N^1N^1$ and $N^0N^0N^0$–$N^2N^2N^2$ systems, up to a temperature of 180°. Similarly, no reaction took place when $N^0N^0N^0N^0$ and $N^1N^1N^1$ were heated together.

Using standard CALINGAERT[11] type equations for $n = 3$, the expected stoichiometries in the equilibrated redistributed product, starting from $N^1N^1N^1$ and $N^2N^2N^2$ in equimolar quantities, are

$$[N^1N^1N^1] = [N^2N^2N^2] = 0.125$$
$$[N^1N^1N^2] = [N^1N^2N^2] = 0.375$$

assuming ideality. However, there seems a bias away from $N^1N^2N^2$ in the work pre-

TABLE II

RELATIVE PROPORTIONS OF CYCLOTRISILAZANES IN THE REDISTRIBUTION OF $N^1N^1N^1$–$N^2N^2N^2$

Compound	*Mole fraction at 24° after 3 weeks*	*Mole fraction at 150° after 4 h*
$N^1N^1N^1$	0.275	0.206
$N^1N^1N^2$	0.147	0.292
$N^1N^2N^2$	0.081	0.206
$N^2N^2N^2$	0.497	0.296

sented here. The stoichiometries obtained for the $N^1N^1N^1$–$N^2N^2N^2$ reaction at room temperature and 150° are given in Table II.

As a check it would be of interest to heat a sample of $N^1N^1N^2$ which should give $[N^1N^1N^1] = 0.296$, $[N^1N^1N^2] = 0.445$, $[N^1N^2N^2] = 0.198$, and $[N^2N^2N^2] = 0.037$. It would also be of importance to check on differences from ideality by obtaining true equilibrium constants.

A similar bias appeared to occur in the two reactions involving $N^3N^3N^3$, but in these cases quantitative data were not obtained.

CONCLUSION

The evidence given above indicates that N-alkyl interchange occurs, without the aid of a catalyst, when N-alkylcyclotrisilazanes are heated together at moderate temperatures.

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CHROM. 4677

REDISTRIBUTION REACTIONS OF SOME STERICALLY HINDERED TETRAALKYLSILANES

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SUMMARY

The range of tetraalkylsilane redistribution reactions has been extended to include reactions of the sterically hindered isopropyl- and isobutylsilanes. Gas chromatography has been used throughout as an analytical tool; because of the extent of chlorosilane formation, a slightly modified procedure has been used for the stoichiometry calculations. Non-statistical redistributions were observed in some of the reactions, especially those involving the longer chain alkyl groups.

Retention volume data for all the alkylsilanes formed in the reactions have been reported, and are presented graphically for a number of cases.

INTRODUCTION

Redistribution reactions involving the aluminium halide catalysed exchange of alkyl groups between central Group IVb elements have been the subject of a number of investigations. The early work on this type of reaction has been reviewed by CALINGAERT AND BEATTY[1], while RUSSELL[2,3] has proposed a reaction mechanism on the basis of kinetic studies involving a number of cocatalysts. In recent years the scope of these reactions has been greatly extended, and now includes the exchange of alkyl groups between different central elements. A large number of Group IVb redistribution reactions have been studied in this department[4,5], and by PHILLIPS and co-workers[6], using gas–liquid chromatography as an analytical technique.

Little work has thus far been reported involving redistributions of sterically hindered tetraalkylsilanes which contain one or more branched-chain substituents. Reactions of trimethylisopropylsilane and trimethylisobutylsilane were included in a previous investigation by POLLARD *et al.*[4].

This communication describes the redistribution reactions of a number of sterically hindered tetraalkylsilanes. Some details of the stoichiometry calculations are given, and various factors involving the aluminium chloride catalyst discussed. The analytical tool used was gas chromatography, and retention data are reported for all tetraalkylsilanes formed in the reactions.

PART 1. REDISTRIBUTION REACTIONS OF TETRAALKYLSILANES

EXPERIMENTAL

Preparation of tetraalkylsilanes

Trimethylisopropylsilane and dimethyldiisopropylsilane were prepared by Grignard syntheses from the appropriate chlorosilanes, using diethyl ether as solvent. Several attempts were made to prepare other branched-chain alkylsilanes by this route, but all were unsuccessful, even when carried out in a high-boiling solvent. The limitations of Grignard procedures in the synthesis of hindered silanes have been noted by several workers[7–9]. In the present work the required hindered silanes were prepared by means of alkyllithium reagents in conjunction with alkylfluorosilanes or silyl hydrides, *e.g.*

$$^{i}PrCl + 2Li \rightarrow {}^{i}PrLi + LiCl$$

$$^{i}PrLi + Et_3SiF \rightarrow Et_3Si^{i}Pr + LiF$$

In the preparation of the alkyllithium reagents, low boiling (30–40°) petroleum ether was employed as the solvent, and the lithium was used in the form of freshly prepared shot[10]. A commercially available solution of *n*-butyllithium in *n*-hexane (Forte Chemical Co.) was used in the preparation of *n*-butylalkylsilanes. Reactions in which silyl hydrides were used instead of fluorosilanes were carried out in a mixed solvent system, the hydride being dissolved in diethyl ether before addition to the hydrocarbon solution of alkyllithium. The various alkylsilane syntheses are summarised in Table I.

TABLE I

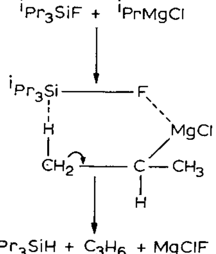
PREPARATION OF SOME TETRAALKYLSILANES

Compound	*Synthetic route*	*Yield (%)*	*B.p. (°C)*
$Me_2Si^{i}Pr_2$	$MeMgI + {}^{i}Pr_2SiCl_2$	22.0	144
$Et_3Si^{i}Pr$	$^{i}PrLi + Et_3SiF$	72.0	169–173
$^{n}Pr_3Si^{i}Pr$	$^{i}PrLi + {}^{n}Pr_3SiF$	61.0	216–218
$^{i}Pr_4Si$	$^{i}PrLi + {}^{i}Pr_3SiH$	11.9	223–227
$^{i}Pr_2Si^{n}Bu_2$	$^{n}BuLi + {}^{i}Pr_2SiF_2$	34.0	64–68 (2 mm)
$^{i}Bu_4Si$	$^{i}BuLi + {}^{i}Bu_2SiF_2$	58.0	247–250
$^{n}BuSi^{i}Bu_3$	$^{n}BuLi + {}^{i}Bu_3SiH$	57.0	251
$^{i}Pr_2Si^{n}(C_5H_{11})_2$	$^{n}(C_5H_{11})Li + {}^{i}Pr_2SiF_2$	26.6	74–78 (5 mm)

Two types of synthesis were used in the preparation of fluorosilanes. In the first, the appropriate Grignard reagent was reacted directly with silicon tetrafluoride:

$$3{}^{i}PrMgCl + SiF_4 \rightarrow {}^{i}Pr_3SiF + 3MgClF$$

In some cases the trialkylsilylhydride was also formed, presumably due to the reaction of fluorosilane with the Grignard reagent, and the abstraction of a β-hydrogen atom.



A similar process has been reported for the reaction of phenyltrichlorosilane with sterically demanding Grignard reagents[11].

In the second method, alkylethoxysilanes were synthesised from tetraethylorthosilicate by Grignard reactions, and then fluorinated with aqueous–alcoholic hydrofluoric acid, using the procedure described by EABORN[12]. Better yields were obtained with this method than with the tetrafluoride reaction.

Gas–liquid chromatography was used throughout this preparative work to monitor the reactions, and to check on the purity of products and intermediates. In addition to the syntheses mentioned above, two preparative-scale redistribution reactions were carried out, and the resulting alkylsilane mixtures separated by preparative gas chromatography. The two redistributions were made using triethylisopropylsilane and diisopropyldi-*n*-butylsilane. Chlorosilane impurities were removed by hydrolysis, followed by treatment with concentrated sulphuric acid, before carrying out the separations (Table II).

TABLE II

YIELDS OF PRODUCTS FROM PREPARATIVE-SCALE REDISTRIBUTION REACTIONS

Starting material, 20 g Et_3Si^iPr		*Starting material, 19 g* $^iPr_2Si^nBu_2$	
Component	*Yield (g)*	*Component*	*Yield (g)*
Et_4Si	5.0	$^iPr_4Si$	0.6
Et_3Si^iPr	3.0	$^iPr_3Si^nBu$	2.5
$Et_2Si^iPr_2$	1.0	$^iPr_2Si^nBu_2$	2.9
$EtSi^iPr_3$	0.1	$^iPrSi^nBu_3$	1.8
$^iPr_4Si$	—	$^nBu_4Si$	0.7

Redistribution reactions of tetraalkylsilanes

All reactions were carried out in sealed micro Carius tubes, heated to the required temperature (usually 220°) in an oven. Before introduction of the catalyst and silane, the tube was flame dried, sealed with a "Subaseal" rubber or silicone seal, and then weighed. Freshly sublimed aluminium chloride was quickly transferred to the tube under a blanket of dry nitrogen, the serum cap replaced, and the tube reweighed. The required weight of alkylsilane (dried over concentrated sulphuric acid) was then added through the serum cap with a 100-μl syringe, the tube reweighed, and then sealed off in a flame. After heating to achieve redistribution the tube was

allowed to cool, opened under dry nitrogen, and sealed with a serum cap. Samples were then extracted for analysis with a microsyringe. In the reactions carried out, a catalyst level of at least 2.3 mole per cent was necessary to achieve complete redistribution.

Analysis of reaction products

Analysis was carried out using both gas density balance and flame ionisation detection systems. The instrument used to obtain full stoichiometric data from the redistributions was a Pye F104, Model 24 gas chromatograph, incorporating a twin-channel flame ionisation detector system. This instrument was calibrated for a series of tetraalkylsilanes and trialkylchlorosilanes, using trimethyl-*n*-pentylsilane as an internal standard. A linear relationship of molar response *versus* number of carbon atoms was obtained above a carbon number of 10.

A Griffin and Gecrge D6 gas density balance chromatograph was used in parallel with the F104 instrument for analysis of the redistribution reactions, in connection with the preparative work, and for obtaining retention data.

Separations of the preparative-scale redistribution reactions mentioned above were carried out using a Wilkens Aerograph Model 705 preparative gas chromatograph.

Silicone gum stationary phases (10–15% on Silocel or Fosasil) were used throughout this work. This type of phase has been found to be very satisfactory in the chromatography of organosilicon compounds[4].

Preparation and nature of the catalyst

In the course of previous studies, POLLARD *et al.*[4] found that incomplete redistribution occurred in the case of trimethylalkylsilanes if the aluminium chloride concentration was less than about 1 mole per cent, even on prolonged heating at 175°. With higher alkylsilanes, catalyst levels of around 2 mole per cent became necessary, and this led to the formation of trialkylchlorosilanes due to the reaction:

$$\geq SiR + Al_2Cl_6 \rightarrow \geq SiCl + R \cdot Al_2Cl_5$$

In contrast to this, PHILLIPS *et al.*[6] stated in a personal communication that they had been able to achieve complete redistribution in milligram-scale reactions using lower catalyst concentrations. It has been suggested that this discrepancy was due to the presence of very small quantities of water in the larger scale reactions. This explanation seems questionable, however, as RUSSELL[2,3] found that water (in small quantities) was in fact a cocatalyst for the reaction. Moreover, this does not explain why larger quantities of catalyst are required for the higher alkylsilane redistributions than for the small alkyl group compounds. Other factors which may be more significant than the presence of water are the purity, physical state, age, and distribution of the catalyst.

In the present work, even using the handling technique described above, complete redistribution of the alkylsilanes studied was not achieved below a catalyst concentration of about 2.3 mole per cent, and often even higher concentrations were required. Identical results were obtained in a series of reactions in which the catalyst was freshly prepared from analytically pure aluminium and dry chlorine.

RESULTS

Calculation of stoichiometry in redistribution reactions

The method of calculation of stoichiometry as developed by CALINGAERT[1] has been summarised in a previous paper[4]. The same equations were used in the present work, most of the reactions involving scrambling tetraalkylsilanes of the general formula $SiR_xR'_{(4-x)}$. A system of this type, involving an equilibrium between five components, can be described by three equilibrium constants, K_1, K_2, K_3, where:

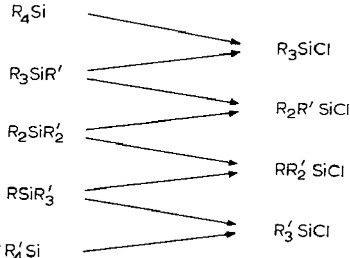
$$K_1 = \frac{[SiR_4] \cdot [R_2SiR'_2]}{[R_3SiR']} \qquad (1)$$

$$K_2 = \frac{[R_3SiR'] \cdot [RSiR'_3]}{[R_2SiR'_2]} \qquad (2)$$

$$K_3 = \frac{[R_2SiR'_2] \cdot [SiR'_4]}{[RSiR'_3]} \qquad (3)$$

The various other equilibria which can be written for the system can be expressed by a combination of eqns. 1, 2 and 3.

In the case of straight-chain tetraalkylsilane redistributions, the concentration of trialkylchlorosilanes is low (less than 2 mole per cent), and these compounds can be ignored in preliminary stoichiometry calculations. In reactions involving branched-chain alkylsilanes chlorosilane formation is more favoured, and the case for excluding these components from the calculations is not as strong. In the redistribution considered here, each reaction mixture included nine major components, *viz.* five tetraalkylsilanes and four trialkylchlorosilanes. As chlorosilane formation in these reactions is essentially irreversible[4], each chlorosilane originated from two different alkylsilanes:



If it is assumed that the Si–R and Si–R' bonds both have equal probability of fission, and also that redistribution of alkylsilanes is complete before chlorosilane formation begins, then the mole fractions of alkylsilanes which have been converted to chlorosilanes can easily be calculated from a knowledge of the predicted distribution.

If the compound $R_2SiR'_2$ was the starting material, the chlorosilanes $R_2R'SiCl$ and RR'_2SiCl would be expected to be formed to some extent as soon as the redistribution commenced, before formation of the other two monochlorosilanes became possible. In fact this sort of bias could not be detected in pilot experiments, in which different starting materials were used to achieve the same redistributed mixture. The assumption that the chlorosilanes originate from a redistributed mixture, therefore, probably has some validity.

The second assumption made, that the Si–R and Si–R′ bonds have equal probability of formation, is not valid in the case of sterically hindered alkylsilanes, and it would be obviously incorrect to use concentrations of alkylsilanes derived for an ideal case in the calculations. The experimentally obtained concentrations for the tetraalkylsilanes were, therefore, used in correcting for the presence of chlorosilanes. The relative concentrations of tetraalkylsilanes were also calculated without taking into account the presence of chlorosilanes. Both sets of results are given below for each system studied. The equilibrium constants were calculated from each series of results, and are also reported. The comparison of the actual product distribution with the statistical distribution is shown graphically, for each system, in Figs. 5, 6 and 7. Most of the results given below were obtained using the Pye F104 gas chromatograph. As a check that results were of the correct order, most of the reactions were also analysed on the Griffin & George D6 gas chromatograph. The results from these analyses (in which the chlorosilane contribution is ignored) are given in the tables below.

Results of redistribution reactions

In Tables III–XI, the meanings of the column headings are as follows:

area = peak area after correction, using the F104 calibration graph,
%(a) = per cent mole, derived from corrected peak area,
%(b) = per cent mole, after correcting for chlorosilanes,
%(c) = per cent mole, ignoring the presence of chlorosilanes,
%(d) = per cent mole in a statistical distribution,
Δ% = difference (mole per cent) between %(b) and %(d),
D6%(c) = per cent mole from D6 analysis.

For the equilibrium constants (calculated from eqns. 1, 2 and 3), value (a) is derived from the %(b) results, and value (b) from the %(c) figures.

The compounds are tabulated in shorthand form, where 1 = Me, 2 = Et, 3 = ^{n}Pr, 3′ = ^{i}Pr, 5 = $^{n}(C_5H_{11})$, etc.

(a) Scrambling reactions of isopropylsilanes

The methyl-isopropyl system. This system was studied by UDEN[13], who reported

TABLE III

REDISTRIBUTION OF $Me_2Si^iPr_2$

Component	*Area*	*%(a)*	*%(b)*	*%(c)*	*%(d)*	*Δ%*	*D6%(c)*
1111	234	5.9	6.7	6.9	6.25	+0.345	7.0
111Cl	75	3.9	—	—			
1113′	881	22.2	27.1	25.8	25.0	+2.1	26.1
113′Cl	163	4.5	—	—			
113′3′	1300	32.8	37.9	38.1	37.5	+0.4	36.8
13′3′Cl	179	4.1	—	—			
13′3′3′	777	19.6	22.8	22.8	25.0	−2.2	23.3
3′3′3′Cl	155	1.9	—	—			
3′3′3′3′	202	5.1	5.5	6.4	6.25	−0.75	5.9

K_1: (a) 0.346, (b) 0.395
K_2: (a) 0.430, (b) 0.405
K_3: (a) 0.308, (b) 0.469

a statistical distribution of products. The results obtained in the present work confirmed this, although the formation of 3'3'3'3' was slightly inhibited (Table III). A catalyst level of 2.2 mole per cent was used. The reaction tube was cooled in liquid nitrogen before opening to prevent loss of tetramethylsilane.

The ethyl-isopropyl system. The results obtained are given in Table IV. A catalyst concentration of 2.3 mole per cent was used.

TABLE IV

REDISTRIBUTION OF Et_3Si^iPr

Component	*Area*	*%(a)*	*%(b)*	*%(c)*	*%(d)*	*Δ%*	*D6%(c)*
2222	1832	28.6	32.8	34.2	31.6	+1.2	33.4
222Cl	555	8.7	—	—	—		
2223'	1990	31.2	38.8	37.0	42.4	−3.4	38.4
223'Cl	329	5.1	—		—		
223'3'	1278	20.0	23.8	23.8	21.2	+2.6	23.4
23'3'Cl	140	2.2		—			
23'3'3'	576	4.1	4.5	4.9	4.65	−0.15	4.8
3'3'3'Cl	Not resolved, coincided with 2223'; assumed to be negligible.						
3'3'3'3'	6	0.1	0.1	0.1	0.35	−0.25	Not detected

K_1: (a) 0.520, (b) 0.591
K_2: (a) 0.308, (b) 0.320
K_3: (a) 0.017, (b) 0.0099

The n-propyl-isopropyl system. Several attempts were made to separate the products from the redistribution of tri-*n*-propylisopropylsilane using packed columns, but all were unsuccessful. Attempts were also made using wide-bore capillary columns, but again insufficient resolution was achieved.

The isopropyl-n-butyl system. The results obtained are given in Table V. It was found with this system that rearrangement was incomplete below a catalyst level of 3.5 mole per cent. As well as the expected products, several peaks were obtained at

TABLE V

REDISTRIBUTION OF $^iPr_2Si^nBu_2$

Component	*Area*	*%(a)*	*%(b)*	*%(c)*	*%(d)*	*Δ%*	*D6%(c)*
3'3'3'3'	452	10.1	12.1	13.1	6.25	+5.85	11.6
3'3'3'Cl	283	6.3	—	—	—	—	—
3'3'3'4	895	20.1	26.4	26.3	25.0	+1.3	26.6
3'3'4Cl	204	4.6	—	—	—	—	—
3'3'44	1160	25.5	33.1	33.4	37.5	−4.1	34.4
3'44Cl	369	8.3	—	—	—	—	—
3'444	752	16.9	23.7	22.1	25.0	−1.3	22.4
444Cl	194	4.3	—	—	—	—	—
4444	173	3.9	4.7	5.1	6.25	−1.55	4.8

K_1: (a) 0.575, (b) 0.632
K_2: (a) 0.571, (b) 0.521
K_3: (a) 0.277, (b) 0.349

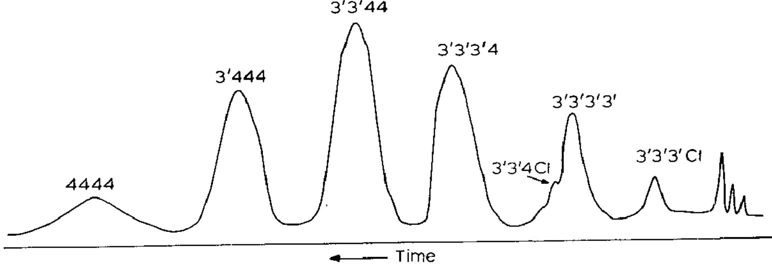


Fig. 1. Chromatogram of redistributed diisopropyldi-*n*-butylsilane products.

low retention volumes (Fig. 1). These were attributed to the presence of *n*-butyl group decomposition products, and possibly also to chlorosilanes. No account was taken of them in the calculations.

The isopropyl-n-pentyl system. The results obtained are given in Table VI. 5 mole per cent catalyst were required to achieve complete distribution in this system.

TABLE VI

REDISTRIBUTION OF $^{i}Pr_2Si(n\text{-}C_5H_{11})_2$

Component	*Area*	*%(a)*	*%(b)*	*%(c)*	*%(d)*	*Δ%*	*D6%(c)*
3'3'3'3'	228	9.2	15.0	16.9	6.25	+8.75	16.2
3'3'3'Cl	328	13.3	—	—	—	—	—
3'3'3'5	345	13.9	31.6	25.6	25.0	+6.6	26.1
3'3'5Cl	430	17.4	—	—	—	—	
3'3'55	435	17.6	31.8	32.2	37.5	−5.7	32.4
3'55Cl	278	11.2	—	—	—	—	
3'555	288	11.6	19.0	21.3	25.0	−6.0	21.5
555Cl	89	3.6	—	—	—	—	
5555	54	2.2	2.8	4.0	6.25	−3.45	3.8

K_1: (a) 0.478, (b) 0.831
K_2: (a) 0.595, (b) 0.526
K_3: (a) 0.247, (b) 0.284

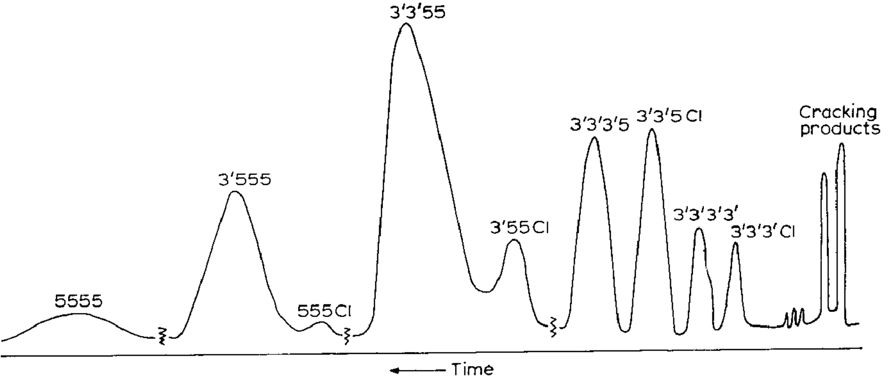


Fig. 2. Chromatogram of the redistributed mixture from diisopropyldi-*n*-pentylsilane.

TABLE VII

REDISTRIBUTION OF Et_4Si–$^iBu_4Si$

Component	*Area*	*%(a)*	*%(b)*	*%(c)*	*%(d)*	*Δ%*	*D6%(c)*
2222	1080	6.2	6.6	6.6	6.25	+0.35	6.2
222Cl	342	2.0	—				
2224′	4220	24.0	26.5	25.9	25.0	+1.5	26.0
224′Cl	387	2.2	—	—			
224′4′	5980	34.1	36.3	36.6	37.5	−1.2	37.0
24′4′Cl	290	1.7	—	—	—		
24′4′4′	4305	24.6	26.3	26.4	25.0	+1.3	25.9
4′4′4′Cl	180	1.0	—	—	—		
4′4′4′4′	735	4.2	4.3	4.5	6.25	−4.95	4.9

K_1: (a) 0.344, (b) 0.360
K_2: (a) 0.530, (b) 0.510
K_3: (a) 0.226, (b) 0.236

As in the scrambling of diisopropyldi-*n*-butylsilane, numerous low retention volume peaks were observed, and were not included in the calculations (Fig. 2).

(b) Scramling reactions of isobutylsilanes

The methyl–isobutyl system. The methyl–isobutyl system was among those studied by UDEN[13], who found that an almost statistical distribution was obtained. No attempt was made to study this system in the present work.

The ethyl–isobutyl system. The results obtained are given in Table VII. This system was derived from scrambling 0.166 g tetraethylsilane and 0.296 g tetraisobutylsilane (0.0023), using 2.2 mole per cent catalyst.

The n-propyl–isobutyl system. The results obtained are given in Table VIII. 0.201 g tetra-*n*-propylsilane and 0.254 g tetraisobutylsilane were scrambled using 2.5 mole per cent catalyst.

The n-butyl-isobutyl system. The results obtained are given in Table IX. In contrast to the lack of success in separating *n*-propyl-isopropylsilane mixtures, all the

TABLE VIII

REDISTRIBUTION OF $^nPr_4Si$–$^iBu_4Si$

Component	*Area*	*%(a)*	*%(b)*	*%(c)*	*%(d)*	*Δ%*	*D6%(c)*
3333	226	5.4	6.1	6.8	6.25	−0.15	6.4
333Cl	160	3.8	—	—	—	—	—
3334	945	22.7	29.2	28.5	25.0	+4.2	27.8
334′Cl	320	7.7	—	—	—	—	—
334′4′	1192	28.7	36.5	36.0	37.5	−1.0	36.8
34′4′Cl	246	5.9	—	—	—	—	—
34′4′4′	795	19.1	24.0	24.0	25.0	−1.0	24.6
4′4′4′Cl	126	3.0	—	—	—	—	—
4′4′4′4′	156	3.7	5.2	4.7	6.25	−1.05	5.4

K_1: (a) 0.261, (b) 0.301
K_2: (a) 0.525, (b) 0.530
K_3: (a) 0.329, (b) 0.294

TABLE IX

REDISTRIBUTION OF $^{n}BuSi^{i}Bu_3$

Component	*Area*	*%(a)*	*%(b)*	*%(c)*	*%(d)*	*Δ%*	*D6(c)*
4'4'4'4'	4860	22.1	26.6	28.7	31.6	−5.0	27.6
4'4'4'Cl	2420	11.0	·	—			
4'4'4'4	7070	32.0	43.8	41.6	42.2	+1.6	41.7
4'4'4Cl	1870	8.5		—			
4'4'44	4155	18.9	25.1	24.6	21.2	+3.9	25.5
4'44Cl	790	3.6		—			
4'444	800	3.6	4.2	4.7	4.65	−0.45	5.2
444Cl	Not resolved, coincided with 4444; assumed negligible						
4444	60	0.3	0.3	0.4	0.35	−0.05	—

K_1: (a) 0.348, (b) 0.406
K_2: (a) 0.292, (b) 0.324
K_3: (a) 0.043, (b) 0.044

n-butyl-isobutylsilane isomers were sufficiently resolved to make quantitative measurements possible. 4.0 mole per cent catalyst was used in the reaction. Comparison of the boiling point ranges for the propyl- and butylsilanes gives some explanation for this difference in resolution. For the propylsilanes the range is 214° ($^{n}Pr_4Si$) to 223° ($^{i}Pr_4Si$), while for the butylsilanes the range is much wider, from 249° ($^{i}Bu_4Si$) to 280° ($^{n}Bu_4Si$).

Two reactions were carried out in which *n*-butyl-triisobutylsilane was redistributed with an equimolar quantity of a tetraalkylsilane, giving reaction mixtures which contained a total of fifteen alkylsilanes. In the analysis of the reaction mixtures not all the by-product trialkylchlorosilanes were resolved, and so only the main products have been considered in the stoichiometry calculations. Both reaction mixtures contained butylsilane cracking products with low retention volumes (see Fig. 3).

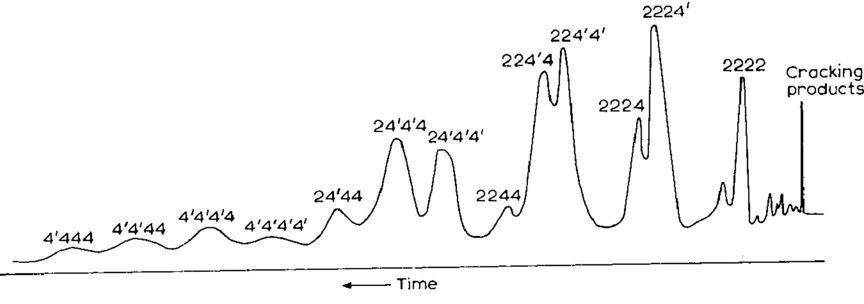


Fig. 3. Chromatogram of products arising from the redistribution between *n*-butyl-triisobutylsilane with tetraethylsilane.

The methyl–n-butyl-isobutyl system. The results obtained are given in Table X. Tetramethylsilane (0.076 g) and *n*-butyl-triisobutylsilane (0.221 g) were scrambled using 2.5 mole per cent catalyst. The reaction tube was cooled in liquid nitrogen before sealing and before opening after equilibration to prevent loss of tetramethylsilane (see Fig. 4).

TABLE X

REDISTRIBUTION OF Me_4Si–$^nBuSi^iBu_3$

Component	*Area*	*%(c)*	*%(d)*	*Δ%*
1111	442	10.3	6.25	+4.05
1114′	680	15.8	18.75	−2.95
1114	560	13.0	6.25	+6.75
114′4′	770	17.9	21.1	−3.2
114′4	633	14.7	14.1	+0.6
1144	156	3.6	2.35	+1.3
14′4′4′	216	5.0	10.55	−5.6
14′4′4	268	6.3	10.55	−4.3
14′44	50	1.2	3.5	−2.3
1444	40	0.9	0.38	+0.5
4′4′4′4′	106	2.5	1.98	+0.5
4′4′4′4	210	4.9	2.61	+2.3
4′4′44	150	3.5	1.32	+2.2
4′444	18	0.4	0.29	+0.2
4444	Not detected		0.02	

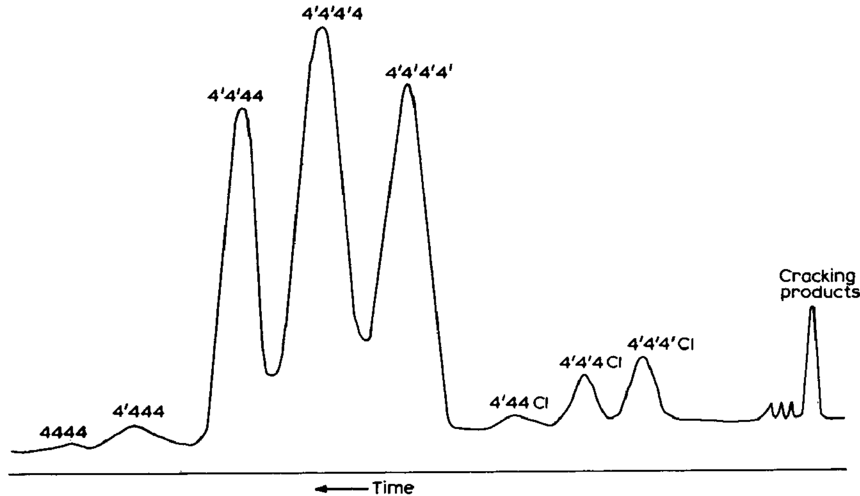


Fig. 4. Chromatogram of the redistribution of *n*-butyl-triisobutylsilane.

The ethyl–n-butyl-isobutyl system. The results obtained are given in Table XI.

DISCUSSION

Near statistical distributions were obtained with the methyl-isopropyl, ethyl-isopropyl, and ethyl-isobutyl systems, although in each case the formation of the most sterically hindered alkylsilane was somewhat inhibited. The isopropyl-*n*-butyl and isopropyl-*n*-pentyl systems both showed marked divergences from ideality, formation of tetraisopropylsilane being favoured in each case. Consideration of molecular models gives no obvious indication why this bias should occur, although the long-chain alkyl substituents have a greater possibility of impeding the approach of

TABLE XI

REDISTRIBUTION OF Et_4Si–$^nBuSi^iBu_3$

Component	*Area*	*%(c)*	*%(d)*	*Δ%*
2222	790	12.8	6.25	+6.5
22224′	1285	20.8	18.75	+2.0
2224	785	12.7	6.25	+6.4
224′4′	1001	16.2	21.1	−4.9
224′4	846	13.7	14.1	−0.4
2244	182	2.9	2.35	+0.5
24′4′4′	436	7.0	10.55	−3.6
24′4′4	519	8.4	10.55	−2.2
24′44	177	2.9	3.5	−0.6
2444	Not detected		0.38	—
4′4′4′4′	37	0.6	1.98	−1.4
4′4′4′4	68	1.1	2.61	−1.5
4′4′44	48	0.8	1.32	−0.5
4′444	7	0.1	0.29	−0.2
4444	Not detected		0.02	—

an incoming group than have small groups such as methyl and ethyl. A contributory factor may be the decrease in concentration of butyl and pentyl groups due to thermal decomposition. It would be instructive to find out if a bias towards the tetraisopropylsilane also occurs in the *n*-propyl-isopropyl system. The *n*-propyl-isobutyl system showed a slight bias away from the isobutylsilanes, and this bias was more pronounced for the *n*-butyl-isobutyl scramble in the case of tetraisobutylsilane. The two *n*-butyl-triisobutylsilane–tetraalkylsilane scrambles both showed a bias away

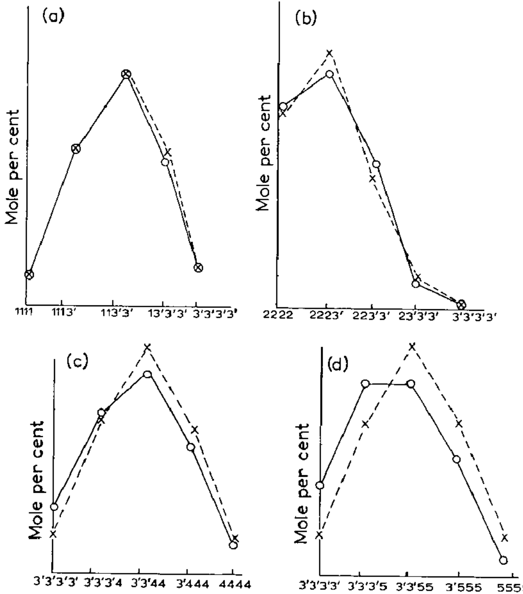
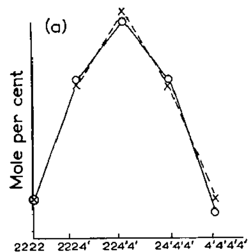


Fig. 5. Product distribution in the isopropylsilane series after redistribution. (a) Methyl-isopropyl system; (b) ethyl-isopropyl system; (c) *n*-butyl-isopropyl system; (d) *n*-pentyl-isopropyl system. ○, experimental; ×, calculated.



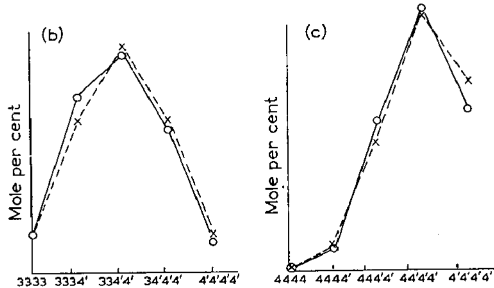
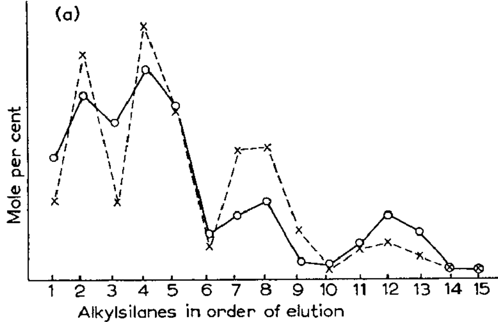


Fig. 6. Product distribution in the isobutylsilane series after redistribution. (a) Ethyl-isobutyl system; (b) *n*-propyl-isobutyl system; (c) *n*-butyl-isobutyl system. ○, experimental; ×, calculated.



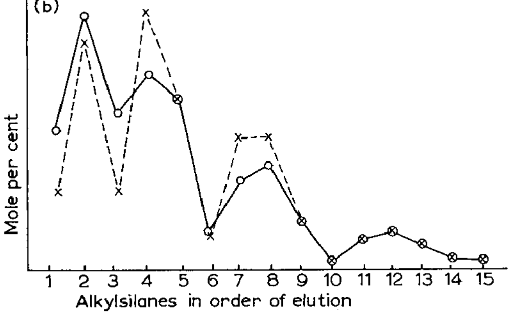


Fig. 7. Product distribution in the *n*-butyl-isobutylsilane series after redistribution. (a) Methyl–*n*-butyl-isobutyl system (Table X); (b) ethyl–*n*-butyl-isobutyl system (Table XI). ○, experimental; ×, calculated.

from the more sterically hindered silanes. Formation of the *n*-butyl compounds was preferred for each set of isomeric tetraalkylsilanes.

Both sets of results for the scrambling reactions gave similar distributions, the most significant difference being that in the concentrations obtained for the R_4Si and R'_4Si species when the chlorosilane correction was made. It should be emphasised that Figs. 5, 6 and 7, which illustrate the relative proportions of products in the scrambled mixtures, are not graphs. The points are connected by lines merely for the sake of clarity. The reactions involving redistribution of three alkyl groups on silicon are described by a total of 39 equilibrium constants[14]. No attempt was made to calculate these.

The results given above were all calculated from one set of GLC data, and for one reaction in each system. Other reactions were carried out for all systems except the last two, and the product distributions showed good agreement with those given above. The maximum variation for any given component was about 6%, which was considered satisfactory in view of the difficulty in obtaining reproducible catalyst conditions. In all cases the deviations from statistical distributions gave a similar pattern to those reported here.

PART 2. RETENTION DATA FOR TETRAALKYLSILANES

EXPERIMENTAL

Specific retention volumes were obtained for the tetraalkylsilanes using the Griffin and George D6 chromatograph, modified as described elsewhere[4], using the following conditions.

Column: 200 × 0.5 cm I.D. U-tube, packed with 15% (w/w) E301 silicone gum (2.52 g) on acid/alkali treated Silocel (36–60 mesh size).

Sample size: 1 μl, measured with a 10-μl Hamilton microsyringe.

Carrier gas: B.O.C. "white spot" nitrogen.

Column pressures (p_i/p_o): 2.01, 1.60, and 1.31.

Temperature (°C): (A) 150°, (B) 175°, (C) 200°.

Column temperature was measured by a copper-constantan thermocouple, and displayed on a potentiometric recorder. Column inlet pressures were measured using a mercury manometer, and the carrier gas flow rate measured with a soap bubble flow meter.

Most of the retention volumes were obtained from chromatographing redistribution reaction products. Component peaks were timed using two stopwatches in alternation. The recommendations proposed for the measurement of retention data[15] were followed as closely as possible throughout this work. The specific retention volumes obtained for the alkylsilanes are given below. In all cases measurements were carried out using at least two values of p_i/p_o (see Table XII).

Plots of log V_G *versus* carbon number for the ethyl-isopropyl- and isopropyl-*n*-butylsilanes gave straight-line graphs at all three temperatures used (Fig. 8). A second type of homologous series is that obtained by varying the number of isobutyl groups in a series of butylsilanes. Plots of log V_G *versus* number of isobutyl groups

TABLE XII

VALUES OF V_G ON 15% (w/w) SILICONE OIL

For other conditions, see text.

Sample type	*Specific retention volume (ml)*			*Mean value*	*log V_G*
	P_i/P_o 2.01	P_i/P_o 1.60	P_i/P_o 1.31		
(A) Temperature, 150°					
1113'	20	19	17	19	1.271
113'3'	32	31	30	31	1.492
13'3'3'	65	64	60	63	1.800
2222	31	29	28	29	1.462
2223'	44	43	42	43	1.634
223'3'	64	61	59	61.5	1.789
23'3'3'	95	87	86	91	1.959
3'3'3'3'	137	135	136	136	2.134
3'3'3'4	202	186	171	186	2.270
3'3'44	292	295	293	293.5	2.468
3'444	422	410	—	416	2.620
1114'	—	12	12	12	1.079
1114	—	18	19	19	1.279
114'4'	41	43	39	41	1.613
114'4	48	49	48	48	1.642
1144	58	56	57	56	1.748
14'4'4'	129	130	128	129	2.111
14'4'4	152	153	142	149	2.173
14'44	167	169	162	166	2.213
1444	228	224	—	226	2.270
2224'	63	60	63	62	1.793
2224	72	67.5	70	70	1.845
224'4'	118	111	110	115	2.061
224'4	135	126	138	132	2.118
2244	154	144	—	149	2.174
24'4'4'	204	191	—	197.5	2.296
24'4'4	232	218		225	2.352
24'44	272	258		265	2.424
2444	352	325		309	2.490
4'4'4'4'	344	312		328	2.516
4'4'4'4	390	350		370	2.568
4'4'44	448	406		427	2.631
4'444	515	489		502	2.702
4444	566	564		565	2.744
(B) Temperature, 175°					
2222		18	17	17.5	1.244
2223'	24	24	24	24	1.380
223'3'	33	34	32	33	1.519
23'3'3'	45	45		45	1.654
3'3'3'3'	63	61	62	62	1.792
3'3'3'4	89	91	89	90	1.954
3'3'44	118	122	—	120	2.080
3'444	163	165	—	164	2.215
3334'	74	70	—	72	1.857
334'4'	91	93	—	92	1.964
34'4'4'	113	113	—	113	2.053
2224'	31	34		32.5	1.512
2224	35	37		36	1.557
224'4'	54	57		55.5	1.744
224'4	61	63		62	1.792
2244	67	69		68	1.833

(continued on p. 202)

TABLE XII *(continued)*

Sample type	*Specific retention volume (ml)*			*Mean value*	*log V_G*
	P_i/P_o *2.01*	P_i/P_o *1.60*	P_i/P_o *1.31*		
24'4'4'	88	90		89	1.950
24'4'4	97	101		99	1.996
24'44	116	113		114.5	2.059
2444	139	140		140	2.146
4'4'4'4'	126	126		126	2.100
4'4'4'4	144	144		144	2.159
4'4'44	176	166		171	2.233
4'444	198	188		193	2.286
4444	228	221	218	222	2.346
(C) Temperature, 200°					
3'3'3'3'	36	36	35	35.5	1.551
3'3'3'4	48	48	48	48	1.682
3'3'44	62	62	62	62	1.792
3'444	83	82	81	82	1.914
3333	36	36	36	36	1.556
3334'	42	41	40	41	1.613
334'4'	49	49	48	49	1.690
34'4'4'	58	59	61	59	1.771
3'3'3'5	89		89	89	1.950
3'3'55	139		132	135.5	2.132
3'555	216		215	215.5	2.333
5555	328		312	320	2.506
2224'	32	31		31.5	1.499
2224	35	34		34.5	1.538
224'4'	48	48		48	1.682
224'4	53	53		53	1.724
2244	60	56		58	1.764
24'4'4'	69	69		69	1.839
24'4'4	80	76		78	1.892
24'44	88	84		86	1.934
2444	100	100		100	2.02
4'4'4'4'	73	73		73	1.864
4'4'4'4	84	79		81	1.909
4'4'44	89	88		88.5	1.947
4'444	98	94		96	1.982
4444	105.5	108	106	106	2.076

were found to be linear for the $1\bar{4}\bar{4}\bar{4}$, $2\bar{4}\bar{4}\bar{4}$, and $\bar{4}\bar{4}\bar{4}\bar{4}$ series (Fig. 9), where $\bar{4}$ refers either to a *n*-butyl or an isobutyl group.

UDEN[13] noted that log V_G *versus* carbon number plots for asymmetric tetra-alkylsilanes showed a slight curvature, asymmetric members of an homologous series having higher retention volumes than expected. In the present work this was found to be the case for the methyl–butyl, ethyl–butyl, and isopropyl-*n*-pentyl (Fig. 10) series of compounds. Curvature was more pronounced for isobutylsilanes than for the corresponding *n*-butylsilanes (Fig. 11).

Plots of log V_G against $1/T$, where T is the absolute column temperature, were non-linear for all the alkylsilanes considered, but especially so for the more asymmetric ethyl-isobutylsilanes (Figs. 12 and 13). In Fig. 12 the values for the ethyl-isopropyl-

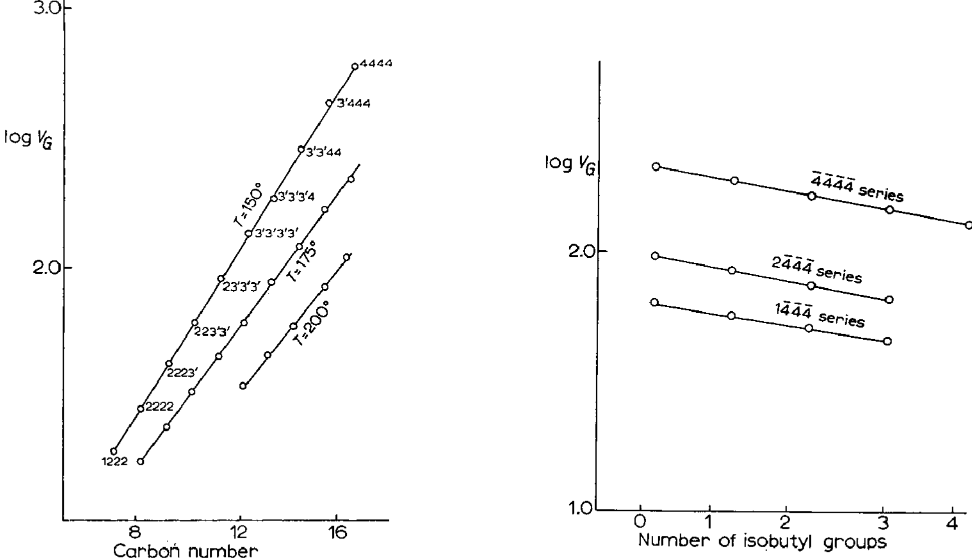


Fig. 8. Plot of log V_G *versus* carbon number for a series of isopropylsilanes at three temperatures.

Fig. 9. Plots of log V_G *versus* number of isobutyl groups for a series of isobutylsilanes.

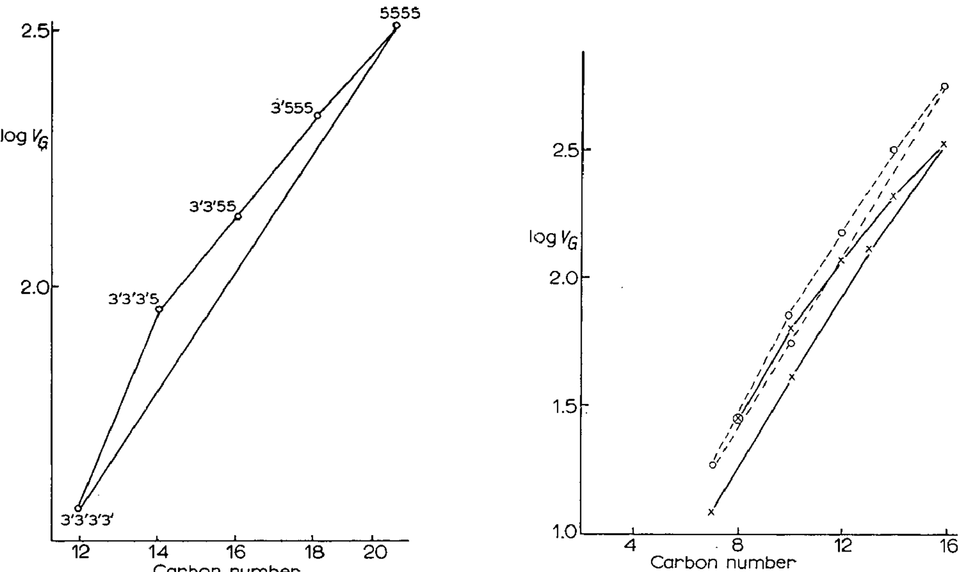


Fig. 10. Plot of log V_G *versus* carbon number for the isopropyl-*n*-pentylsilanes.

Fig. 11. Plots of log V_G *versus* carbon number for the butylsilanes. ○, *n*-butylsilanes; ×, isobutylsilanes.

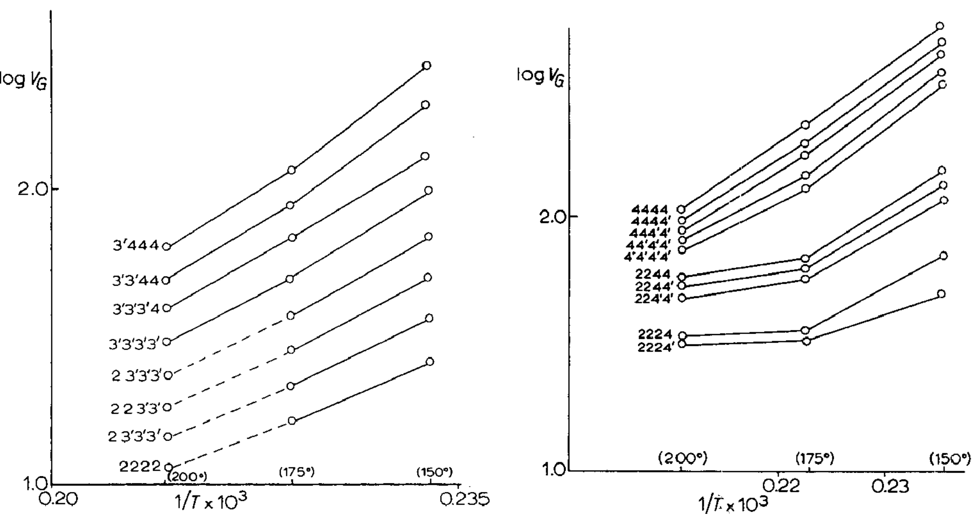


Fig. 12. Plots of log V_G *versus* $1/T$ for the isopropylsilanes.

Fig. 13. Plots of log V_G *versus* $1/T$ for the isobutylsilanes.

silanes at 200° were obtained by extrapolation of the relevant log V_G–carbon number plot in Fig. 8.

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CHROM. 4665

TRENNUNG UND BESTIMMUNG VON CARBAMAT- UND HARNSTOFF-HERBIZIDEN DURCH REAKTIONS-GASCHROMATOGRAPHIE

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SUMMARY

Separation and determination of carbamate and urea herbicides by reaction gas chromatography

The behaviour of a number of carbamate and urea herbicides during thermal degradation in the interior of the gas chromatographic apparatus and during chromatography through a KOH-layered reaction separation column is studied. Methods are developed for the direct gas chromatographic analysis of the herbicidal active substances proximpham, propham, chlorpropham, fenuron, monuron, diuron, monolinuron, linuron, and metobromuron. Results show that the rapid separation and quantitative determination of these herbicides is possible.

EINLEITUNG

Die zunehmende Anwendung der Carbamat- und Harnstoffherbizide schafft das Bedürfnis nach unkomplizierten, rationellen Analysenmethoden für diese Verbin-

TABELLE I

CHEMISCHE STRUKTUR EINIGER CARBAMAT- UND HARNSTOFFHERBIZIDE

X-C₆H₄-NH—CO—N(CH₃)R			X-C₆H₄-NH—CO—OR		
Wirkstoff	*X*	*R*	*Wirkstoff*	*X*	*R*
Fenuron	—	$-CH_3$	Propham (IPC)	—	$-CH(CH_3)_2$
Monuron	4-Cl	$-CH_3$	Proximpham	—	$-N=C(CH_3)_2$
Monolinuron	4-Cl	$-OCH_3$	Chlorpropham (CIPC)	3-Cl	$-CH(CH_3)_2$
Metobromuron	4-Br	$-OCH_3$			
Diuron	3,4-Cl,Cl	$-CH_3$			
Linuron	3,4-Cl,Cl	$-OCH_3$			

* Leiter: Dr. A. JUMAR.

dungsklassen. In jüngster Zeit werden vielfach zur Erweiterung des herbiziden Wirkungsspektrums Gemische der chemisch nahe verwandten Wirkstoffe (Tabelle I) in Form von Kombinationspräparaten eingesetzt. Dadurch werden solche analytischen Methoden notwendig, die zugleich qualitative und quantitative Informationen liefern, so dass die Gaschromatographie in den Mittelpunkt des Interesses rückt.

Von den in der Tabelle I genannten Wirkstoffen sind jedoch nur Propham und Chlorpropham unzersetzt und ohne vorherige chemische Veränderung chromatographierbar[1–4]. Alle übrigen bisher geprüften N-Phenylcarbamate und Phenylharnstoffe erleiden bei den erforderlichen hohen Injektor- und Säulentemperaturen eine thermische Zersetzung, die allerdings in den meisten Fällen unvollständig und schlecht reproduzierbar, also für eine quantitative Auswertung wenig geeignet ist.

Um diesen Schwierigkeiten auszuweichen, wurde vorgeschlagen, die N-Trimethylsilylderivate der Herbizide zu chromatographieren, was bei Propham, Chlorpropham, Monuron und Diuron gelang[5], oder aber die Wirkstoffe vor der gaschromatographischen Bestimmung zu den entsprechenden Anilinen zu hydrolysieren[6,7]. Durch Bromierung der Aniline[8] oder durch Bildung ihrer 2,4-Dinitrophenylderivate[9] lässt sich ihre Elektronenaffinität erhöhen und die Nachweisempfindlichkeit bei Verwendung des Elektroneneinfangdetektors steigern. Da die chemische Veränderung der Carbamat- und Harnstoffherbizide vor der Injektion in den Gaschromatographen arbeitsaufwendig ist, die Gefahr von Wirkstoffverlusten in sich birgt und zusätzlich Komplikationen durch Reagenzienüberschüsse entstehen lässt, untersuchten wir die Möglichkeiten der direkten Gaschromatographie der Verbindungen durch ihre quantitative Spaltung innerhalb der gaschromatographischen Apparatur. Versuche in dieser Richtung wurden mit Phenylharnstoffherbiziden bereits von Henkel[10] unternommen. Dabei ergaben sich nur kleine, quantitativ nicht reproduzierbare Peaks, die nach Meinung des Autors von den entsprechenden Anilinen herrührten. Da die Bemühungen um eine vollständigere thermische Spaltung zu keinen brauchbaren Ergebnissen führten, wurden die Wirkstoffe im Gemisch mit methanolischer Kalilauge oder noch vorteilhafter zusammen mit Tetramethylammoniumhydroxid injiziert, wobei die Aniline in etwa 75%iger Ausbeute entstanden.

Eine grössere Gruppe von substituierten Harnstoffherbiziden wurde später von McKone und Hance[11] ebenfalls mit dem Ziel der direkten Chromatographie der unveränderten Verbindungen bearbeitet. Tatsächlich entstanden bei hohen Temperaturen des Injektors (265°) und der Säule (150°) reproduzierbare, allerdings sehr temperaturabhängige Peaks, die den intakten Wirkstoffen zugeschrieben wurden. Es fällt aber auf, dass alle die Verbindungen, die im Phenylrest gleichartig substituiert sind, auch gleiche Retentionszeiten aufwiesen. So liessen sich Monolinuron, Monuron und Buturon, die in *p*-Stellung durch Cl substituiert sind, ebensowenig voneinander trennen wie die 3,4-dichlorsubstituierten Wirkstoffe Diuron, Linuron, Neburon, Metoxymarc, Benzomarc und Bayer 43975. Dieses Verhalten deutet darauf hin, dass entgegen der Ansicht der Autoren eine thermische Zersetzung stattgefunden hat, die zu den gleichen Spaltprodukten führte.

EXPERIMENTELLES

Es wurde ein Doppelsäulen-Gaschromatograph der Firma Varian-Aerograph, Model 204-1B, mit linearer Temperaturprogrammierung und FID benutzt. Für Ar-

beiten mit empfindlichen Substanzen lässt sich der Injektorraum mit einem Glaseinsatz versehen.

Sämtliche Untersuchungen wurden mit Hilfe von 5 ft. × $\frac{1}{8}$ in. Glassäulen durchgeführt. Als Säulenfüllung dienten: (1) 3% Dow-11 auf 80–100 mesh Aeropak (silanisiertes Chromosorb W der Firma Varian-Aerograph); (2) 2% Carbowax 20M auf 0.1–0.2 mm alkalibehandeltem Porolith (VEB Berlin-Chemie). Vor dem Imprägnieren mit Carbowax 20M wird das Porolith nach der Vorschrift von FELTKAMP UND THOMAS[12] in folgender Weise mit KOH belegt: 90 g Porolith werden mit einer Lösung von 30 g KOH in 600 ml Methanol versetzt und im Rotationsverdampfer bis zur Trockene eingeengt.

THERMISCHE SPALTUNG VON CARBAMAT- UND HARNSTOFFHERBIZIDEN

Wie wir an anderer Stelle[13] bereits mitteilten, zerfällt der Wirkstoff Proximpham (Tabelle I) beim Erhitzen auf Temperaturen über 90° in die Spaltprodukte Phenylisocyanat und Acetonoxim.

$$C_6H_5\text{–NH–CO–O–N} = C(CH_3)_2 \rightarrow C_6H_5\text{–NCO} + (CH_3)_2C = NOH$$

Der Grad dieser Spaltung lässt sich auch in der gaschromatographischen Apparatur bei hinreichend hohen Temperaturen so vervollständigen, dass eine quantitative Bestimmung des Proximphams durch Auswertung des Isocyanat- oder des Oximpeaks möglich wird.

Unsere Versuche ergaben, dass andere Carbamat- und Harnstoffherbizide zwar deutlich thermostabiler sind als das Proximpham, so dass nur unvollständige thermische Zersetzungen beobachtet wurden, dass aber die Richtung der Aufspaltung in allen untersuchten Fällen (Tabelle II) die gleiche ist: Bei der thermischen Zersetzung der N-Phenylcarbamate entstehen die entsprechenden Phenylisocyanate und ali-

TABELLE II

THERMISCHE SPALTUNG EINIGER CARBAMAT- UND HARNSTOFFHERBIZIDE

Bedingungen: 5 ft. × 1/8 in. I.D. Glassäule mit 3% Dow-11 auf Aeropak; Injektor 350°, Detektor 240°; Trägergas 30 ml N_2/min, Wasserstoff 25 ml/min, Luft 250 ml/min.

Wirkstoff	*Säulentemperatur (°C)*	*Gesamtretentionszeit (sec)*				
		Peak des aromatischen Spaltproduktes	*Vergleichswerte*			
Propham	75	80				
Proximpham	75	80	Phenylisocyanat	80	Anilin	107
Fenuron	75	80				
Chlorpropham	105	83	3-Chlorphenylisocyanat	83	3-Chloranilin	124
Monolinuron	105	94	4-Chlorphenylisocyanat	94	4-Chloranilin	142
Linuron	140	60	3,4-Dichlorphenylisocyanat	60	3,4-Dichloranilin	110

phatischen Alkohole, aus den Phenylharnstoffen werden Phenylisocyanate und aliphatische Amine gebildet:

$$X\text{-}C_6H_4\text{-}NH\text{—}CO\text{—}OR \longrightarrow X\text{-}C_6H_4\text{-}NCO + ROH$$

$$X\text{-}C_6H_4\text{-}NH\text{—}CO\text{—}N(CH_3)R \longrightarrow X\text{-}C_6H_4\text{-}NCO + NH(CH_3)R$$

Die Identifizierung der Phenylisocyanate gelang gaschromatographisch anhand ihrer Retentionszeiten, die sich bei Verwendung einer Dow-11-Säule von den Retentionszeiten der entsprechenden Aniline deutlich unterscheiden (Tabelle II).

Zur Bestätigung dieses Befundes wurden die gaschromatographischen Phenylisocyanat-Fraktionen mit Hilfe eines Fraktionssammlers aufgefangen und dünnschichtchromatographisch geprüft. Da die direkte Unterscheidung zwischen den Phenylisocyanaten und Anilinen wegen ihres sehr ähnlichen Retentionsverhaltens nicht gelang, wurden die Dünnschichtplatten vor der Entwicklung des Chromatogramms in eine mit konzentriertem Ammoniak beschickte Entwicklungskammer gestellt. Dabei setzten sich die Phenylisocyanate zu den entsprechenden Phenylharnstoffen um, die sich leicht von den zugehörigen Anilinen trennen liessen. Die bei der thermischen Spaltung entstehenden Alkohole bzw. Amine wurden IR-spektrographisch identifiziert, indem die Wirkstoffe trocken erhitzt und die Zersetzungsprodukte in eine Gaszelle eingeleitet wurden.

Die Zielsetzung unserer Arbeiten bestand darin, Unterschiede im Verhalten der Carbamat- und Harnstoffherbizide bei der Gaschromatographie festzustellen und zur separaten Bestimmung der einzelnen Wirkstoffe in Gemischen auszunutzen. Dabei gingen wir von folgenden Befunden aus:

Das Carbamoyloxim Proximpham lässt sich quantitativ thermisch spalten und durch Auswertung des Phenylisocyanat-Peaks bestimmen.

Die Carbamate Propham und Chlorpropham kann man bei günstiger Wahl der Arbeitsbedingungen unzersetzt chromatographieren.

Die Phenylharnstoffe sind nicht unzersetzt chromatographierbar. Sie können also nur auf dem Wege über ihre Spaltprodukte direkt bestimmt werden.
Es wurde angestrebt, Propham und Chlorpropham auch in Gemischen mit Phenylharnstoffherbiziden als intakte Verbindungen zu chromatographieren, weil dadurch ihre Identifizierung und separate Bestimmung in solchen Wirkstoffkombinationen möglich wird.

Bei der Suche nach den optimalen Arbeitsbedingungen für die direkte Gaschromatographie dieser beiden Carbamate wurde beobachtet, dass ihre thermische Spaltung nicht nur durch hohe Temperaturen begünstigt, sondern auch durch die heissen Metalloberflächen im Injektor und in der Säule katalytisch gefördert wird. So wurde z.B. bei der Gaschromatographie einer benzolischen Lösung von Chlorpropham (Sdp. 229°) festgestellt, dass zur Vermeidung der thermischen Spaltung des Wirkstoffes die ziemlich enge Temperaturspanne des aus Edelstahl bestehenden Injektors von 195–205° eingehalten werden muss. Bei niedrigerer Temperatur verdampft der Wirkstoff zu langsam, so dass ein breiter, unsymmetrischer Peak entsteht; bei höherer Tempe-

TABELLE III

GRAD DER THERMISCHEN SPALTUNG EINIGER CARBAMAT- UND HARNSTOFFHERBIZIDE BEI VERSCHIEDENEN INJEKTORTEMPERATUREN IM VOLLGLASSYSTEM (GEMESSEN AM PEAK DES ENTSPRECHENDEN ISOCYANATES)

Spaltung vollständig, +++; stark, ++; schwach, +; keine Spaltung, —. Versuchsbedingungen wie bei Tabelle II.

Wirkstoff	*Injektortemperatur (°C)*		
	400	*375*	*350*
Proximpham	+++	+++	+++
Propham	+	—	—
Chlorpropham	+	—	—
Fenuron	++	+	—
Monuron	—	—	—
Diuron	—	—	—
Monolinuron	++	+	—
Linuron	++	+	—
Metobromuron	+	—	—

ratur macht sich die thermische Zersetzung des Chlorprophams durch das Auftreten eines Signals bemerkbar, das vom 3-Chlorphenylisocyanat herrührt. Mit Hilfe eines Glaseinsatzes im Injektor und durch Verwendung von Glassäulen (Vollglassystem) lassen sich die katalytischen Effekte soweit eliminieren, dass die Injektortemperatur bis 350° erhöht werden kann, ohne eine Spaltung der von uns untersuchten Wirkstoffe hervorzurufen (Tabelle III).

Unter diesen Bedingungen wird also Proximpham quantitativ zu Phenylisocyanat und Acetonoxim zersetzt, Propham und Chlorpropham werden unzersetzt chromatographiert, wogegen bei den Phenylharnstoffen Fenuron, Monuron, Diuron, Monolinuron, Linuron und Metobromuron keine Spaltung zum entsprechenden Iso-

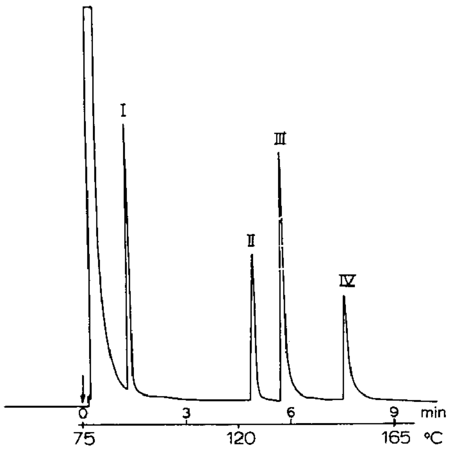

Fig. 1. Gaschromatogramm der quantitativen Bestimmung eines Herbizidgemisches aus Proximpham (I), Propham (III) und Chlorpropham (IV) in Benzol. Das Signal des Proximphams wird durch Phenylisocyanat hervorgerufen. Als innerer Standard dient Diphenyl (II). Bedingungen: 5 ft. × 1/8 in. I.D. Glassäule mit 3% Dow-11 auf Aeropak; Säulentemperatur programmiert von 75–165°, Heizrate 10°/min, Injektor (mit Glaseinsatz) 340°, Detektor 235°; Trägergas 30 ml N_2/min.

cyanat eintritt. Dadurch ist die gaschromatographische Bestimmung von Proximpham, Propham und Chlorpropham allein oder in ihren vier möglichen Kombinationen als Zwei- oder Dreistoffgemische durchführbar. Die Analyse dieser drei Wirkstoffe gelingt auch dann, wenn die Gemische ausserdem die in der Tabelle III aufgeführten Phenylharnstoffherbizide enthalten. Als Beispiel ist in Fig. 1 das Gaschromatogramm einer Bestimmung der Wirkstoffe in der Kombination Proximpham–Propham–Chlorpropham wiedergegeben.

BESTIMMUNG DER N-PHENYLCARBAMAT- UND PHENYLHARNSTOFFHERBIZIDE DURCH REAKTIONS-GASCHROMATOGRAPHIE

Die direkte gaschromatographische Bestimmung der Phenylharnstoffherbizide gelingt nur dann, wenn sie innerhalb der gaschromatographischen Apparatur in flüchtige Spaltprodukte oder andere flüchtige Derivate umgewandelt werden. Das Einspritzen der Wirkstoffe im Gemisch mit alkoholischer Kalilauge bzw. wässriger Tetramethylammoniumhydroxid-Lösung[10] oder Zusatzeinspritzungen von Ammoniak[14] bringen keine befriedigenden Ergebnisse, weil die Spaltung der Wirkstoffe nicht vollständig ist und die quantitativen Resultate infolgedessen schlecht reproduzierbar sind. Wir griffen auf die seit langem bekannten Umsetzungen der Urethane, Harnstoffe und Isocyanate mit Calciumhydroxid zurück, bei denen durch einfaches Erhitzen der beiden Reaktionspartner in guten Ausbeuten die entsprechenden Amine entstehen[15]. In Abwandlung dieser Methode belegten wir das Trägermaterial mit Kaliumhydroxid und imprägnierten es anschliessend mit der Trennflüssigkeit. Bei der Chromatographie der Carbamat- und Harnstoffherbizide durch die so präparierten Säulen und bei Injektortemperaturen von etwa 350° werden die Wirkstoffe mit Ausbeuten zwischen 85 und 100% zu den Anilinen umgesetzt:

$$X\text{-}C_6H_4\text{-}NH\text{—}CO\text{—}N(CH_3)R + 2\ KOH \longrightarrow X\text{-}C_6H_4\text{-}NH_2 + CH_3HR + K_2CO_3$$

$$X\text{-}C_6H_4\text{-}NH\text{—}CO\text{—}OR + 2\ KOH \longrightarrow X\text{-}C_6H_4\text{-}NH_2 + ROH + K_2CO_3$$

Alle Reaktionsprodukte wurden auf gaschromatographischem oder IR-spektroskopischem Wege bzw. durch chemische Reaktionen identifiziert.

Zur Vervollständigung der Reaktion wurden Zusatzeinspritzungen von Wasser, Tetramethylammoniumhydroxid[10] und Ammoniak[14] erprobt. Durch Wasser werden in einigen Fällen (Chlorpropham, Proximpham, Monolinuron und Metobromuron) die Ausbeuten der Aniline auf 100% gesteigert. Tetramethylammoniumhydroxid-Lösung bewirkt eine unkontrollierte Wirkstoffspaltung, die sich im Auftreten mehrerer Peaks äussert. Sie erzeugt ausserdem ein starkes Eigensignal, das die quantitative Auswertung stört. Dagegen werden durch eine Zusatzeinspritzung von Ammoniak bei allen untersuchten Wirkstoffen 100%ige und gut reproduzierbare Anilinausbeuten erzielt, ohne dass die Bestimmung durch Nebensignale beeinträchtigt wird. Die praktische Durchführung erfolgt so, dass nacheinander etwa gleiche Volumina der Wirkstoff-

TABELLE IV

MÖGLICHKEITEN DER DIREKTEN GASCHROMATOGRAPHIE EINIGER CARBAMAT- UND HARNSTOFF-HERBIZIDE AN ZWEI VERSCHIEDENEN TRENNSÄULEN

Wirkstoff	*Inerte Trennsäule 3% Dow-11/Aeropak 80–100 mesh Vollglassystem*	*Reaktions-Trennsäule 2% Carbowax-20 M/Porolith 0.1–0.2 mm, alkalibehandelt*
Propham	intakt	Anilin
Proximpham	Phenylisocyanat	Anilin
Fenuron	—	Anilin
Chlorpropham	intakt	3-Chloranilin
Monuron	—	4-Chloranilin
Monolinuron	—	4-Chloranilin
Diuron	—	3,4-Dichloranilin
Linuron	—	3,4-Dichloranilin
Metobromuron	—	4-Bromanilin

lösung und einer konzentrierten Ammoniaklösung in die Injektionsspritze gesaugt und dann gemeinsam eingespritzt werden.

Durch die in der Säule stattfindende Umwandlung des Kaliumhydroxids zu Kaliumcarbonat kommt es im Laufe der Zeit zu einer Verkrustung am Säulenanfang, so dass die ersten 5 cm der Säulenfüllung nach einigen Wochen ausgewechselt werden sollten. Obgleich diese Beobachtung zeigt, dass die Zersetzung zu den Anilinen überwiegend am Säulenanfang stattfindet, ist es vorteilhaft, den gesamten Trägerstoff der Säulenfüllung mit KOH zu belegen, weil dadurch das Tailing der Anilinpeaks unterdrückt wird.

Da die hier verwendete Säule eine chemische Reaktion und eine Trennung der Reaktionsprodukte bewirkt, benutzen wir dafür die Bezeichnung "Reaktions-Trennsäule" und unterscheiden sie dadurch von der im vorangehenden Abschnitt beschriebenen inerten Trennsäule.

TABELLE V

BESTIMMUNGSMÖGLICHKEITEN EINIGER CARBAMAT- UND HARNSTOFFHERBIZIDE IN ZWEIKOMPONENTEN-GEMISCHEN

2 = beide Komponenten bestimmbar (Reaktions-Trennsäule); 1 = eine Komponente bestimmbar (inerte Trennsäule); 0 = nur Bestimmung der Gesamtmenge des substituierten Anilins möglich (Reaktions-Trennsäule).

	Metobromuron	*Linuron*	*Monolinuron*	*Diuron*	*Monuron*	*Fenuron*	*Chlorpropham*	*Propham*
Proximpham	2	2	2	2	2	1	2	2
Propham	2	2	2	2	2	1	2	
Chlorpropham	2	2	1	2	1	2		
Fenuron	2	2	2	2	2			
Monuron	2	2	0	2				
Diuron	2	0	2					
Monolinuron	2	2						
Linuron	2							

GASCHROMATOGRAPHISCHE ANALYSE VON WIRKSTOFF-KOMBINATIONEN AUS CARBAMAT- UND HARNSTOFFHERBIZIDEN

In der Tabelle IV sind die in den beiden vorigen Abschnitten geschilderten Methoden der direkten Gaschromatographie von Carbamat- und Harnstoffherbiziden gegenübergestellt. Daraus ist zu ersehen, dass in allen bisher untersuchten Fällen eine Einzelbestimmung der Wirkstoffe möglich ist. Für die gleichzeitige Bestimmung mehrerer Wirkstoffe in Kombinationen ist es ausserdem notwendig, dass die verwendeten Säulen auch in der Lage sind, die injizierten Wirkstoffe bzw. ihre Spaltprodukte

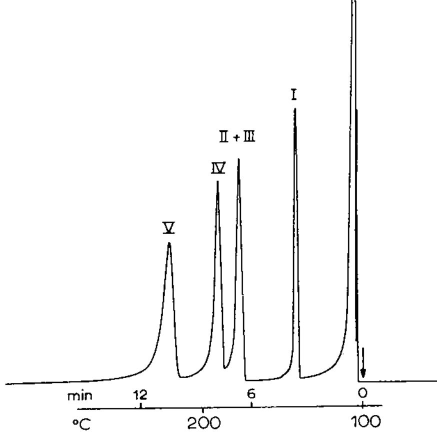

Fig. 2. Gaschromatogramm der benzolischen Lösung einer Mischung aus Anilin (I), 3-Chloranilin (II), 4-Chloranilin (III), 4-Bromanilin (IV) und 3,4-Dichloranilin (V). Bedingungen: Reaktions-Trennsäule; Säulentemperatur programmiert von 100–200°, Heizrate 12°/min, Injektor 340°, Detektor 190°; Trägergas 24 ml N_2/min.

zu trennen. Die erfolgreiche Trennung des Gemisches Propham–Chlorpropham–Proximpham in einer inerten Dow-11-Säule wurde in Fig. 1 bereits dargestellt. Die Trennung der Aniline in der mit Carbowax 20M belegten Reaktions-Trennsäule ist in Fig. 2 wiedergegeben. Nachteilig ist, dass es nicht gelang, 3-Chloranilin und 4-Chloranilin voneinander zu trennen. Für dieses Problem wurde auch von andere Seite[16] keine Lösung gefunden.

Die kombinierte Anwendung der beiden beschriebenen Methoden ermöglicht die Analyse vieler Zweistoff-Gemische, wobei die Wirkstoffbestimmung in den meisten dieser Gemische allein mit Hilfe der Reaktions-Trennsäule gelingt. In der Tabelle V sind alle Zweistoff-Gemische dargestellt, die sich aus den von uns bearbeiteten Carbamat- und Harnstoffherbiziden kombinieren lassen. Von den insgesamt 36 möglichen Kombinationen kann man 30 allein durch die quantitative Zersetzung zu den Anilinen analysieren. Nur in sechs Fällen versagt diese Methode, weil die beiden Wirkstoffe gleichartig substituierte Aniline oder ein Gemisch aus 3- und 4-Chloranilin liefern. Jedoch kann man noch in vier dieser restlichen Kombinationen wenigstens einen der beiden Wirkstoffe—nämlich Propham, Chlorpropham und Proximpham—mit Hilfe der beschriebenen inerten Trennsäule bestimmen. Ausserdem lässt sich in den Kombinationen Proximpham–Fenuron und Propham–Fenuron auch das Fenuron

über die Bestimmung der gesamten Anilinmenge durch Differenzbildung erfassen.

Die praktische Bedeutung dieser Methoden ergibt sich aus der zunehmenden Anwendung von Kombinationspräparaten aus Carbamat- und Harnstoffherbiziden in der Landwirtschaft. Z.B. enthält das britische Verzeichnis der amtlich anerkannten Pflanzenschutzmittel von 1969 (Lit. 17) fünf derartige Präparate mit den Wirkstoff-Kombinationen Chlorpropham–Propham, Chlorpropham–Diuron, Chlorpropham–Fenuron, Chlorpropham–Linuron und Monolinuron–Linuron. Alle diese Präparate lassen sich durch die Anwendung der hier beschriebenen Methoden gaschromatographisch analysieren. Auch Kombinationspräparate aus drei Wirkstoffen, die bisher nur vereinzelt angewendet werden[17], sind der direkten gaschromatographischen Bestimmung zugänglich. Fig. 3 zeigt die Gaschromatogramme einer Analyse der Wirkstoff-

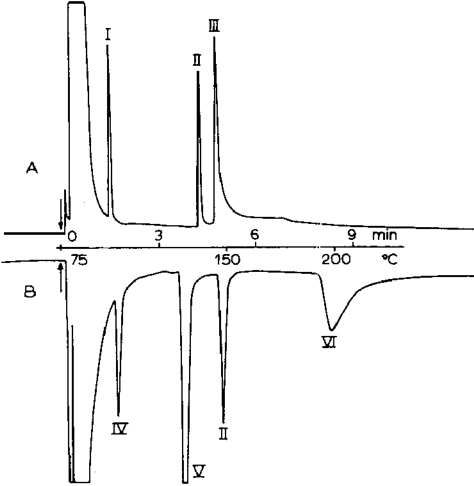

Fig. 3. Gaschromatogramm der Simultanbestimmung eines Herbizidgemisches aus Proximpham (I), Propham (III) und Diuron (VI) an der inerten Trennsäule (A) und der Reaktions-Trennsäule (B). I = Phenylisocyanat aus Proximpham; II = Diphenyl (innerer Standard); III = Propham; IV = Acetonoxim aus Proximpham; V = Anilin aus Proximpham und Propham; VI = 3,4-Dichloranilin aus Diuron; Bedingungen für A und B: Säulentemperatur programmiert von 75–220°, Heizrate 15°/min, Injektor (A mit Glaseinsatz) 340°, Detektor 235°; Trägergas 24 ml (A) und 60 ml (B) N_2/min. Die Lösung des Wirkstoffgemisches wurde in die Reaktions-Trennsäule (B) zusammen mit einem gleichgrossen Volumen 25%iger wässriger Ammoniaklösung injiziert.

kombination Proximpham–Propham–Diuron bzw. Proximpham–Propham–Linuron. Darin wurden Proximpham (als Phenylisocyanat) und Propham (als unveränderte Verbindung) im Vollglassystem mit einer Dow-11-Säule bestimmt. Durch eine zweite Einspritzung des Gemisches in die Reaktions-Trennsäule wurde der Diuron- bzw. Linuron-Gehalt der Probe anhand des 3,4-Dichloranilin-Peaks ermittelt.

ZUSAMMENFASSUNG

Es wird das Verhalten einer Anzahl von Carbamat- und Harnstoffherbiziden bei der thermischen Spaltung im Gaschromatographen sowie bei der Chromatographie

durch eine mit KOH belegte Reaktions-Trennsäule studiert. Daraus werden Methoden zur direkten gaschromatographischen Analyse der herbiziden Wirkstoffe Proximpham, Propham, Chlorpropham, Fenuron, Monuron, Diuron, Monolinuron, Linuron und Metobromuron entwickelt. Sie ermöglichen die schnelle Trennung und quantitative Bestimmung einer Vielzahl von Kombinationen dieser Herbizide.

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CHROM. 4681

AN ELECTRON-CAPTURE GAS CHROMATOGRAPHIC METHOD FOR THE DETERMINATION OF SOME CARBAMATE INSECTICIDES AS 2,4-DINITROPHENYL DERIVATIVES OF THEIR PHENOL MOIETIES*

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SUMMARY

A method is described for the analysis of residues of insecticidal carbamates which involves hydrolysis to a phenol and concurrent formation of the corresponding electron-capturing 2,4-dinitrophenyl ether. Free phenols do not interfere. The application of this method to the determination of carbamates in river waters and vegetables is described.

INTRODUCTION

The introduction and widespread acceptance of the carbamate pesticides has led to a need for specific and sensitive methods for the analysis of their residues in soils, waters and foodstuffs. The carbamate esters used fall into two general classes, *viz.*

(i) R–NH–CO_2–Alkyl (herbicides) and

(ii) CH_3–NH–CO_2–R′ (insecticides),

where R and R′ are substituted aryl or heterocyclic ring systems.

A procedure for the analysis of residues of herbicidal carbamates has been presented previously[1]. The current paper is concerned with the determination of four insecticidal carbamates, *viz.* propoxur, butacarb, carbaryl and methiocarb (a molluscicide).

There are several colorimetric[2,3] and TLC[4,5] methods available for the analysis of the insecticidal carbamates. The former procedures, which involve hydrolysis to the corresponding phenol followed by formation of a derivative amenable to spectrophotometric determination, have poor specificity. TLC methods, in which the intact carbamate or its phenol hydrolysis product are visualised, are prone to interference from co-extractives and require rigorous clean-up procedures. A GLC method incor-

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Propoxur	Carbaryl	Butacarb	Methiocarb
(Arprocarb)	(Sevin)	(RD-14639)	(Mesurol)
(Unden)	(UC-7744)		(Bayer 37344)
(Bayer 39007)			(H-321)

porating a sensitive and specific detector would be preferable[6], since it would give more precise quantitative data and be less susceptible to interference from co-extractives. Direct GLC is precluded because of the tendency of some free carbamates to on-column breakdown and the poor response to them of the detectors available. The detection limit using an electron-capture detector was 20–100 ng, while using a caesium bromide thermionic detector, with gas flow rates and the inter-electrode distance adjusted for maximum sensitivity to nitrogen, the detection limit was 50–100 ng.

Determination of traces of these carbamates by GLC therefore requires an indirect procedure which again generally involves hydrolysis to the phenol followed by derivative formation. The derivative must be amenable to GLC and should be able to be detected at the nanogram level. A method in which the esterified phenol is brominated[7] has only been applied to carbaryl; a sensitive method utilising the trichloroacetylated phenol[8] requires the laboratory preparation of an acetylating agent which, being susceptible to hydrolysis, is not very stable. The electron-capturing properties of halomethyldimethylsilyl phenol derivatives have not allowed estimation of less than 10 ng of carbamate[9]. We found dimethylthiophosphorylation[10] to give poor yields with the less acidic phenols; in addition the presence of excess reagent, which could not be removed, gave a large background current with the thermionic detector, making quantitative interpretation of the chromatograms difficult.

This paper describes a quantitative and qualitative method for the analysis of residues of insecticidal carbamates in the presence of free phenols. Hydrolysis of the carbamate to the phenol and formation of the corresponding 2,4-dinitrophenyl ether is effected concurrently. The derivative, extracted into hexane, is identified and determined by GLC with electron-capture detection.

EXPERIMENTAL

REINHEIMER *et al.* have described a procedure[11] which has been used for the preparation of 1-fluoro-2,4-dinitrobenzene (FDNB) derivatives of phenols[12]. A method for the analysis of residues of carbamates involving the reaction with FDNB of the phenols formed by alkaline hydrolysis of the insecticides seemed promising.

The yields of 2,4-dinitrophenyl ethers, obtained when the carbamates under review were heated in a buffer with a 1% (w/v) solution of FDNB in acetone, varied

markedly with changes in the volume of reagent and the pH of the buffer. However, 0.5 ml of FDNB solution and a phosphate buffer of pH 11.0 gave good and consistent results. At pH 11.0 amines do not react with FDNB, thus there was no interference from the amine hydrolysis products of the herbicidal carbamates or the allied substituted ureas such as propham and metobromuron.

Several organophosphorus pesticides, *e.g.* fenchlorphos, dichlofenthion and bromophos, can hydrolyse to phenols yielding FDNB derivatives with similar retention times to those derived from the carbamates. They did not interfere, however, presumably because of their failure to hydrolyse under such mild conditions.

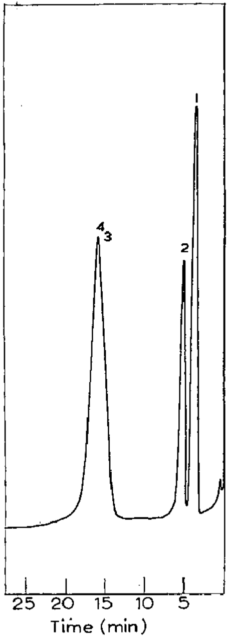

Fig. 1. Gas chromatographic separation of 2,4-dinitrophenyl derivatives of phenols. For GLC conditions, see Table I. 1 = 2-isopropoxyphenol DNP derivative; 2 = 3,5-di-*tert.*-butylphenol DNP derivative; 3 = 1-naphthol DNP derivative; 4 = 3,5-dimethyl-4-methylthiophenol DNP derivative.

Free phenols, especially those corresponding to the parent carbamate and which therefore may be present as breakdown products, are a potential source of interference. Their removal using extraction procedures based on the acidity of phenols did not prove satisfactory and hence elimination of the phenols by selective oxidation was tried. Of the several oxidants used, *i.e.* potassium permanganate, ferric chloride, potassium dichromate, nitric acid, hydrogen peroxide, ammonium persulphate and ceric sulphate, only the last removed phenols completely with no loss of any of the carbamates. Even with ceric sulphate, satisfactory results were only obtained if about 20% acetone was present in the aqueous reaction phase. Breakdown of the carbamates was then completely inhibited, while under the conditions of the procedure 500 μg of phenol gave a response equivalent to only 0.5 μg of the corresponding carbamate.

Gas chromatography

The dinitrophenyl ethers were gas chromatographed at 211°, a slightly lower temperature than used previously[1] for the 2,4-dinitrophenyl derivatives of aromatic amines; all other conditions were, however, the same. Good separation of the 2-isopropoxyphenyl, 3,5-di-*tert*.-butylphenyl and 1-naphthyl ethers was achieved, but the 1-naphthyl and the 3,5-dimethyl-4-methylthiophenyl ethers were not resolved (Fig. 1). Reaction of a mixture of these two ethers with peracetic acid resulted in total oxidation of the thio-linkage and allowed estimation of the individual derivatives by difference.

Extraction and clean-up

River water, after removal of free phenol, was analysed directly by chloroform extraction and derivative formation. Some sources of analytical grade chloroform required redistillation to prevent almost complete loss of the butacarb; even then recoveries of this compound were not very good. A comparison of the IR spectra of the redistilled and unredistilled chloroform did not indicate the presence of any impurities in the analytical grade reagent which could have caused the loss of butacarb.

Plant materials require a more rigorous clean-up. Hydrolysis of the carbamates followed by steam distillation gave very poor yields. Apart from this, a clean-up involving a preliminary hydrolysis was unacceptable since it would preclude differentiation between carbamates and free phenols. Most of the coextracted plant material could be removed by coagulation[13] and filtration. Free phenols were then oxidised and the carbamates extracted. Butacarb gave very poor yields, but since it is used mainly in sheepdips it is unlikely to be present in vegetable material.

PROCEDURE

Reagents

The following reagents were applied: Buffer solution pH 11.0; 8.2 ml of 0.1 *N* sodium hydroxide and 100 ml of 0.05 *M* disodium hydrogen phosphate diluted to 200 ml with deionised water. 1-Fluoro-2,4-dinitrobenzene solution; 1 g of FDNB dissolved in 100 ml of acetone. Coagulating solution; 1.25 g of ammonium chloride and 2.5 ml of orthophosphoric acid (s.g. 1.75) dissolved in 1 l of deionised water. Ceric sulphate solution; 20 mg of ceric sulphate dissolved in 20 ml of approximately 4 *N* sulphuric acid (prepare freshly as required). Hydrogen peroxide solution; 16 ml of 100 volumes hydrogen peroxide dissolved in 100 ml of glacial acetic acid. Redistilled chloroform. Redistilled hexane. Anhydrous sodium sulphate, granular.

Water

To 1 l of the sample of water, contained in a 2-l separating funnel, add the freshly prepared ceric sulphate solution. After 15 min dissolve 20 g of anhydrous sodium sulphate in the water and extract with three 50-ml portions of redistilled chloroform, shaking for 1 min for each extraction. Dry the extracts by passage through a column containing 15 g of granular anhydrous sodium sulphate. Combine the dried extracts and evaporate to a small volume in a Kuderna–Danish evaporator fitted with a 10-ml pear-shaped flask, and then reduce the volume still further using a micro-

Snyder column. Finally take to dryness in a gentle stream of air, hydrolyse and form the derivative as described below.

Vegetable material

Macerate 50 g of vegetable tissue for 2 min with each of three separate 100-ml portions of acetone; centrifuge after each operation. Add the combined supernatant extracts to 1 l of deionised water containing 20 g of anhydrous sodium sulphate and extract with three 50-ml portions of redistilled chloroform, shaking for 1 min for each extraction. Pass the extracts down a column containing 15 g of granular anhydrous sodium sulphate into a Kuderna–Danish evaporator. Reduce the volume to 5 ml, wash with chloroform into a 150-ml beaker and evaporate to dryness in a gentle stream of air. Redissolve the extracted vegetable matter in 50 ml of acetone and add 50 ml of coagulating solution. Cover with a watch glass and allow to stand overnight. Filter the coagulated solution, under vacuum, through a tightly packed filter paper pulp pad about 0.5 cm thick contained in a coarse sintered glass filter funnel. Wash the beaker with 30 ml of coagulating solution and pass the washings through the filter pad. Transfer the filtrate, which should be almost colourless, to a 250-ml separating funnel and add 20 ml of freshly prepared ceric sulphate solution. After 15 min, add 10 ml of propan-2-ol to minimise emulsion formation. Extract with redistilled chloroform and evaporate to dryness as described above for water samples.

Hydrolysis and derivative formation

Pipette 0.5 ml of 1% (w/v) FDNB in acetone into the pear-shaped flask containing the cleaned-up residue and add 10 ml of the buffer solution. Allow the mixture to react in a water bath at 50° for 30 min. Transfer the yellow reaction mixture to a 100-ml separator and shake for 1 min with 10 ml of redistilled hexane. Discard the aqueous layer and dry the hexane phase by passage through a column containing about 5 g of granular anhydrous sodium sulphate. (Any emulsion in the hexane layer may be dispersed by shaking with about 1 g of anhydrous sodium sulphate just prior to passing through the drying column.) Inject 5 μl of the hexane extract on to the GLC column described in Table I.

Analysis of a mixture of methiocarb and carbaryl

If peaks corresponding to methiocarb or carbaryl are obtained the following

TABLE I

GAS CHROMATOGRAPHIC PROPERTIES OF 2,4-DINITROPHENYL DERIVATIVES OF PHENOLS

Column: glass 140 cm in length 1.5 mm I.D.; 1.0% GE-XE 60 and 0.1% Epikote 1001 on 60–80 mesh Chromosorb G AW/DMCS at 211°. Carrier gas: Nitrogen 180 ml/min.

Phenol	*Retention time (min) on GE-XE 60 column*	*Detection limit (ng)*
2-Isopropoxyphenol	3.5	0.1
3,5-Di-*tert.*-butylphenol	5.5	0.2
1-Naphthol	16.2	0.2
3,5-Dimethyl-4-methylthiophenol	15.2	0.2

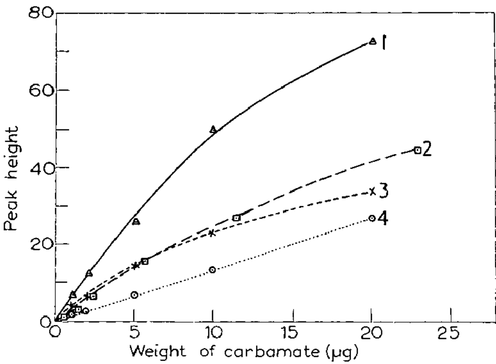

Fig. 2. Calibration curve, prepared by subjecting standard amounts of carbamate to hydrolysis and derivative formation. 1 = propoxur; 2 = methiocarb; 3 = butacarb; 4 = carbaryl.

procedure is adopted: Take 5 ml of the hexane extract to dryness in a gentle stream of air and add 3 ml of the hydrogen peroxide solution. Heat the mixture for 15 min at 50°. Wash into a 100-ml separating funnel with enough saturated sodium bicarbonate solution to give a neutral solution. Extract with 5 ml of redistilled hexane and dry by passage down a column containing 4 g of granular anhydrous sodium sulphate. Inject 5 µl of this solution on to the GLC column. The thiophenyl derivative will have been removed and any remaining peak will be due to carbaryl. Any methiocarb present may be estimated from the difference between the two chromatograms.

RESULTS

The calibration curves shown in Fig. 2 were plotted by subjecting standard amounts of the insecticides to hydrolysis and derivative formation. These curves were

TABLE II

RECOVERIES OF CARBAMATES FROM VARIOUS SPIKED SUBSTRATES

Sample	*Carbamate*	*Number of determinations*	*Mean recovery (%)*	*Relative mean deviation (%)*
Thames river water, 1 l spiked with 10 µg of carbamate	Propoxur	12	94	2.5
	Butacarb	6	41	15
	Methiocarb	6	97	4
	Carbaryl	6	63	5
Peas, 50 g spiked with 10 µg of carbamate	Propoxur	8	87	6
	Methiocarb	4	89	5
	Carbaryl	4	87	5
Lettuce, 50 g spiked with 10 µg of carbamate	Propoxur	8	96	7
	Methiocarb	4	97	4
	Carbaryl	4	82	0
Apple, 50 g spiked with 10 µg of carbamate	Propoxur	6	100	9
	Methiocarb	4	94	5
	Carbaryl	4	94	5

used in the calculation of the percentage recoveries (Table II) of carbamate from spiked samples of water, peas, apples and lettuce.

The water taken from the Thames in Central London gave several interfering peaks before reaction with ceric sulphate. The mean recovery of butacarb varied between 40% and 65% from day to day, but on any particular day the relative mean deviation was never greater than 15%. No reason could be found for the generally poor recoveries of butacarb nor for the daily fluctuations, which were not observed with the other three carbamates.

Incomplete coagulation occurred in the presence of 50 ml of acetone and a small amount of coextractive remained in solution resulting in a rather broad injection peak, which made measurement of the propoxur peak difficult. If the presence of this compound is indicated a more accurate analysis may be obtained by using 15 ml of acetone instead of 50 ml. The early peak due to coextractives is then greatly reduced with no reduction in the propoxur recovery; however, the recoveries of methiocarb and carbaryl are reduced.

The method described allows the analysis of insecticidal carbamates in river waters at levels down to about 0.005 p.p.m. and in plant material down to 0.1 p.p.m. The method for plant material was unsuccessful when applied to soil samples, and for these more stringent clean-up procedures would be required.

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CHROM. 4662

COLUMN CHROMATOGRAPHY ON POLYSTYRENE RESIN USING AQUEOUS SYSTEMS

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SUMMARY

Chromatography on the polystyrene resin Amberlite XAD-2, using water or dilute aqueous solutions as eluants, provides useful separations of biologically important compounds of low molecular weight. Adsorption effects govern separations. Compounds with aromatic or non-polar groups are retarded on the resin, while ionic or polar compounds pass through the column with little or no retention. The importance of pH is discussed. A nucleoside may be separated from the corresponding nucleotide or free base, the order of elution being nucleotide–base–nucleoside. Pyrimidine nucleosides may be separated from the strongly adsorbed purine nucleosides and amino acids may be separated into groups. Protonation of a molecule resulted in a smaller elution volume.

INTRODUCTION

Chromatography on cross-linked dextran (Sephadex*) and cross-linked polyacrylamide (Bio-Gel) has been utilized extensively for the separation of a large variety of organic compounds. Relatively little information is available about chromatography on cross-linked polystyrene resins which do not contain ion-exchange groups, particularly when using aqueous eluants.

The principle of gel filtration developed by PORATH AND FLODIN[1] was applied by VAUGHAN[2] to a non-aqueous system to fractionate polystyrene on columns of polystyrene–divinylbenzene resin with benzene as eluant. Lipid mixtures have also been separated on polystyrene resins with benzene[3]. CORTIS-JONES[4] separated a number of organic compounds of low molecular weight on polystyrene resins with benzene or other organic solvents as eluants. Macroreticular polystyrene gels have been developed by MOORE[5] for the determination of molecular weight distribution of polymers in non-aqueous media.

Few attempts have been made to use polystyrene resins for chromatography in

* Mention of commercial names does not imply endorsement by the U.S. Department of Agriculture.

aqueous media. CORTIS-JONES[4] reported that substances as widely different in molecular weight as egg albumin and glucose could not be separated on polystyrene resin with aqueous eluants. However, WHEATON AND BAUMAN[6] found that non-ionic organic compounds of low molecular weight could be separated by elution with water through columns of cross-linked polystyrene ion-exchange resins such as Dowex 50.

Gel filtration effects were observed during chromatography with water as eluant of oligosaccharides[7,8] and polyhydric alcohols[9] on Dowex 50 cation-exchange resin. HOUGH *et al.*[10] eluted raffinose, sucrose, and glucose, in that order, with water from an anion-exchange resin Permutit De-Acidite, but observed no separation on Permutit polystyrene beads.

Separation of monosaccharides analogous to partition chromatography was observed on cation-exchange resins with water[8,11] and on anion-exchange resin with aqueous ethanol[12] as eluant. Anomeric glycosides were resolved with water on Dowex 1 X2 [OH^-] although free sugars were not eluted from the column[13].

Salting-out chromatography[14] was useful in separating alcohols, ethers, amines, aldehydes, and ketones. Non-ionic compounds were eluted from columns of cation- or anion-exchange resins with electrolyte solutions in order of their increasing hydrophobic character. Ionic and non-ionic materials were separated on ion-exchange resins by ion exclusion[15].

Recently new resins, polystyrene cross-linked with divinylbenzene, have become available under the name of Amberlite XAD. These stable non-ionic resins are in the form of beads each of which is an agglomeration of a large number of microspheres. This structure imparts to the resin macroreticular porosity, uniform pore size distribution, and high surface area. These resins are designed to remove water-soluble organic materials from aqueous solution by adsorption. Van der Waals forces are considered to be involved in the adsorption mechanism[16]. The hydrophobic portion of the solute molecule is selectively adsorbed on the polystyrene surface of the bead while the hydrophilic portion is oriented toward the aqueous phase.

RILEY AND TAYLOR[17] employed Amberlite XAD-1 polystyrene resin for concentration of trace organic compounds such as surfactants, insecticides, and dyes in sea water. We have reported previously the use of Amberlite XAD-2, a higher surface area analog of Amberlite XAD-1, for separation of water-soluble meat flavor precursors[18] and for removal of excess picric acid used to deproteinize tissue extracts[19]. In the present study we report on the separation of a number of compounds of low molecular weight found in tissue extracts on columns of Amberlite XAD-2 resin with aqueous solutions as eluants.

EXPERIMENTAL

Polystyrene resin, Amberlite XAD-2 (20–50 mesh, Rohm and Haas Co.), was washed extensively with methylene chloride–methanol (1:3), methanol, and finally water to remove UV absorbing impurities. A slurry of resin in water was poured into glass columns fitted with sintered glass supports to give resin beds of 1.5 × 43 cm, 1.6 × 40 cm, 1.5 × 84 cm, and 2.9 × 34.5 cm. Elution was generally carried out with water under gravity flow. When 0.001 *N* HCl or 0.001 *N* NaCl was used as eluant, the column was equilibrated with the eluant prior to sample application.

Purine and pyrimidine derivatives as well as amino acids were obtained from

Sigma Chemical Co., St. Louis, Mo.; creatinine from Fisher Scientific Co., Fair Lawn, N. J.; glucose from J. T. Baker Chemical Co., Phillipsburg, N. J.

Solutions of standard compounds (0.5–10 mg) were prepared in water with the addition of hydrochloric acid or sodium hydroxide when necessary to effect solution.

The column effluent was monitored by means of a Gilford Model 2000 multiple absorbance recording spectrophotometer to detect UV absorbing compounds. The wavelength was set at the absorbance maximum for each compound. Fractions of 2.5–4.5 ml were collected with a Beckman Model 132 fraction collector at a flow rate of 1–2 ml/min.

Amino acids were estimated by the ninhydrin procedure of COCKING AND YEMM[20] and identified by thin-layer chromatography. Glucose was estimated by the anthrone procedure of TOENNIES AND KOLB[21].

RESULTS AND DISCUSSION

Our experiments showed that useful separations may be obtained on columns of polystyrene resin using water and dilute aqueous solutions as the eluting media. Tables I–IV list the elution volumes of standard compounds on Amberlite XAD-2 columns.

It has been shown[19] that amino acids can be separated into two groups on Amberlite XAD-2. Thus, leucine, isoleucine, phenylalanine, and tyrosine were separated from the other amino acids found in meat extract. Table I shows the elution volumes of a number of amino acids from a 1.6 × 40 cm column of Amberlite XAD-2. Most amino acids elute from the column early, while those possessing large alkyl chains (leucine) or aromatic groups (tyrosine) are retarded. TLC of the eluted fractions showed that the two groups of amino acids were almost completely separated.

TABLE I

ELUTION VOLUMES (ml) OF STANDARD COMPOUNDS FROM AN AMBERLITE XAD-2 1.6 × 40 cm COLUMN

Compound	*Sample medium[a]/eluant*		
	0.4 N HCl/ water	*0.001 N NaCl/ 0.001 N NaCl*	*0.4 N HCl/ 0.001 N NaCl*
Glycine	51	55	45
Glutamic acid	51		48
Histidine · HCl	51		45
Leucine	81		75
Tyrosine	81		75
Lysine · HCl		55	
Taurine		55	
Methionine		76	
Inosine		200	
Hypoxanthine			72
Glucose		55	

[a] Solutions (1 ml) of 3–6 mg of each compound were applied to the column.

TABLE II

ELUTION VOLUMES (ml) OF STANDARD COMPOUNDS FROM AN AMBERLITE XAD-2 1.5 × 43 cm COLUMN

Eluant: water; sample medium: HCl, pH 1.

Compound	*Elution volume*
Glycine	60
Glucose	72
Inosinic acid } Hypoxanthine }	96[a]
Leucine	108
Inosine	232
Guanosine	236

[a] One peak was recorded at 247 mμ for samples containing a mixture of inosinic acid and hypoxanthine. Inosinic acid was eluted on the leading edge of the peak.

Elution of amino acids with water or with 0.001 *N* sodium chloride gave similar separations. In mixtures of inosine, hypoxanthine and amino acids, inosine was completely separated while hypoxanthine was eluted together with the second group of amino acids.

Previously CLEAVER AND CASSIDY[22] showed that adsorption of amino acids on polystyrene-based ion exchangers was independent of true ion exchange. According to MOORE AND STEIN[23] the polystyrene matrix of the ion exchanger plays an important role in the resolution of amino acids. Thus, glycine, alanine, valine, and leucine, amino acids of identical charge, are separated on Dowex 50 as a result of greater affinity of the resin for longer aliphatic side chains.

TABLE III

ELUTION VOLUMES (ml) OF STANDARD COMPOUNDS FROM AN AMBERLITE XAD-2 1.5 × 84 cm COLUMN

Eluant: water.

Compound	*Sample medium*		
	HCl[a]	*NaOH*	*Water*
Glucose	110		
Glycine	108	108[b,c]	107
Isoleucine	156		
Uracil	163	164[c]	
Uridine	235	227[c]	
Inosine		408[b]	421
Adenosine			~490[e]
Hypoxanthine } Uric acid }		180[b,d]	188

[a] Sample solution, pH 1.
[b] Sample solution, pH 13.
[c] Sample solution, pH 8.
[d] One peak was recorded at 260 mμ for a mixture of hypoxanthine and uric acid, with uric acid being eluted on the leading edge.
[e] Very diffuse peak.

The order of elution of nucleic acid derivatives from XAD-2 resin (nucleotide–base–nucleoside) was different from that observed on elution from Sephadex[24] or Bio-Gel[25] (nucleotide–nucleoside–base). Ionization rather than molecular weight apparently influences the separation on polystyrene.

On a small column of resin, 1.5 × 43 cm (Table II), inosine was separated from hypoxanthine, but inosinic acid was not resolved from hypoxanthine. On a column 2.9 × 34.5 cm, containing a larger amount of resin, similar sample load gave well separated peaks for inosinic acid, hypoxanthine, and inosine.

Purine and pyrimidine derivatives may also be resolved on Amberlite XAD-2 (Table III). Pyrimidines tend to be eluted somewhat sooner than purines, *e.g.* uracil ahead of hypoxanthine, and uridine ahead of inosine. This trend was observed on Sephadex[24,26] and Bio-Gel[25,27] columns.

CORTIS-JONES[4] has reported that with organic solvents as eluants the presence of hydroxyl groups tends to cause a molecule to be eluted earlier from a polystyrene resin column than a molecule of higher molecular weight but having a fewer number of hydroxyl groups.

In the present work, using aqueous systems, hydroxyl groups were found to affect the elution of a molecule from the resin. Glucose was eluted from columns of Amberlite XAD-2 without noticeable retardation. Also, uric acid, containing three hydroxyl groups, was eluted ahead of hypoxanthine, containing one hydroxyl group. Uric acid was not resolved from hypoxanthine on a 1.5 × 84 cm column but quite well resolved on a 2.9 × 34.5 cm column.

The role of pH was found to be very important in the chromatography of ionizable compounds on polystyrene resin in aqueous media. Not only was the pH of the eluant important in determining the elution volumes of compounds, but also the pH of the sample solution applied to the column. When creatinine in 0.05 *N* HCl was applied to a polystyrene column and eluted with water it emerged from the column as a sharp peak at 153 ml. Similarly, on chromatography of creatinine dissolved in 0.001 *N* HCl and eluted with the same solvent, a sharp peak at 170 ml was obtained. However, when a solution of creatinine in water was applied to the column and eluted with water, the creatinine emerged as a somewhat broad peak at 294 ml. In acidic solution creatinine (pK 4.8) exists as a cation and is not significantly adsorbed by the resin. In neutral solution creatinine is in the form of uncharged free base and is strongly adsorbed by the resin since it is potentially an aromatic system (imidazole).

KWON[28] has reported similar results in the case of malonaldehyde chromatographed on Sephadex G-10 where the elution volume was dependent on the pH of the eluant. In acidic solvents a chelate structure was considered to be present for malonaldehyde, which, owing to its pseudo-aromatic nature, was more strongly adsorbed to the column material than the linear molecule in neutral or basic solutions.

From the consideration of their pK values[29] it is evident that inosine, guanosine, and adenosine are still unionized in 0.001 *N* HCl and are strongly adsorbed on Amberlite XAD-2. The elution volumes of these compounds are quite large and the peaks very broad. Apparently purines in the free base form tend to be strongly adsorbed on polystyrene resin.

According to SWEETMAN AND NYHAN[26] protonation decreases adsorption. The elution volume of adenine (pK 4.2) from Sephadex G-10 was decreased when the

TABLE IV

ELUTION VOLUMES (ml) OF STANDARD COMPOUNDS FROM AN AMBERLITE XAD-2 2.9 × 34.5 cm COLUMN

Compound	*Eluant/sample medium*				
	Water/ water	*Water/ acid*	*Water/ base*[a]	*0.001 N HCl/ 0.001 N HCl*	*0.1 N HCl/ 0.1 N HCl*
Glucose	135	145[b]			
Glycine		137[c]	136	140	
Histidine	142	140[c]			
Leucine	211				
5′-Inosinic acid	137	169[b]		191	
Hypoxanthine		248[b]	243	256	
Tyrosine		248[b]			
Creatinine	294	153[c]		170	
Uric acid			127[d]		
5′-Adenylic acid				192	
Adenine				204	
Cytosine				148	
Cytidine				181	
Guanine				199	
Uridine				316	
Inosine	579	580[c]		568	453
Guanosine				657	351
Adenosine				735	327

[a] Sample in NaOH solution, pH 10.
[b] Sample solution in 0.1 *N* HCl.
[c] Sample solution in 0.05 *N* HCl.
[d] The column was washed with water after chromatography of a sodium hydroxide-containing sample. An initial run of uric acid following chromatography of an acid-containing sample yielded an elution volume of 173 ml.

eluant was changed from pH 7 to pH 5, while no effect was observed on the elution volumes of hypoxanthine, xanthine, and guanine (p*K* 2.0, 0.8 and 3.3, respectively). This reasoning can be applied to the present experiment. When inosine, guanosine, and adenosine were eluted from Amberlite XAD-2 with 0.1 *N* HCl, markedly smaller elution volumes were obtained together with sharpening of the peaks as compared to elution with water or 0.001 *N* HCl using the same column (Table IV).

Inosinic acid also exhibited differences in elution volumes depending on whether the sample was dissolved in water or in dilute acid. For inosinic acid dissolved in water the elution volume (Table IV) was 137 ml, while for inosinic acid dissolved in 0.1 *N* HCl the elution volume was 169 ml. A larger elution volume (191 ml) was observed for inosinic acid when elution was carried out with 0.001 *N* HCl. In acid solution the unionized form of the molecule may predominate over the charged species which are not adsorbed by the resin.

In the case of uracil and uridine the elution volumes were unchanged for samples of pH 1 or pH 8. Elution volumes of glycine and hypoxanthine were also independent of sample pH as well as of the eluting medium.

Anomalous behavior of uric acid during chromatography on Amberlite XAD-2 was noted. Sodium hydroxide was used to dissolve samples containing uric acid. In

the first chromatographic run uric acid was eluted with water at 173 ml, after glycine (139 ml). In a subsequent run performed directly after the initial one uric acid was eluted from the column at 125 ml, ahead of glycine, the elution volume of which was unaffected (140 ml). It was also noted that the effluent was not basic as expected. When a column of Amberlite XAD-2 was treated with 0.5 bed volumes of 1 N sodium hydroxide and subsequently washed with water, the residual sodium hydroxide was difficult to remove. No satisfactory explanation for these observations can be offered at this time, although it appears that sodium hydroxide is adsorbed by the polystyrene resin. It has been reported[24] that hydroxyl ions are adsorbed to Sephadex.

As had been noted previously in the case of gel filtration[30], the peaks that elute from the column early are quite sharp, but those that elute late become progressively more diffuse due to strong interaction with the resin phase. A similar trend was noted with Amberlite XAD-2. For example, a compound not retained on the resin—glucose (6.64 mg)—could be eluted in a 70-ml-wide band, while a compound showing strong adsorption—inosine (2.27 mg)—was eluted in a 270-ml-wide band from a 1.5 × 43 cm column of XAD-2 with 0.001 N HCl or water as eluant.

The results indicate that useful separations of water-soluble organic compounds may be achieved on polystyrene resin by taking advantage of adsorption effects. Since chromatography may be carried out with water or very dilute electrolyte solutions, the use of polystyrene resin may be more advantageous than other resins where solutions of high ionic strength are necessary to elute compounds from the column.

Amberlite XAD-2 may be conveniently employed for fractionation of nucleic acid derivatives, particularly for resolution of base–nucleoside pairs, and for separation of purine nucleosides from pyrimidine nucleosides. This resin may also be useful for desalting of water-soluble aromatic compounds.[18] Further experiments with aqueous eluants of various pH values will undoubtedly result in other useful separations on columns of Amberlite XAD-2.

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GEL PERMEATION CHROMATOGRAPHY OF THE CYCLIC MONOMERS AND OLIGOMERS IN NYLON 6 AND NYLON 66

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SUMMARY

The cyclic monomers and oligomers in nylon 6 and nylon 66 were separated and determined by gel permeation chromatography. The gels used in the experiment were Sephadex G-15, G-25 and Bio-Gel P-4. The ethanol extracts of nylon 6 and nylon 66 were dissolved in 0.1 N hydrochloric acid which was used as an eluant. The effluent was introduced into a flow cell and the absorbance at 210 mμ was measured continuously by means of a spectrophotometer. The method is applicable to 1–5 mg samples or less and the time needed for chromatography is less than 6 h. The presence of linear oligomers does not affect the determination of the cyclic oligomers.

Linear relationships between log M and several elution values, *viz.* V_e, V_e/V_t, V_e/V_0, K_d, and $V_e/(V_0 + V_i)$, have been confirmed for the cyclic monomer and oligomers of nylon 6. Deviation from linearity of the elution values of the cyclic dimer of nylon 66 implies that the dimension of the dimer is contracted in a solution compared with others.

INTRODUCTION

In most polyamides, cyclic monomers (*e.g.*, caprolactam for nylon 6), cyclic oligomers (cyclic dimer, trimer, etc.) and linear monomers and oligomers were considered to be the equilibrium products of polymerization. In numerous publications regarding the estimation of the monomer and oligomer in nylon 6, methods, often involving special techniques, for their determination have generally been based on weight differences.

ONGEMACH *et al.* determined the caprolactam (monomer) content in aqueous extracts of nylon 6 by gas chromatography[1] and the oligomer content, with that of the monomer[2], by differential refractometry and IR spectroscopy. According to BUKAČ *et al.*[3], the cyclic oligomer content could be estimated from the difference between percentage of the aqueous extracts and that of the monomer obtained by UV

and IR spectrophotometry. ANTON[4] directly determined the cyclic oligomer content in an aqueous extract of nylon 6 by IR spectrophotometry. By these methods it has not been possible to differentiate each cyclic oligomer (cyclic dimer, trimer, etc.) from the cyclic oligomer mixture in the sample.

Fractional sublimation[5] was applied for the differentiation and quantitative determination of the cyclic dimer to tetramer of nylon 6. Paper chromatography was also employed for differentiating the cyclic oligomers in nylon 6 (ref. 6) and nylon 66 (ref. 7). The cyclic oligomers separated on the paper chromatogram were determined colorimetrically at 420 mμ after color development of the spots by means of chlorine gas and *o*-tolidine/potassium iodide solution[6,8,9]. There is little information in the literature on the quantitative determination of the individual oligomers except these two methods (fractional sublimation and paper chromatography), which are lengthy, complicated and time-consuming.

The technique of gel permeation chromatography (GPC) has proved to be a versatile tool for the fractionation of a homologous series of macromolecules according to molecular size[10]. This technique is applicable to oligomer separation. Low-molecular-weight homologs in poly(ethylene glycol) were separated by GPC using cross-linked dextran gels (Sephadex)[11,12]. KUSCH AND ZAHN[13] isolated the higher oligoamides in several polyamides on a preparative scale by gel filtration of the polyamide extracts on Sephadex G-25 and Bio-Gel P-10. By their method, the extracts were dissolved in 30–50% acetic acid and loaded onto a column of gels (4 cm in diameter, 500 cm long). DETERMANN *et al.*[14] prepared a copolymer of methyl methacrylate and ethylene glycol dimethacrylate that was used for fractionation of low-molecular-weight polystyrenes. HEITZ *et al.*[15] fractionated oligophenylenes and oligourethanes using several cross-linked gels. The use of particular dextran gels for separations according to size was introduced by PORATH AND FLODIN[16].

The method described in this paper enables quantitative separation of the cyclic monomers and oligomers in nylon 6 and nylon 66. The ethanol extracts of nylon 6 and nylon 66 were dissolved in 0.1 *N* HCl, separated into fractions by GPC and then detected by UV spectrophotometry. The gels used in this study were the commercially available, cross-linked dextrans Sephadex (Pharmacia, Uppsala, Sweden) and the cross-linked polyacrylamide Bio-Gel (Bio-Rad Laboratories, Calif., U.S.A.).

EXPERIMENTAL

Materials

The gels used were Sephadex G-25 Fine (particle size, 20–80 μ), G-15 (40–120 μ) and Bio-Gel P-4 (200–400 mesh). These gels were used without further screening. Their regain in water was determined experimentally in our laboratory. The eluant was 0.1 *N* HCl in water; its pH was approx. 1.1. Cyclic oligoamides of nylon 6 and cyclic mono- and oligoamides of nylon 66 were obtained by evaporating the corresponding ethanol extracts. The cyclic dimer and trimer of nylon 6 were obtained from the oligomer mixture by fractional sublimation in accordance with the method of HEIKENS[5]. The cyclic monomer of nylon 66 was extracted with aceton from the oligomer mixture.

Packing

The dry gels were suspended in 0.1 *N* HCl and left overnight. The columns used were cylindrical glass tubes 15.6 mm in diameter and 100 (and 150) cm in length. The bottom ends were connected to 3-cm-long capillaries with a 2-mm bore by means of silicone rubber stoppers. Before packing, the columns were mounted vertically, and small pieces of glass wool were laid over the outlet capillaries. The swollen gels were then packed into the columns by the method of WIDÉN AND ERIKSSON[17]. After the packing, the columns were percolated overnight with the eluant to be used to stabilize the beds and to remove the dissolved materials in the gels. The tops of the column tubes were connected to 1-l Mariotte bottles through polyvinylchloride tubes inserted through silicone rubber stoppers.

Elution

A 5–30 mg portion of the cyclic mono- and oligoamides was weighed into a 10-ml flask and the flask was filled with 0.1 *N* HCl. If necessary the solution in the flask was heated in a water bath at 80°. Application of the sample solution was carried out by the method of FLODIN[18]. A 1-ml portion of the sample solution was introduced onto the column. A constant flow rate was regulated by means of the Mariotte bottle; the flow rate was kept at 30 ml/h for the Sephadex columns and 20 ml/h for the Bio-Gel column. Elution was carried out at constant temperature, and the volume of the eluates was checked by means of a graduated cylinder. Blue Dextran 2000 (Pharmacia) was applied to determine the void volume (V_0) of the columns and to check the homogeneity of packing.

Detection

A Hitachi double-beam grating spectrophotometer Model 124 equipped with a flow cell was used. The bottom end of the column tube was connected to the flow cell through a teflon tube. Cyclic mono- and oligoamides appearing in the effluent were determined by measuring the absorption at 210 mμ. A Hitachi QPD_{34} recorder was used for the continuous recording of the absorption at 210 mμ. Blue Dextran 2000 was determined at 620 mμ.

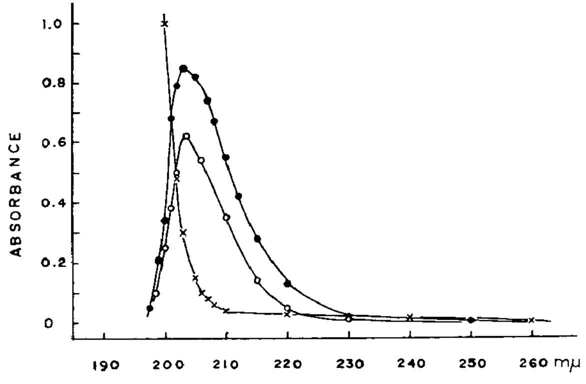

Fig. 1. UV absorption spectra for caprolactam and the cyclic dimer of nylon 6. Cell length: 1 cm. Reference side: for caprolactam and dimer, 0.1 *N* HCl; for 0.1 *N* HCl, H_2O. ●, Caprolactam, 0.0015% in 0.1 *N* HCl; ○, dimer, 0.005% in 0.1 *N* HCl; ×, 0.1 *N* HCl.

RESULTS AND DISCUSSION

The UV absorption spectra of the cyclic monomer and dimer of nylon 6 in 0.1 *N* HCl are shown in Fig. 1. The molar absorption coefficients (ε) of each cyclic monomer and oligomer at 210 mμ and 215 mμ were measured and listed in Table I. In computing the molar absorption coefficient, the assumption has been made that one mole of each oligomer is 113 g, considering that the absorbance in this region

TABLE I

THE MOLAR ABSORPTION COEFFICIENTS OF EACH CYCLIC MONOMER AND OLIGOMER

Monomer and oligomer	*Molar absorption coefficient, ε (l/mole[a]·cm)*		
	At 210 mμ	*Correction factor*	*At 215 mμ*
Nylon 6 monomer	2800	0.35	1470
dimer	980	1	390
trimer	970	1	460
tetramer	970	1	440
Nylon 66 monomer	1810	0.69	870
dimer and trimer (mixture)	1250	1	490
ε-Aminocaproic acid	68		55

[a] As $-(CH_2)_5CONH-$ unit (for nylon 66, half of the $-CO(CH_2)_4CONH-(CH_2)_6NH-$ unit is considered).

would be proportional to the quantity of the amide linkage. From the UV spectra, 210 mμ was selected for the detection of the monomer and oligomers. The value obtained by multiplying the absorbance of the monomer by the correction factor in Table I gives the impression that the ratios of absorbances of each monomer and oligomer represent their weight ratios.

The elution curves of the cyclic monomer and oligomer of nylon 6 and nylon 66 on G-15, G-25 and P-4 are shown in Fig. 2. Even though there was some overlap in elution peaks, the separation seems to be sufficient for the purpose of quantitative analysis. The variations in particle size and flow rate proved to be the most important factors for the efficiency of the column[18]. Better column efficiency can be obtained by using a small particle size. On Sephadex G-25 Superfine a better separation between the two adjacent peaks was obtained than on Sephadex G-25 Fine, but the time required for one cycle was longer because of the lower flow rate. Lengthening the gel head in the column also improved the separation, and at the same time a broadening of the peak width occurred.

The volume of sample solution injected governs the peak width[18]. A series of solutions ranging from 3 to 20 mg of the cyclic dimer of nylon 6 per 10 ml of 0.1 *N* HCl was prepared, and 1 ml of each solution was injected onto the column. The width of the elution peak at the base remained constant regardless of the weight of the dimer injected. This result implies that the apparent separability between the two adjacent peaks would improve when the sample concentration in the solution was

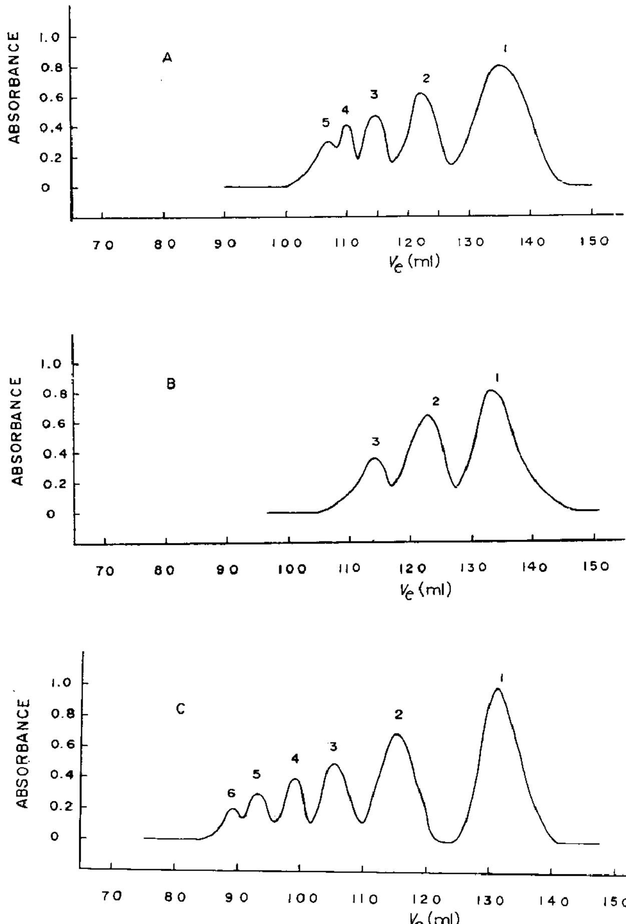

Fig. 2. Elution chromatograms of the cyclic monomers and oligomers of nylon 6 and nylon 66. (A) Separation of monomer and oligomers in nylon 6 on G-15 (column 15A). Concentration: 5 mg/ml. (B) Separation of monomer and oligomers in nylon 66 on G-25 (column 25A). Concentration: 4 mg/ml. (C) Separation of monomer and oligomers in nylon 6 on P-4 (column P4). Concentration: 3.5 mg/ml. 1 = cyclic monomer; 2 = cyclic dimer; 3 = cyclic trimer; 4 = cyclic tetramer; 5 = cyclic pentamer; 6 = cyclic hexamer.

increased in an appropriate range. This conclusion was verified by the experiments.

The column constants showing the column performance for Sephadex G-15, G-25 and Bio-Gel P-4 are listed in Table II. V_0 was experimentally determined as the elution volume for Blue Dextran 2000 (mol. wt. 2,000,000). V_i was calculated from the water regain (W_r), and the dry weight of gels (a): $V_i = a \cdot W_r$. W_r was determined by centrifuging the swollen gels and by weighing the centrifuged swollen gels before and after drying.

The volume in a teflon tube between the bottom end of the column and the inlet of the flow cell was subtracted from the apparent elution volume. The corrected elution volumes (V_e) of each monomer and oligomer are presented in Table III. The

TABLE II

COLUMN CONSTANTS

Column symbol	15A	15B	25A	25B	P4
Type of gel	G-15	G-15	G-25	G-25	P-4
Column head (cm)	89.6	149	91	147	87
Dry weight of gel, a (g)	54	90	36.2	58.6	40
Total volume of gel bed, V_t (ml)	171	286	174	281	166
Void volume, V_0 (ml)	67	111[a]	69	112[a]	52
Inner volume, V_i (ml)	70	117[a]	83	133[a]	80
Volume of gel phase, V_x (ml)	104	175[a]	105	169[a]	114
Volume of gel matrix, V_g (ml)	34	58	22	36	34
Water regain, W_r (g/g of dry gel)	1.3		2.3		2.0
Bed volume per g dry gel (ml)	3.2		4.8		4.2

[a] Calculated in proportion to V_t of 15A and 15B, 25A and 25B.

TABLE III

THE ELUTION VOLUMES, V_e (ml), OF CYCLIC MONOMERS AND OLIGOMERS

Oligomer		*Type of gel/column symbol*					
		G-15/ 15A	*G-15/ 15B*	*G-25/ 25A*	*G-25/ 25B*	*P-4/P4*	*Mol. wt.*
Nylon 6	monomer	135	234	144	243	131	113
	dimer	122	211	133	223	115	226
	trimer	115	192	125	210	105	339
	tetramer	110	180	120	201	99	452
	pentamer	107	172	117	193	93	565
	hexamer					89	678
Nylon 66	monomer	122		133	222	117	226
	dimer	114		123	205	104	452
	trimer	104		114	190	90	678
ε-Aminocaproic acid				131			

TABLE IV

PER CENT OF CYCLIC MONOMERS AND OLIGOMERS IN THE ETHANOL EXTRACTS AND IN POLYMERS[a]

Oligomer	*Nylon 6*		*Nylon 66*	
	In extract	*In polymer*	*In extract*	*In polymer*
Monomer	82.5	8.70	42.3	0.67
Dimer	7.1	0.75	43.0	0.68
Trimer	4.5	0.47	14.7	0.23
Tetramer	2.8	0.30		
Pentamer	1.6	0.17		
Hexamer[b]	1.5	0.16		

[a] Nylon 6 and nylon 66 were prepared at the laboratory.
[b] Included oligomers larger than hexamer.

elution volume of ε-aminocaproic acid (ACA), which appeared in nylon 6 as a linear monomer, is near that of the cyclic dimer of nylon 6. However, the quantity of ACA is less than 0.1% (ref. 19) and in addition the molar absorption coefficient of ACA is small (Table I), so that the presence of ACA does not affect the determination of the cyclic dimer.

The cyclic monomer and oligomer mixtures in nylon 6 and nylon 66 were obtained by extracting nylon 6 and nylon 66 with ethanol for 24 h followed by evaporation of the ethanol. From the elution chromatograms of these mixtures, peak areas (= absorbance × ml) were calculated and the peak area of the monomer was corrected by multiplying the peak area by the correction factor given in Table I. The ratios of each peak area to the total peak area show the ratios of the monomer and individual oligomers in the mixture by weight. The monomer and oligomer contents in the polymer were also calculated on the basis of the weight of the extracts. The results are shown in Table IV. Accuracy and precision data are listed in Table V. In conclusion, it has been shown that with relatively simple equipment and the GPC technique, the presence of as little as 0.1% cyclic oligomers can be detected in nylon 6 and nylon 66.

Several authors have investigated the correlation between molecular weight and elution behavior. For example, linear proportionality between V_e/V_x and log M (ref. 20), V_e/V_0 and log M (refs. 21 and 22), and V_e and log M (ref. 23) was observed. DETERMANN AND MICHEL[24] investigated these relations systematically and proposed the following equation:

$$\log M = \log M_0 - (6.062 - 5.00d)\ (V_e/V_0)$$

In the course of our experiments several relationships were compared: molecular weight or logarithm of molecular weight were plotted against V_e, V_e/V_t, V_e/V_0, K_d, or $V_e/(V_0 + V_i)$. Linear relationships between log M and these elution values have been confirmed within experimental error for the monomer and oligomers of nylon 6 and between molecular weight and these elution values for nylon 66. Some examples for these relations are drawn in Fig. 3. Assuming linearity up to the upper limit, one should be able to calculate the exclusion limits of different gels. The exclusion limits for the cyclic oligomer of nylon 6 fall somewhere between 4600 and 5500 (G-15), 7300 and 9500 (G-25), and 3000 and 3400 (P-4). These limits vary with the elution functions.

TABLE V

RECOVERY EXPERIMENT OF THE CYCLIC MONOMER AND OLIGOMERS OF NYLON 6

Material	*Added (%)*	*Found (%)*	*Relative error (%)*
Monomer	20.7	22.3	+ 7.7
Dimer	39.5	36.9	− 6.6
Trimer	39.8	40.8	+ 2.5
Monomer	20.2	17.5	−13.4
Dimer	39.8	38.7	− 2.8
Trimer	40.0	43.8	+ 9.5

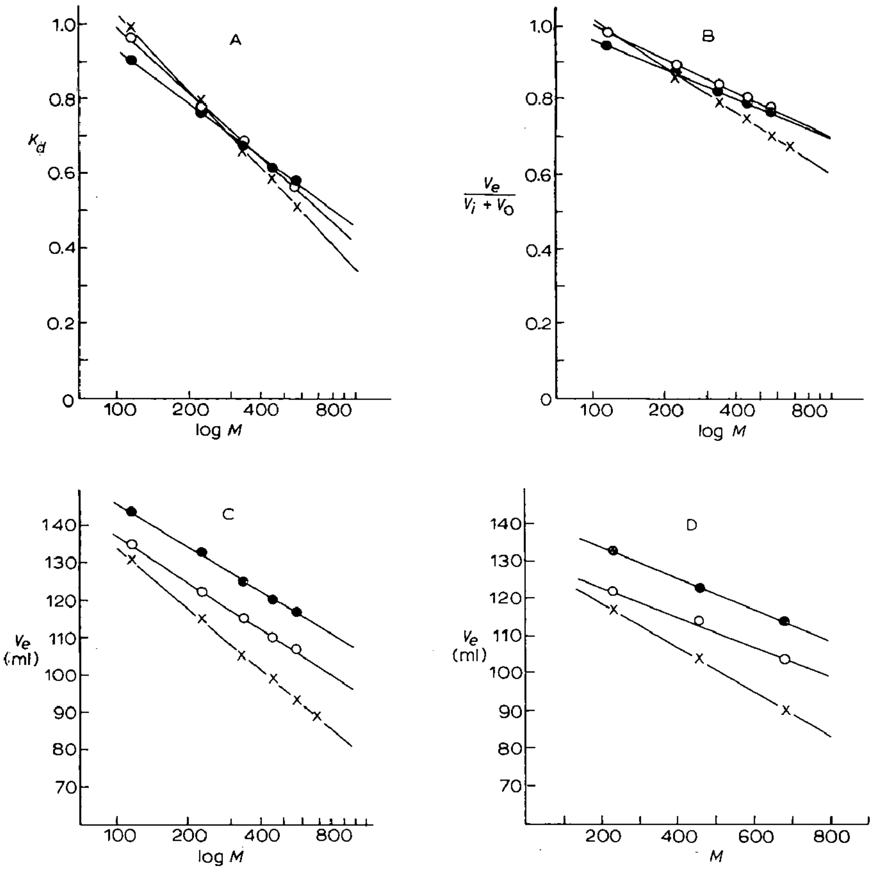

Fig. 3. Correlations between the elution volumes and molecular weights of cyclic monomers and oligomers on G-15 (column 15A), G-25 (column 25A), and P-4 (column P_4). (A) K_d *vs.* log M for nylon 6 monomer and oligomers. (B) $V_e/(V_i + V_0)$ *vs.* log M for nylon 6 monomer and oligomers. (C) V_e *vs.* log M for nylon 6 monomers and oligomers. (D) V_e *vs.* M for nylon 66 monomer and oligomers. ●, G-25; ○, G-15; ×, P-4.

SIEGEL AND MONTY[25] stated that it is not the molecular weight, but the Stoke radius which governs the elution position in some special cases. The cyclic dimer of nylon 66 seems to correspond to this special case. The elution volumes for the cyclic monomer and trimer of nylon 66 are nearly coincident with those for the cyclic dimer and hexamer of nylon 6, which have the same molecular weights. The cyclic dimer of nylon 66 seems to have a higher elution volume than what can be expected from its molecular weight in comparison with the cyclic tetramer of nylon 6. This implies that the dimension of the cyclic dimer of nylon 66 is contracted in a solution compared with others.

The volume available for a solute in the gel phase was determined from the elution volume, the void volume and the inner volume of the gel column. From this point of view, the term of $V_e/(V_0 + V_i)$ is newly introduced in order to predict the elution behavior. The values of $V_e/(V_0 + V_i)$ of the same oligomer on Sephadex G-15

and G-25 columns are almost identical and so are the values of K_d. This function thus seems to be appropriate along with K_d. However, this is an empirical result and it is not certain whether this is an equation of universal validity, since the experiments were only done with one kind of sample and two types of gels in our laboratory. Further systematic investigation will be needed in this respect.

It is well-known that in aqueous solutions aromatic substances are retarded on Sephadex gels of the G-series[26]. This effect is particularly noticed in the highly cross-linked gels of G-10, 15 and 25. During our experiments, water was used as the eluant at first and the retardation effect was observed, especially in caprolactam. The separation between two adjacent peaks was very poor. Using 0.1 N HCl as the eluant, the retardation effect became far less pronounced and the solubility of the oligomers was also greatly improved.

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SEPARATION OF PRIMARY AROMATIC AMINES ON ALGINIC ACID AND CARBOXYMETHYLCELLULOSE COLUMNS

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SUMMARY

The chromatographic behavior of primary aromatic amines in columns of alginic acid and carboxymethylcellulose was investigated extensively. Aqueous solutions of mineral acids and organic acids, water and aqueous-organic solvents were used as eluents.

The relation between the data obtained on a thin layer and those obtained in a column was examined.

INTRODUCTION

The column chromatographic separation of primary aromatic amines has mostly been studied using Celite, silica gel and Teflon-6 as adsorbents[1-3]. Ion-exchange chromatography in this field seems only to have been used by TOMPSETT[4], who takes advantage of the high affinity of polystyrene resins for aromatic substances to separate a group of primary aromatic amines from other substances, present in biological materials, on strong cation exchangers and, more recently, on anion exchangers[5]. We have consequently considered it useful, as a logical extension of research already begun in this sector[6], to undertake a systematic investigation of the behavior of aromatic amines on columns of weak cation exchangers.

The study was limited to the use of alginic acid and carboxymethylcellulose, two exchangers that had already been successfully employed in thin-layer chromatography in the separation of the same aromatic amines[6]. We have thus been able to study any relationship which might exist between the data obtained by the two different chromatographic techniques and to verify whether separations obtainable on thin layers could be reproduced on a larger scale on a column of the same adsorbent.

EXPERIMENTAL

The alginic acid employed was obtained as described in a preceding investigation[7], but had a larger particle size (50–150 mesh) than that used in TLC (*viz.* passed mesh sizes greater than 150) so as to have a greater eluent flow rate. Columns having

a cross-section of 0.94 cm^2 and filled with 4 g of exchanger were used. 2 g of the exchanger in the acid form, obtained by treatment of CMCNa (ref. 8) with 1 *M* HCl and successive washing with water until the chloride ions disappeared, were used for the carboxymethylcellulose columns.

The eluent flow rate was found to be greater in the case of alginic acid columns (1 ml every 30 sec) than in the case of CMC columns (1 ml every 60 sec). In the case of the alginic acid, this rate remained practically constant whereas it decreased in the CMC columns to half its initial value.

The amine solutions were prepared by dissolving the sample in the same solvent as was used in the column (water; 0.1 *M* HCl; 1 *M* acetic acid and 1 *M* monochloroacetic acid in water and in 50% isopropyl alcohol). The amine concentration used was 1 g/l with the exception of the following:

(a) *o*- and *m*-nitroaniline (0.5 g/l), *p*-nitroaniline (0.3 g/l) in all solvents excepting water;

(b) arsanilic acids, *p*-aminoacetophenone and *o*-nitroaniline (0.25 g/l), *m*- and *p*-nitroaniline (0.2 g/l) in water.

The volume of solution introduced into the column was in the majority of cases 0.05 ml, as can be seen from the quantity of amine employed (see Table I).

In the case of 4-aminosalicylic acid, a solution having a concentration of 5 g/l (1 ml of which was introduced in the column so as to obtain the maximum quantity reported in Table II) was also employed.

Amine solutions that decompose in air (*e.g.* phenylenediamines and *p*-aminodimethylaniline) were prepared immediately before use.

The elution curves were obtained by colorimetry, using *p*-dimethylaminobenzaldehyde to reveal the aromatic amines[6]. The total quantity of amine eluted was determined spectrophotometrically after diazotization with nitrous acid followed by coupling with N-(1-naphthyl)ethylenediamine[9]. Fig. 1 shows the calibration curves of some of the amines. To obtain reproducible results, the spectrophotometric mea-

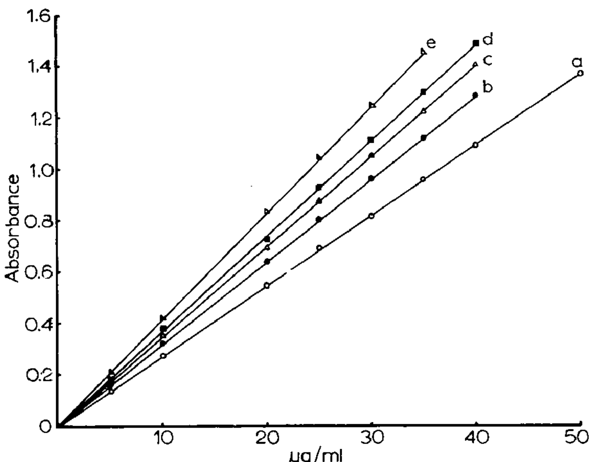

Fig. 1. Calibration curves for some aromatic amines. (a) *o*-Nitroaniline; (b) *m*-nitroaniline, sulfanilamide and *p*-aminohippuric acid; (c) *m*-aminobenzoic acid; (d) *p*-aminobenzoic acid; (e) *p*-nitroaniline.

surements must be made at least 60 min after the addition of the coupling reagent.

As 4-aminosalicylic acid does not react with N-(1-naphthyl)ethylenediamine, it was determined indirectly by taking advantage of the formation of molybdenum blue[10].

RESULTS AND DISCUSSION

Alginic acid columns

Elution with 1 M acetic acid. In thin-layer chromatography of aromatic amines and if 1 *M* acetic acid is used as eluent the diamines show a greater affinity for alginic acid than the monoamines, as indicated by their R_F value ($\leq$ 0.06). This characteristic is enhanced in a column, permitting the quantitative separation of the diamines from the monoamines.

o-Phenylenediamine, although in fact it has the same R_F value as the naphthylamines (0.06), is completely and distinctly separated from the latter. It should also be noted that the naphthylamines, while having the lowest R_F values of the monoamines, give an elongated elution curve which develops over a volume range of about 100 ml (see Fig. 2). For this reason they do not lend themselves to quantitative determination.

It is possible to deduce the following from the monoamine elution curves shown in Fig. 2:

In general the resolving power of a column is less with respect to that of a thin

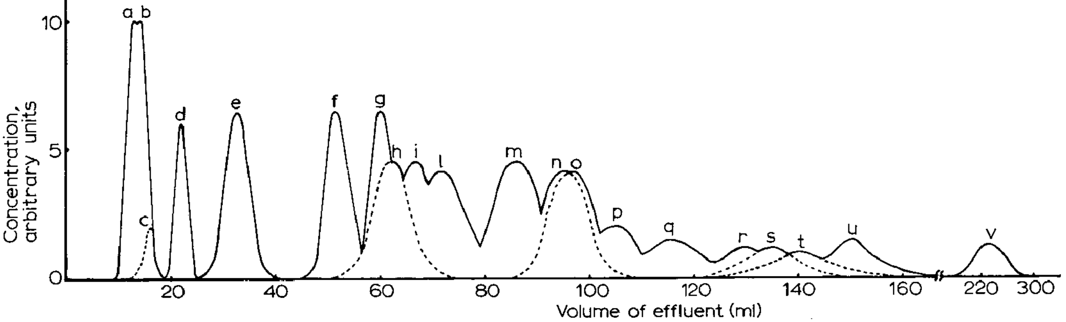

Fig. 2. Elution curves for aromatic amines on an alginic acid column with 1 *M* acetic acid as eluent. (a) Sulfanilic, methanilic and orthanilic acids; (b) *o*-arsanilic acid; (c) *o*-nitroaniline; (d) *p*-nitroaniline; (e) *p*-arsanilic acid; (f) 4-aminosalicylic acid; (g) *o*-aminobenzoic acid; (h) sulfanilamide; (i) *p*-aminoacetophenone; (l) 5-aminosalicylic acid; (m) *p*-aminobenzoic acid; (n) *o*-chloroaniline; (o) *p*-aminohippuric acid; (p) *m*-nitroaniline; (q) aniline; (r) *o*- and *p*-toluidine; (s) *m*-aminobenzoic acid; (t) *o*-anisidine and *o*-aminophenol; (u) *m*- and *p*-aminophenol; (v) α- and β-naphthylamine.

layer when the R_F values are high (of the order of 0.5 or greater), while it is greater when the R_F values are low. In this last case, separations not foreseeable from the R_F values are sometimes possible, as for example in the separation of *o*-chloroaniline (R_F = 0.13) and *p*-aminobenzoic acid (R_F = 0.12) from a group of amines (such as toluidines, aminophenols, *o*-anisidine and *p*-aminobenzoic acid) having R_F values ranging from 0.08 to 0.10.

TABLE I

SEPARATION OF THE ISOMERS OF SOME AROMATIC AMINES ON AN ALGINIC ACID COLUMN WITH 1 M ACETIC ACID AS ELUENT

Compound used	*Weight of compound placed on column (μg)*	*Volume range of the eluate (ml)*	*Recovery of base (%)*[a]
o-Nitroaniline	10	13–19	93 ± 4
p-Nitroaniline	30	19–25	95 ± 3
m-Nitroaniline	30	95–115	92 ± 4
o-Aminobenzoic acid	50	54–68	94 ± 3
p-Aminobenzoic acid	50	74–98	94 ± 3
m-Aminobenzoic acid	50	120–150	91 ± 4
o-Arsanilic acid	50	10–18	95 ± 3
p-Arsanilic acid	50	25–40	94 ± 3
p-Arsanilic acid	100	22–45	96 ± 3
4-Aminosalicylic acid	50	42–60	93 ± 4
5-Aminosalicylic acid	50	60–80	94 ± 4

[a] The reported data are the means of several determinations.

Contrary to what might be predicted from the thin-layer data, the separation of amines containing sulfonic groups ($R_F = 0.96$) from *o*-arsanilic acid ($R_F = 0.82$) and from *o*-nitroaniline ($R_F = 0.55$), is not possible.

The possibilities of separating isomeric aromatic amines on alginic acid are illustrated in Table I where data on the volume range of the eluate, the quantity of substance employed, and the percentage base recovered are reported. It can be seen from these data that a good separation among the isomers can be obtained, though, in the case of nitroanilines, the separation of the *ortho*- from the *para*-isomer is critically bound to the quantity of substance indicated in Table I. In fact, 250 μg of *o*-nitroaniline are eluted between 10 and 22 ml (recovery of the base 85%, calculated by direct spectrophotometric determination of the colored solution at 285 or 415 mμ) whereas 150 μg of *p*-nitroaniline pass between 10 and 32 ml (recovery of the base 92%, calculated by direct spectrophotometric determination at 385 mμ).

In addition, in this case the height of the column (that is, the quantity of exchanger used) is also a determining factor in that with 3 g of alginic acid, there is a partial overlap of the elution curves of the two nitroanilines even with the quantities reported in Table I.

The feasibility of a clean separation of 4-aminosalicylic acid from *m*-aminophenol should also be noted as it can be used for the purification of the acid. From the data reported in Table II, it can be seen that it is possible that considerable quantities of acid are used, notwithstanding the reduced dimensions of the column adopted by us, as the *m*-aminophenol is completely held back, even if present in a quantity less than 1% with respect to the 4-aminosalicylic acid.

TABLE II

SEPARATION OF *m*-AMINOPHENOL FROM 4-AMINOSALICYLIC ACID ON AN ALGINIC ACID COLUMN WITH 1 *M* ACETIC ACID AS ELUENT

Compound used	*Weight of compound placed on column (μg)*	*Volume range of the eluate (ml)*	*Recovery of base (%)*[a]
m-Aminophenol	50	140–165	90 ± 5
4-Aminosalicylic acid	50	42–60	93 ± 4
4-Aminosalicylic acid	1000	32–75	97 ± 2
4-Aminosalicylic acid	5000	26–90	98 ± 2

[a] The reported data are the means of several determinations.

Elution with 0.1 M hydrochloric acid. The great affinity of diamines for alginic acid can be considerably reduced by using hydrochloric acid as eluent, analogous to that which is observed on a thin layer. The elution curves in Fig. 3 show that the diamines are rapidly eluted with 0.1 *M* hydrochloric acid. On the column, *p*-aminodimethylaniline is eluted together with the *o*-phenylenediamine even though these two amines have considerably different R_F values on thin layers (respectively 0.36 and 0.63). Furthermore, *o*-phenylenediamine is incompletely separated from the *meta*- and *para*-isomers, in contrast to the clean separation observed on thin layers (*m*- and *p*-phenylenediamine have R_F values of 0.28 and 0.27, respectively).

The elution curve for the naphthylamines, which presents a much widened maximum when eluted with 1 *M* acetic acid, is shown in Fig. 3, as is that of aniline, which can be separated from 4-aminodiphenylamine and from benzidine.

Elution with 1 M monochloroacetic acid. The use of monochloroacetic acid is less efficient in the separation of monoamines as compared with acetic acid in that it lowers their affinities for alginic acid. For example, aniline which shows a considerable affinity for the exchanger with acetic acid, is now eluted between 20 and 30 ml. In practice all monoamines are eluted between a minimum of 9 ml and a maximum of 35

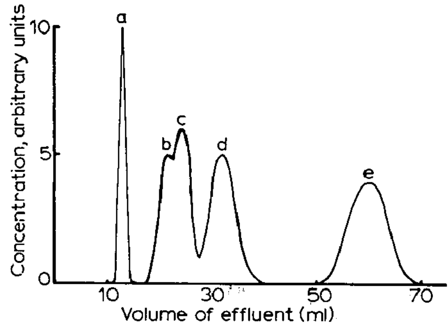

Fig. 3. Elution curves for aromatic amines on an alginic acid column with 0.1 *M* HCl as eluent. (a) Aniline; (b) *p*-aminodimethylaniline; (c) α- and β-naphthylamine, *o*-phenylenediamine, N-phenyl-*p*-phenylenediamine; (d) *m*- and *p*-phenylenediamine; (e) benzidine.

ml. However, the use of monochloroacetic acid for diamines and in particular for the separation of *o*-phenylenediamine from the *meta*- and *para*-isomers is interesting. The *ortho*-isomer elutes between 46 and 65 ml and the other two between 95 and 120 ml.

Elution with aqueous-organic solvents. To complete the picture of the behavior of aromatic amines in a column we deemed it useful to employ aqueous-organic solutions as eluents even though the results obtained on thin layers with eluents of this type were not very satisfactory.

A solution of 1 *M* monochloroacetic acid in 50% isopropyl alcohol was used. The separations obtainable on a column are not as good as those which might have been expected on the basis of the behavior of the amines on thin layers. For example 4-aminosalicylic acid, although it has an R_F of 0.28, elutes in the same volume range as those amines having an $R_F \geq 0.45$, and that is between 9 and 20 ml.

Carboxymethylcellulose columns

Elution with water. Carboxymethylcellulose shows, with respect to alginic acid, a lower affinity for aromatic amines and therefore has fewer possibilities of application in column chromatography. For this reason water was used as the eluent since it has, with respect to other solvents, a smaller leveling capacity[6].

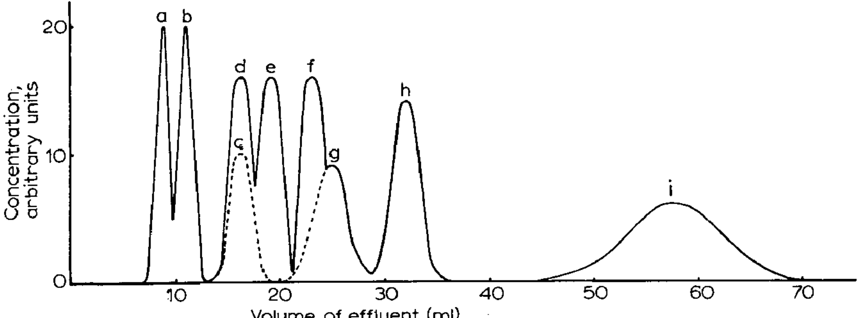

Fig. 4. Elution curves for aromatic amines on a carboxymethylcellulose column with water as eluent. (a) Sulfanilic, methanilic and orthanilic acids; (b) *o*- and *p*-arsanilic acids; (c) *o*-nitroaniline and *o*-aminobenzoic acid; (d) 4-aminosalicylic acid, and *p*-nitroaniline; (e) sulfanilamide, and *p*-aminohippuric acid; (f) *p*-aminoacetophenone; (g) *p*-aminobenzoic acid; (h) *m*-nitroaniline; (i) *m*-aminobenzoic acid.

As the elution curves reported in Fig. 4 show, the resolving power of the column increases considerably for R_F values ≤ 0.2. The clean separation of *m*-aminobenzoic acid ($R_F = 0.14$) from other amines and in particular from *p*-aminobenzoic acid ($R_F = 0.28$) and from *m*-nitroaniline ($R_F = 0.25$) should be noted.

As on alginic acid, it is also possible to separate the isomers of aminobenzoic acid on carboxymethylcellulose, as shown by the data reported in Table III.

The separation of the *ortho*- from the *para*-isomer, however, is critically bound to the quantity of amine indicated in Table III. It should, however, be pointed out that the use of water as eluent presents considerable advantages in that it permits us to obtain amine solutions in the absence of other ions.

This characteristic, notwithstanding the low flow rate of the eluent in such columns, could be used to advantage to obtain a solution having a primary aromatic

TABLE III

SEPARATION OF *o*-, *m*- AND *p*-AMINOBENZOIC ACID ON A CARBOXYMETHYLCELLULOSE COLUMN WITH WATER AS ELUENT

Compound used	*Weight of compound placed on column* (μg)	*Volume range of the eluate (ml)*	*Recovery of base* (%)[a]
o-Aminobenzoic acid	12.5	12–20	94 ± 3
p-Aminobenzoic acid	12.5	20–30	94 ± 3
m-Aminobenzoic acid	16.5	45–70	91 ± 4

[a] The reported data are the means of several determinations.

amine composition different from its initial one. A possible application would therefore be in concentration processes.

Elution with 1 M acetic acid. Amines that are held back strongly on the column when eluted with water and do not appear in Fig. 4, can be successively eluted with 1 *M* acetic acid. In this way it is possible to recover them in an interval of 8–30 ml and, at the same time, effect their separation from benzidine and the *meta*- and *para*-isomers of phenylenediamine.

Comparison of column and thin-layer data

The existence of a relation between the R_F value and the volume of the eluent needed to obtain the maximum concentration of an eluted ion in the effluent (V_{max}) has been assumed, even though, recently, it has been observed that results obtained on ion-exchange papers do not necessarily imply analogous results on a column[11]. In the present case considerable differences in the chromatographic behavior of the same amines, when the two techniques were employed, were also observed. It is not to be doubted, however, that a relation does exist between the two types of data. We therefore endeavoured to verify whether the nature of this relation was purely qualitative,

TABLE IV

VOLUME OF EFFLUENT RELATIVE TO THE PEAK OF THE ELUTION CURVE (V_{max}) AND R_F VALUE FOR SOME AROMATIC AMINES ON ALGINIC ACID WITH 1 *M* ACETIC ACID AS ELUENT

Compound used	V_{max}	R_F	$1/(R_F - 1)$
o-Arsanilic acid	14.0	0.82	0.22
o-Nitroaniline	16.0	0.55	0.82
p-Nitroaniline	22.0	0.47	1.13
p-Arsanilic acid	32.5	0.41	1.44
4-Aminosalicylic acid	52.0	0.26	2.85
o-Aminobenzoic acid	60.5	0.23	3.35
Sulfanilamide	62.5	0.22	3.55
p-Aminoacetophenone	67.0	0.21	3.77
5-Aminosalicylic acid	71.5	0.20	4.00
p-Aminobenzoic acid	86.0	0.16	5.25

semiquantitative, or rather quantitative by taking advantage of the equation:

$$V_{\max} = V_{\text{int}} + \frac{A_l}{A_s} g \left(\frac{1}{R_F} - 1\right) \qquad (1)$$

where

V_{int} = interstitial volume of the column;
A_l/A_s = cross sectional areas ratio of mobile and stationary phase on thin layer;
g = weight of the exchanger in column.

This relation was obtained by equating the two expressions that give the molar distribution coefficient K_d (amount of ion per gram of resin/amount of ion per ml of

TABLE V

VOLUME OF EFFLUENT RELATIVE TO THE PEAK OF THE ELUTION CURVE ($V_{\max}$) AND R_F VALUE FOR SOME AROMATIC AMINES ON CARBOXYMETHYLCELLULOSE WITH WATER AS ELUENT

Compound used	V_{max}	R_F	$1/(R_F - 1)$
p-Arsanilic acid	11.0	0.85	0.18
o-Nitroaniline	16.5	0.52	0.92
4-Aminosalicylic acid	16.5	0.50	1.00
p-Nitroaniline	16.5	0.47	1.08
o-Aminobenzoic acid	16.5	0.44	1.27
Sulfanilamide	19.0	0.43	1.32
p-Aminohippuric acid	19.0	0.43	1.32
p-Aminoacetophenone	23.0	0.35	1.86
p-Aminobenzoic acid	25.0	0.28	2.57
m-Nitroaniline	32.0	0.25	3.00
m-Aminobenzoic acid	57.5	0.14	6.15

solution) in a column ($V_{\max} = V_{\text{int}} + K_d g$) and on ion-exchange papers $\{K_d = 1/(R_F - 1)\, A_l/A_s\}$[12]. From the data reported in Tables IV and V and from the corresponding diagrams in Figs. 5 and 6, one can deduce the following:

Eqn. 1 seems to fit for both exchangers, under the experimental conditions we operated, in the range of R_F values between 0.2 and 0.8 only.

The V_{int} values obtained from Figs. 5 and 6 (7 ml in both cases) and, therefore, the values of the corresponding ratios A_l/A_s (0.6 for alginic acid and 0.7 for carboxy-

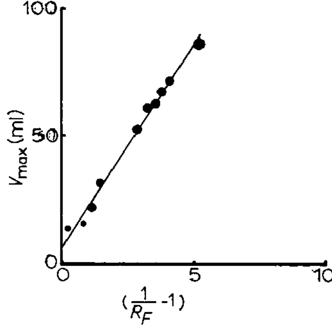

Fig. 5. Relationship between $V_{\max}$ and $1/(R_F - 1)$ for some aromatic amines on alginic acid with 1 *M* acetic acid as eluent.

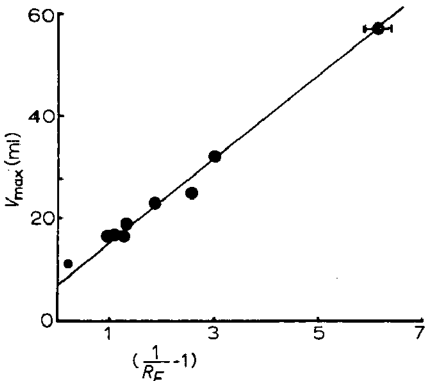

Fig. 6. Relationship between V_{max} and $1/(R_F - 1)$ for some aromatic amines on carboxymethylcellulose with water as eluent.

methylcellulose) seem reliable even if considerably different from those found for sulfonic resins (0.3–0.4)[13].

The A_l/A_s values obtained from the same diagrams (3.95 for alginic acid and 3.27 for carboxymethylcellulose) are comparable with those determined experimentally using ion-exchanger papers[14] and thin layers of Dowex 50 X4[15].

The fact that eqn. 1 is only fit to a limited extent (in the R_F range mentioned above) can be explained by bearing in mind that, for R_F values > 0.8, the solvent front influences the chromatographic behavior of compounds[12] considerably and that, for R_F values < 0.2, the enormous increase in the percentage error in the determination of these R_F values must also be taken into consideration. Another factor which cannot be neglected is the variation of the A_l/A_s ratio along the layer. It can therefore be concluded that, notwithstanding the great limitations indicated, eqn. 1 is on the whole verified and can serve as a convenient reference point for the transposition of a compound studied on layers of weak cation exchangers to a column.

CONCLUSIONS

The examination of the behavior of primary aromatic amines on chromatographic columns has permitted the realization of important separations both among isomers and among groups of amines having different acid–base characteristics, such as diamines from monoamines.

We have also been able to establish that the recovery of various amines is on the whole satisfactory and could therefore be used to advantage for preparative purposes. Finally, it has been verified that a quantitative relation exists between the data obtained on a thin layer and that obtained on a column, although this only applies to a definite R_F range.

ACKNOWLEDGEMENT

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CHROM. 4680

DÜNNSCHICHTCHROMATOGRAPHISCHE TRENNUNG DER AMINE ALS β-AMINOVINYL-*o*-HYDROXYPHENYLKETONE

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SUMMARY

Thin-layer chromatography of amine derivatives in the form of β-aminovinyl o-hydroxyphenyl ketones

Thin-layer chromatographic separation and identification of amines and amino compounds containing other functional groups in the form of enamine derivatives (β-aminovinyl *o*-hydroxyphenyl ketones) is described. Optimal separation is obtained on Kieselgel G using the following developing solvent systems: (1) ethyl acetate–benzene (1:5), (2) chloroform–xylene (8:2), (3) acetone–xylene (1:9). The difference in spot coloration in UV light of the aliphatic (willow-green) and aromatic (orange) enamines enables, together with R_F values, a quick identification of the compounds investigated. The sensitivity of the method is 0.1–1 μg. This new analytical procedure is highly specific and of general use. It also represents a valuable complementary method to the previously described general method of identification of amines in the form of crystalline derivatives with ω-formyl-*o*-hydroxyacetophenone or chromone and to the thin-layer and paper chromatography of amines in which ω-formyl-*o*-hydroxyacetophenone was used as a detecting reagent.

EINLEITUNG

Die Dünnschichtchromatographie (DC) der Amine wurde in der Form von folgenden Gruppen von Derivaten durchgeführt: Sulfonamide[1–4], N-substituierte 2,4-Dinitroaniline[5,6], 3,5-Dinitrobenzamide[3,7,8] und π Komplexe mit nitro- und chloroaromatischen Verbindungen[9,10]. Die DC-Trennungen von Benzamid- und Acetyl-Derivaten[1,3,11,12] wurden nur an einigen Beispielen beschrieben.

Von den vielen Amin-Reagenzien finden, in der chromatographischen und präparativen Identifikation, nur wenige eine gleichzeitige Anwendung[1–3,5,7,8].

Die primären und sekundären aliphatischen und aromatischen Amine und Verbindungen, welche noch andere funktionelle Gruppen enthalten, reagieren sehr leicht mit guter Ausbeute mit ω-Formyl-*o*-hydroxyacetophenon oder mit Benzo-γ-pyron und bilden Enamin-Derivate, nämlich β-Aminovinyl-*o*-hydroxyphenylketone[13].

$$\text{o-HO-C}_6\text{H}_4\text{-CO-CH}_2\text{-CHO} + \text{RNH}_2\ (\text{RR'NH}) \longrightarrow \text{o-HO-C}_6\text{H}_4\text{-CO-CH=CH-NHR}\ (\text{-NRR'}) \longleftarrow \text{RNH}_2\ (\text{RR'NH}) + \text{Chromon}$$

Die Reaktionsgeschwindigkeit der Enamin-Bildung ist sehr gross. Die Verbindungen sind zitronengelb oder orangegelb und zeigen eine sehr starke Fluoreszenz im UV-Licht. Die Reaktion hat einen allgemeinen Verlauf und wurde zur präparativen Identifizierung der Amine angewandt[14]. In einer früheren Mitteilung haben wir das ω-Formyl-*o*-hydroxyacetophenon als ein neues Aminreagenz auch in der Papier- und Dünnschichtchromatographie beschrieben[15].

In der vorliegenden Arbeit wurde die Anwendung der β-Aminovinyl-*o*-hydroxyphenylketone (Enamine) in der DC zur Identifizierung der aliphatischen und aromatischen Amine und Verbindungen, die ausser der Amingruppe noch andere funktionelle Gruppen enthalten, untersucht. Neben der Ermittlung der optimalen Trennbedingungen (Fliessmittelzusammensetzung, Aktivierung, Kammersättigung) wurde die Nachweisgrenze auch Gegenstand der vorliegenden Untersuchungen.

EXPERIMENTELLER TEIL

Geräte und Materialien

Glasplatten in der Grösse 20 × 20 cm wurden mit einem Streichgerät der Fa. Camag in einer Dicke von 0.3 mm auf die übliche Art und Weise beschichtet. Als Sorptionsmittel wurde Kieselgel D-5 der Fa. Camag verwendet. Die gestrichenen Platten wurden einen Tag lang an der Luft getrocknet. Die aktivierten Kieselgel-Platten wurden durch Erwärmen während 1 h bei 110° hergestellt.

Alle Lösungsmittel wurden destilliert und die Enamin-Derivate kristallisiert. Xylol (p.a.) wurde als eine Mischung von *o*-, *m*- und *p*-Isomeren benutzt (Sdp. 138.0–142.0°).

Es wurden Enamin-Lösungen in bestimmter Konzentration vorbereitet: 0.05 g Enamin wurde je nach Löslichkeit in 5 ml Äthanol oder 5 ml Aceton gelöst. Von den 1%-igen Stammlösungen wurden aufeinanderfolgend 0.1%-ige (0.5 ml 1%-ige Lösung + 4.5 ml Äthanol) und 0.01%-ige (0.5 ml 0.1%-ige Lösung + 4.5 ml Äthanol) äthanolische Enamin-Lösungen angefertigt. Die Enamin-Derivate der β-Naphthylamin und *p*-Aminobenzofenon wurden in Chloroform, dagegen die Enamine der Piperazin und *p*-Aminobenzoësäure in Dimethylformamid (DMF) gelöst. Die Löslichkeit der Enamine wurde in der Tabelle I vermerkt.

Die Enamin-Lösungen wurden mit einer Mikropipette TL-705 der Fa. Camag auf die Startpunkte der Platten aufgetragen. Die durchschnittliche Grösse der Flecken auf den Kieselgel-Schichten der aufgetragenen Enamine betrug 3–4 mm.

Die Entwicklungskammern waren mit Filtrierpapier ausgekleidet.

Die Lokalisierung der Enamine auf den entwickelten Platten erfolgte durch Betrachten der Platten unter einer Analysen-Stablampe Pl 335–360 μm (Original Hanau) oder einer Quarzlampe Q-400 (ŁZWAE).

Bestimmung der Nachweisgrenze von Enaminen auf den DC-Platten ohne Entwicklung

Die Enamine wurden in Mengen von 1 μg (1 μl 0.1%-iger Lösung), 0.5 μg (5 μl

0.01%-iger Lösung), 0.2 μg (2 μl 0.01%-iger Lösung), 0.1 μg (1 μl 0.01%-iger Lösung) und 0.05 μg (0.5 μl 0.01%-iger Lösung) auf die Kieselgel-Schichten aufgetragen. Die Platten wurden an der Luft kurz getrocknet und gleich im UV-Licht betrachtet.

Entwicklung der Chromatogramme

Die Enamin-Lösungen in bestimmter Konzentration wurden mit einer Mikropipette quantitativ auf die Startpunkte der Platten, 20 mm von der Kante entfernt aufgetragen. Auf einer Platte wurden 12 Enamin-Derivate einzeln und ihre Mischung, von einem Startpunkt, chromatographiert. Von den mehreren untersuchten Fliessmitteln erwiesen sich die folgenden als besonders geeignet: (1) Äthylacetat–Benzol (1:5), (2) Chloroform–Xylol (8:2) und (3) Aceton–Xylol (1:9).

Für eine Trennung (Laufstrecke 17 cm) wurde in der Regel 1 h benötigt. Nach dem Entwickeln wurden die Platten an der Luft kurz getrocknet und gleich im UV-Licht betrachtet.

Die Messungen der R_F-Werte und die Trennung der Mischungen wurden für alle Enamine in der gleichen Temperatur zweimal auf den unaktivierten und zweimal auf den aktivierten Platten (1 h, Temp. 110°) in drei Fliessmitteln durchgeführt.

ERGEBNISSE

Für 54 Enamine (β-Aminovinyl-*o*-hydroxyphenylketone) wurden die kleinsten Substanzmengen festgelegt, die auf Chromatogrammen, ohne dass diese entwickelt wurden, eine deutliche Fluoreszenz des Fleckes im UV-Licht geben. Auf dieser Grundlage wurde die Brauchbarkeit des angewandten Verfahrens zur Identifizierung sowohl der primären und sekundären Amine als auch der noch andere funktionelle Gruppen enthaltenden Verbindungen nachgewiesen.

Nach dem Auftragen von 0.1 μg Enamin auf die Platten wurde für 46 Amine eine gute Sichtbarkeit festgestellt. Morpholin-, Piperazin-, Benzyl- und *n*-Amylamin-Enamine geben bei Verwendung von 0.2 μg fluoreszierende Flecken, die *p*-Anisidin- und N-Methylanilin-Derivate wurden dagegen erst in einer Menge von 0.5 μg sichtbar. β-Naphthylamin- und *p*-Nitroanilin-Enamine geben nach dem Auftragen von 0.6 μg deutlich sichtbare Flecken. Die kleinsten Mengen der auf die Platten aufgetragenen Enamine, welche ohne Entwicklung im UV-Licht noch gut sichtbar sind, wurden in der Tabelle I angegeben (Nachweisgrenze).

Im Verlauf weiterer Untersuchungen wurden die Empfindlichkeit der Methode und die R_F-Werte für die untersuchten Enamine mit den folgenden drei entwickelnden Lösungsmittelgemischen festgestellt: (1) Äthylacetat–Benzol (1:5), (2) Chloroform–Xylol (8:2) und (3) Aceton–Xylol (1:9). Die beste Differenzierung der R_F-Werte wurde bei den Laufmitteln 1 und 3 erzielt.

Die Reproduzierbarkeit der R_F-Werte ist gut. Die Abweichungen in den Versuchen waren nicht grösser als 0.02 der R_F-Werte. Es wurde nachgewiesen, dass die Aktivierung der Platten und die aufgetragenen Mengen der Enamine auf die R_F-Werte keinen Einfluss ausüben. Bei der Entwicklung der Chromatogramme ist jedoch sorgfältig auf die stabile Temperatur zu achten. Die in der Tabelle I angegebenen R_F-Werte in den drei Laufmitteln stellen einen Durchschnittswert aus vier Einzeltrennungen dar.

TABELLE I

DC DER AMIN-DERIVATE (β-AMINOVINYL-o-HYDROXYPHENYLKETONE) AUF KIESELGEL D-5-SCHICHTEN

Fliessmittel: (1) Äthylacetat–Benzol (1:5), (2) Chloroform–Xylol (8:2), (3) Aceton–Xylol (1:9).

Nr.	*Amin*	*R_F-Werte im Fliessmittel*			*Nachweisgrenze vor dem Entwickeln (µg)*	*Nachweisgrenze nach dem Entwickeln (µg)*			*Löslichkeit*[a]
		1	*2*	*3*		*1*	*2*	*3*	
1	Äthylamin	0.35	0.20	0.38	0.1	0.5	0.5	1.0	A
2	*n*-Propylamin	0.44	0.23	0.47	0.1	1.0	0.5	1.0	
3	Isopropylamin	0.43	0.29	0.47	0.1	1.0	1.0	1.0	
4	*n*-Butylamin	0.44	0.25	0.49	0.1	1.0	0.5	0.2	
5	Isobutylamin	0.50	0.31	0.52	0.1	0.5	0.5	1.0	
6	*n*-Amylamin	0.53	0.28	0.55	0.2	4.0	4.0	3.0	
7	*n*-Hexylamin	0.54	0.30	0.56	0.1	1.0	0.5	1.0	
8	Cyclohexylamin	0.58	0.36	0.55	0.1	1.0	0.5	1.0	A
9	Benzylamin	0.54	0.29	0.50	0.2	1.0	0.5	1.0	A
10	Äthanolamin	0.04	0.00	0.02	0.1	0.5	0.5	0.2	
11	1-Aminopropanol-2	0.06	0.00	0.07	0.1	1.0	0.5	0.2	
12	Allylamin	0.43	0.26	0.44	0.1	0.5	0.5	0.5	
13	Dimethylamin	0.14	0.07	0.18	0.1	0.5	0.2	0.2	
14	Diäthylamin	0.23	0.11	0.29	0.1	0.2	0.2	0.5	
15	Di-*n*-propylamin	0.36	0.12	0.40	0.1	0.2	0.2	0.5	
16	Diisopropylamin	0.29	0.09	0.35	0.1	0.2	0.1	0.1	
17	Di-*n*-butylamin	0.50	0.16	0.49	0.1	0.1	0.1	0.1	
18	Diisobutylamin	0.49	0.16	0.47	0.1	0.2	0.2	0.2	
19	Diäthanolamin	0.00	0.00	0.00	0.1	0.1	0.1	0.1	
20	N-Methylbenzylamin	0.41	0.17	0.37	0.1	0.2	0.2	0.2	
21	Pyrrolidin	0.21	0.07	0.24	0.1	0.2	0.2	0.2	A
22	Piperidin	0.30	0.10	0.32	0.1	0.5	0.2	0.5	A
23	Piperazin	0.16	0.15	0.15	0.2	1.0	0.5	1.0	DMF
24	Morpholin	0.10	0.03	0.14	0.2	0.5	0.5	0.5	
25	Ephedrin	0.10	0.00	0.11	0.1	0.2	0.2	0.1	
26	Anilin	0.64	0.54	0.63	0.1	1.0	1.0	1.0	A
27	*o*-Toluidin	0.64	0.73	0.69	0.1	1.0	1.0	1.0	A
28	*m*-Toluidin	0.63	0.75	0.68	0.1	1.0	1.0	1.0	A
29	*p*-Toluidin	0.67	0.55	0.64	0.1	1.0	1.0	1.0	A
30	*o*-Chloranilin	0.60	0.66	0.58	0.1	0.5	0.5	0.2	A
31	*m*-Chloranilin	0.63	0.63	0.65	0.1	1.0	1.0	1.0	A
32	*p*-Chloranilin	0.66	0.64	0.65	0.1	0.5	1.0	0.2	A
33	*o*-Bromanilin	0.58	0.61	0.60	0.1	1.0	1.0	1.0	A
34	*m*-Nitroanilin	0.57	0.41	0.54	0.1	1.0	1.0	1.0	A
35	*p*-Nitroanilin	0.56	0.33	0.53	0.6	4.0	4.0	4.0	
36	*m*-Aminophenol	0.30	0.06	0.22	0.1	0.5	0.5	0.5	
37	*m*-Aminoacetophenon	0.43	0.25	0.43	0.1	1.0	1.0	1.0	A
38	*p*-Aminobenzophenon	0.58	0.25	0.51	0.1	1.0	1.0	1.0	Ch
39	*p*-Anisidin	0.56	0.47	0.54	0.5	1.0	1.0	1.0	A
40	Phenetidin	0.65	0.71	0.66	0.1	1.0	1.0	1.0	A
41	2,5-Dimethoxyanilin	0.57	0.40	0.54	0.1	1.0	1.0	1.0	A
42	Anästhesin	0.57	0.37	0.53	0.1	1.0	1.0	1.0	A
43	2-Aminopyridin	0.49	0.30	0.55	0.1	1.0	1.0	1.0	A
44	3-Aminopyridin	0.55	0.32	0.54	0.1	1.0	1.0	1.0	A
45	2-Amino-3-methylpyridin	0.53	0.37	0.59	0.1	0.5	0.5	0.2	
46	2-Amino-4-methylpyridin	0.50	0.27	0.56	0.1	1.0	1.0	0.2	A
47	2,6-Diaminopyridin	0.30	0.12	0.27	0.1	1.0	1.0	0.2	A
48	*m*-Phenylendiamin	0.58	0.36	0.53	0.1	0.5	0.5	0.2	A
49	α-Naphthylamin	0.66	0.64	0.64	0.1	1.0	1.0	1.0	
50	β-Naphthylamin	0.65	0.62	0.64	0.6	2.0	2.0	2.0	Ch
51	*p*-Aminobenzoesäure	0.17	0.11	0.17	0.1	1.0	1.0	0.5	DMF
52	*p*-Aminophenylessigsäure	0.05	0.00	0.03	0.1	0.2	0.2	0.2	
53	N-Methylanilin	0.51	0.25	0.48	0.5	1.0	0.5	0.5	
54	Äthylanilin	0.52	0.21	0.43	0.1	1.0	1.0	1.0	

[a] 1%-ige Lösungen wurden in Aceton (A), Chloroform (Ch) oder Dimethylformamid (DMF) vorbereitet.

In Vorversuchen wurde festgestellt, dass die auf den Platten vor der Entwicklung im UV-Licht gut sichtbaren Enaminmengen auf den entwickelten Chromatogrammen unzureichend sichtbar waren. Aus diesem Grunde wurden die geprüften Enamine auf die Chromatogramme in grösseren Mengen aufgetragen. Es wurde festgestellt, dass 0.1 bis 1 μg Enamin auf entwickelten Chromatogrammen im UV-Licht genügend deutlich fluoreszierende Flecken bilden. Für manche Amine erwies sich die Anwendung von 1 μg Derivat noch unzureichend.

Im Verlauf der Untersuchungen beobachtete man auf den Chromatogrammen verschiedene, von der Menge der aufgetragenen Substanz und der Art der entwickelnden Lösungsmittel unabhängige Fluoreszenz der Enamine. Enamine der primären und sekundären aliphatischen und heterocyclischen Amine sind im UV-Licht als blassgrüne, die der primären und sekundären aromatischen Amine als orangefarbige Flecken sichtbar. Der deutliche Unterschied der Fleckenfärbung im UV-Licht der Derivate aliphatischer und aromatischer Amine ermöglicht eine sofortige Differenzierung dieser beiden Aminklassen.

SCHLUSSFOLGERUNGEN UND DISKUSSION

Der Dünnschichtchromatographievorgang der Enamine (β-Aminovinyl-*o*-hydroxyphenylketone), die in der Reaktion von Aminen mit ω-Formyl-*o*-hydroxyacetophenon oder Benzo-γ-pyron entstehen, wurde untersucht.

Die DC der Enamine wurde nach der üblichen Methodik durchgeführt. Auf den mit Kieselgel bedeckten Platten wurden die R_F-Werte der 54 Enamine in drei Lösungsmittelgemischen festgelegt. Die Flecken der aliphatischen und heterocyclischen Enamin-Derivate weisen im UV-Licht blassgrüne Färbung auf, während die Enamine der aromatischen Amine orangefarbig fluoreszieren. Die verschiedene Färbung der Flecken kann, neben den R_F-Werten, als zusätzlicher wertvoller Faktor, der die Identifizierung der Amine in Gestalt ihrer Derivate erleichtert, ausgenutzt werden.

Die Nachweisgrenze vor der Entwicklung der Chromatogramme liegt zwischen 0.1–0.6 μg je Enamin Derivat pro Flecken, nach der Entwicklung der Chromatogramme ist sie kleiner und beträgt 0.1–1 μg Enamin ($2 \cdot 10^{-4}$ μMol–$1 \cdot 10^{-3}$ μMol Amin/Fleck).

Die Empfindlichkeit der von uns angewandten Methode der chromatographischer Identifizierung der Amine als β-Aminovinyl-*o*-hydroxyphenylketone übertrifft andere bisher übliche Methoden zur Identifizierung von Aminen in Gestalt der Derivate[2,9]. Von den wenigen Veröffentlichungen, deren Verfasser die kleinsten Mengen von Amin-Derivaten angeben, sind es lediglich SEILER UND WIECHMANN[1], die die biogene Amine in Form ihrer 1-Dimethylaminonaphthalen-5-sulfonyl- (DANS-) Derivate in einer Menge von 10^{-4} μMol und in manchen Fällen sogar $5 \cdot 10^{-6}$ μMol im Fleck identifizieren. Dieses Verfahren ist aber schwer durchführbar und die verwendete DANS-Cl-Verbindung ist ein unspezifisches Reagenz—es bildet Derivate nicht nur mit Aminen sondern auch mit Alkoholen und anderen Verbindungen.

SCHWARZ *et al.*[5], die die niedrigeren aliphatischen Amine identifizieren, tragen die 2,4-Dinitroaminophenyl-Derivate in einer Menge von 10^{-3} μMol auf Chromatogramme auf. Die Empfindlichkeit der Auftrennung von ausschliesslich aromatischen Aminen in Gestalt von *p*-Toluolsulfonamiden[2] ist zweimal kleiner.

Die in dieser Arbeit beschriebene Anwendung der DC von Amin-Derivaten (β-Aminovinyl-*o*-hydroxyphenylketone), zur Identifizierung der primären und sekundären aliphatischen und aromatischen Amine und deren Derivate, der heterocyclischen und isocyclischen Amine und der Aminoverbindungen, die in ihrer Struktur noch andere funktionelle Gruppen enthalten, wurde am umfangreichen Versuchsmaterial durchgeführt. Das Verfahren ist spezifisch und hat für alle β-Aminovinyl-*o*-hydroxyphenylketone einen generellen Charakter. Es stellt eine wertvolle Vervollständigung der bereits beschriebenen allgemeinen Methode der präparativen Identifizierung von Aminen in Form der Enamine, die mit ω-Formyl-*o*-hydroxyacetophenon oder mit Chromon entstehen[14], und der DC und PC Analyse, in der das ω-Formyl-*o*-hydroxyacetophenon als Anfärbereagenz verwendet ist[15].

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ZUSAMMENFASSUNG

Es wird die dünnschichtchromatographische Auftrennung und Identifizierung der Amine und der Aminoverbindungen, die in ihrer Struktur noch andere funktionelle Gruppen enthalten, in Form der Enamin-Derivate (β-Aminovinyl-*o*-hydroxyphenylketone) beschrieben. Die besten Trennergebnisse erzielt man auf Kieselgelschichten mit folgenden Fliessmitteln: (1) Äthylacetat–Benzol (1:5), (2) Chloroform–Xylol (8:2) und (3) Aceton–Xylol (1:9). Der verschiedene Farbton der Flecken, im UV-Licht, der aliphatischen (blassgrün) und aromatischen (orange) Enamine ermöglicht, neben dem R_F-Wert, eine schnelle Identifikation der untersuchten Verbindungen. Die Nachweisgrenze liegt bei den meisten Enaminen zwischen 0.1–1 μg pro Flecken. Die neue analytische Methode ist allgemein, hat einen spezifischen Charakter und bildet eine wertvolle Vervolständigung der schon früher von uns beschriebenen präparative Identifikation der Amine mit ω-Formyl-*o*-hydroxyacetophenon oder Chromon und der dünnschicht- und papierchromatographische Analyse der Amine, in der das ω-Formyl-*o*-hydroxyacetophenon als Anfärbereagenz verwendet wird.

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THIN-LAYER CHROMATOGRAPHIC SEPARATION OF GLYCOLIPIDS IN ANIMAL LIPID MIXTURES

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SUMMARY

A method of thin-layer chromatography was used for the separation of glycolipids from lipid extracts of animal tissues. The technique of two-step development was applied. In the first step glycolipids are separated from phospholipids; the second step makes possible the separation of different glycolipid classes, *viz.* ceramide mono-, tri- and tetrahexosides, sulphatides and monogalactosyl diglycerides. Gluco- and galactolipids were separated on plates partially impregnated with sodium borate. The separation of psychosine, sphingosine and ceramide is also described.

INTRODUCTION

A wide variety of glycolipids is present in lipid extracts from animal tissues. In addition to ceramide monohexosides (cerebrosides) and sulphate esters of cerebrosides (sulphatides), which are abundant in the peripheral and the central nervous system, more complex glycolipids like ceramide di-, tri- and tetrahexosides have been found in many tissues[1]. Complex glycolipids containing sialic acid (gangliosides) are also extracted with chloroform–methanol mixtures. In the partition procedure of FOLCH *et al.*[2] gangliosides are retained in the upper aqueous phase. As thin-layer chromatography (TLC) of gangliosides is not considered in this study, the term "glycolipids" will be confined to the less polar glycolipids recovered in the lower chloroform phase, which contains a mixture of glycolipids and phospholipids of comparable polarities and similar chromatographic mobilities. Therefore, in many tissues, the unfavourable ratio of glycolipids to some common phospholipids makes difficult the direct TLC analysis of glycolipids. The separation of some glycolipids (cerebrosides) may be obtained with one- and two-dimensional systems commonly used in TLC of polar lipids[3–7]. However, in most cases, before a successful TLC analysis can be done, a separation of glycolipids from other lipids by mild alkaline hydrolysis, silicic acid and Florisil column chromatography or by a combination of these procedures is required. These methods have been recently discussed in detail by RENKONEN AND VARO[8].

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A different approach was reported by SKIPSKI *et al.*[9]. In the TLC technique described by these authors the presence of phospholipids and neutral lipids does not interfere with the separation of glycolipids. This advantage was based mainly on the incorporation of pyridine and acetone into the developing solvent mixtures.

In the present study a solvent mixture containing pyridine and acetone was also used as the first solvent in a two-step TLC. This solvent permits the efficient separation of glycolipids from phospholipids. The second development with a solvent mixture of appropriate composition separates different glycolipid classes, *viz.* ceramide, mono-, di-, tri- and tetrahexosides, sulphatides and monogalactosyl diglycerides. The use of chromatoplates partially impregnated with sodium borate permitted the separation of ceramide monoglucosides (glucocerebrosides) from ceramide monogalactosides (galactocerebrosides). The TLC separation of lipids encountered in studies of glycolipid metabolism, *i.e.* sphingosine, psychosine and ceramide, is also described.

MATERIAL AND METHODS

Standard lipids

Ceramide monogalactosides (galactocerebrosides, a mixture of kerasin and phrenosin) and sulphatides were isolated from bovine brain sphingolipids by silicic acid column chromatography[10]. Ceramide monoglucosides (glucocerebrosides) and ceramide dihexosides (lactosides) were prepared from rat spleen by the following procedure. After mild alkaline hydrolysis[11] and acetone precipitation the crude sphingolipids were loaded on the Florisil column (10 mg of lipid/g of adsorbant). Cholesterol and other less polar lipids were washed through with chloroform (ten column volumes) and the glucocerebrosides were subsequently eluted with chloroform–methanol (70:30) (five column volumes). Ceramide dihexosides were recovered in the next fraction eluted with the same solvent (five column volumes). Further purification of ceramide dihexosides was carried out by silicic acid column chromatography[12]. Monogalactosyl diglyceride was isolated from spinach leaf lipids by chromatography on a silicic acid column[13] and purified by TLC on Silica Gel G with chloroform–acetone–methanol–20% aq. ammonia–water (60:40:20:2:2) as developing solvent. Ceramides were obtained after hydrolysis of beef brain cerebrosides[14] and separated into the normal and hydroxy fatty acid containing fractions by TLC on Silica Gel G with chloroform–acetone–ethanol–20% aq. ammonia–water (70:40:6:1:1). Ceramide tri- and tetrahexosides (crude fractions) were prepared by silicic acid column chromatography[12] using stroma from human erythrocytes as starting material. Sphingosine and psychosine were prepared as described previously[15]. Gangliosides were from Koch-Light Laboratories Ltd. (Colnbrook, Bucks., Great Britain) and cardiolipin from Sylvana Chemical Co. (Millburn, U.S.A.). Lipid extracts from rat brain and human serum were prepared by the method of FOLCH *et al.*[2].

Preparation of plates and spotting of samples

Glass plates 20 × 24 cm were coated with a slurry prepared by mixing 54 g of Silica Gel H (Merck, Darmstadt, G.F.R.) and 6 g of finely ground Florisil (Florisil 60/100 mesh, purchased from Serlabo, Paris, France) with 135 ml of water. No attempts have been made to determine the mesh size of this material, but reproducible results were obtained when Florisil was milled in a small electric blender for 5 min.

In our hands this material was more satisfactory than finer-mesh magnesium silicate preparations commercially available. The plates were coated with a fixed-distance (0.5 mm) Desaga spreader and dried in air. For the separation of gluco- and galacto-cerebrosides (system D, see below) the chromatoplates prepared as described were partially impregnated with sodium borate. The lower part of the layer (5 cm from the bottom) was protected with a metal sheath; then the plates were sprayed with a 1.5% solution of sodium borate and dried in air. The chromatoplates were activated immediately before use by heating at 110° for 30 min. The samples were applied 1.5 cm from the bottom edge as series of spots to give a 1.5-cm-long narrow band.

Development of chromatograms

One-dimensional TLC was performed in chambers (21 × 9 × 23 cm) lined with Whatman No. 3 paper. The solvent mixtures were added 30 min before development and the paper lining was soaked with the solvent. A two-step ascending technique of development has been applied. In all TLC systems described the first solvent was chloroform–acetone–pyridine–20% aq. ammonia–water (20:30:60:2:2). The drying of chromatoplates between the first and the second solvent was carried out in a stream of air for 20 min.

System A. In system A the plate was developed with the first solvent for 10 cm from the bottom. The second solvent, chloroform–acetone–methanol–acetic acid–water (65:35:11:4:1.5) was allowed to run to the top of the plate.

System B. In system B the front of the first solvent was 9 cm above the bottom. After drying the lower part of the adsorbent, a 2.5-cm band starting at the bottom of the plate was scraped off and development proceeded with chloroform–methanol–water (65:25:4) to the top of the plate.

System C. In system C the plate was developed with the first solvent for 8 cm and dried. The adsorbent was scraped from the plate up to a level of 2.5 cm from the bottom and the chromatogram developed with chloroform–acetone–methanol–water (65:30:12:2) to the top of the plate.

System D. In system D the first solvent was allowed to ascend to a level of 8 cm from the bottom of the plate. The chromatogram was dried and the lower part of the layer was removed (2.5 cm from the bottom). The second development was carried out with chloroform–acetone–methanol–acetic acid–water (68:26:12:5:3) up to the top of the plate.

Detection of lipid fractions on thin-layer plates

Orcinol–sulphuric acid spray[16] was used for detection of lipids containing a carbohydrate moiety. For the non-specific detection of lipids by charring, the phosphoric acid–copper acetate reagent[17] was used.

RESULTS AND DISCUSSION

The first solvent, chloroform–acetone–pyridine–20% aq. ammonia–water, which is common for all systems described in this paper, permits a complete separation of glycolipids from phospholipids. Glycolipids, together with cholesterol and other less polar lipids, move near the solvent front; phospholipids stay at the origin

(cardiolipin moves very little) as well as gangliosides, and fatty acids migrate to the area very close to the origin. Of considerable importance for studies involving the use of radioactive precursors is that several non-lipidic compounds (glucose, galactose, glucose- and galactose-1-phosphate, UDP-glucose, UDP-galactose) remain at the origin or move well behind glycolipids.

In system A cerebrosides (kerasin and phrenosin), sulphatides and monogalactosyl diglyceride are clearly separated from each other and from the rest of lipids present in the extracts of animal tissues (Fig. 1). As much as 2.5 mg of total lipids may be chromatographed in this system; when greater amounts were applied, a streaking with the first solvent and bad resolution was observed. This system was successfully applied in our laboratory for rapid quantitative analysis of cerebrosides and sulphatides in brain.

System B was developed primarily for the analysis of neutral ceramide glycosides. Ceramide mono-, di-, tri-, and tetrahexosides were clearly separated as shown in Fig. 2. A rapid qualitative analysis of glycolipids can be carried out on as little as

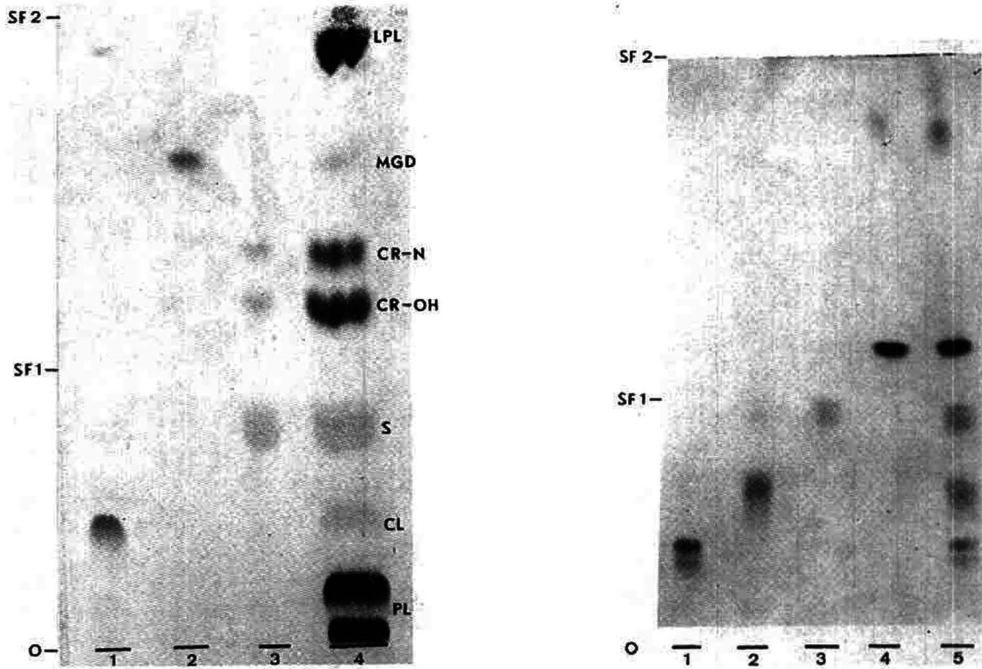
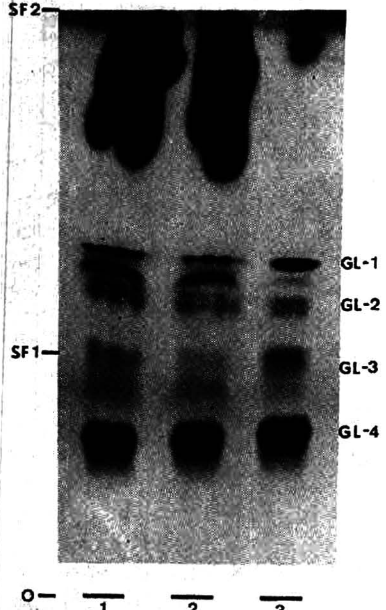


Fig. 1. TLC of lipids extracted from rat brain and of reference lipids in system A. (1) Ceramide with hydroxy fatty acids (upper spot, 40 μg) and cardiolipin (lower spot, 40 μg); (2) monogalactosyl diglyceride (40 μg); (3) galactocerebrosides (upper two spots, 40 μg) and sulphatides (lower spot, 20 μg); (4) lipid extract from rat brain (2 mg of total lipids). Abbreviations: LPL = less polar lipids; MGD = monogalactosyl diglyceride; CR-N = cerebrosides with normal fatty acids; CR-OH = cerebrosides with hydroxy fatty acids; S = sulphatides; CL = cardiolipin; PL = phospholipids; O = origin; SF_1 = first solvent front; SF_2 = second solvent front. Detection: phosphoric acid–copper acetate spray.

Fig. 2. Separation of neutral ceramide glycosides in system B. (1) ceramide tetrahexosides (30 μg); (2) ceramide trihexosides (40 μg); (3) ceramide dihexosides (30 μg); (4) ceramide monohexosides (glucocerebrosides, 40 μg); (5) mixture of 1–4. Abbreviations: O = origin; SF_1 = first solvent front; SF_2 = second solvent front. Detection: phosphoric acid–copper acetate spray.



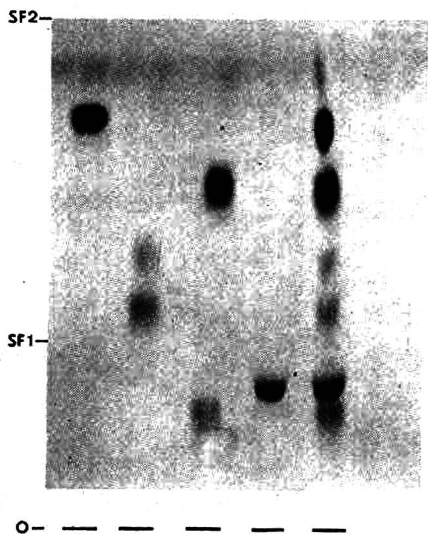


Fig. 3. Separation of human serum glycolipids in system B. (1) and (2): total serum lipids (11 mg, equivalent to 3 ml of serum); (3) mixture of reference compounds (40 μg each). Abbreviations: GL-1 = glucocerebroside; GL-2 = ceramide dihexoside; GL-3 = ceramide trihexoside; GL-4 = ceramide tetrahexoside; O = origin; SF_1 = first solvent front; SF_2 = second solvent front. Detection: phosphoric acid–copper acetate spray.

Fig. 4. TLC of reference lipids in system C. (1) ceramide with hydroxy fatty acids (40 μg); (2) galactocerebrosides (50 μg); (3) sphingosine (upper spot, 40 μg) and psychosine (lower spot, 40 μg); (4) sulphatides (40 μg); (5) mixture of 1–4. Abbreviations: O = origin; SF_1 = first solvent front; SF_2 = second solvent front. Detection: phosphoric acid–copper acetate spray.

3 ml of plasma (Fig. 3). As the glycolipid content of plasma is very small, a large amount of total lipids had to be applied in this case. The application of 10–15 mg of total lipids could be achieved by using thick layers (1 mm) and spotting the sample as a rather large band (1.5 × 1 cm).

System C separates cerebrosides and sulphatides as well as several lipids which may be involved in glycolipid metabolism, *i.e.* ceramides, sphingosine and psychosine (Fig. 4). This system was particularly useful for the rapid separation of labelled lipids during the studies of glycolipid biosynthesis[15]. A more simple TLC method giving a good separation of ceramides, cerebrosides, sphingosine, fatty acids and psychosine was recently reported[18]; however, it is difficult to apply this system in the presence of phospholipids and sulphatides.

In system D the use of plates partially impregnated with sodium borate permitted the separation of gluco-, and galactocerebrosides. TLC of gluco-, and galactocerebrosides on borate-impregnated plates was described previously[19,20], but these techniques are not convenient for analysis of more complex lipid mixtures. With system D a complete resolution of ceramides, monogalactosyl diglyceride, glucocerebrosides, galactocerebrosides, sulphatides and psychosine was obtained (Fig. 5). When a second solvent of somewhat different composition was introduced (chloro-

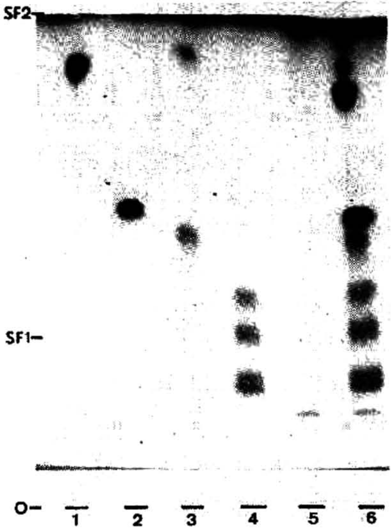


Fig. 5. TLC of reference lipids in system D. (1) ceramide with hydroxy fatty acids (40 μg); (2) monogalactosyl diglyceride (40 μg); (3) ceramide with normal fatty acids (upper spot, 20 μg) and glucocerebrosides (lower spot, 20 μg); (4) galactocerebrosides (upper two spots, 60 μg) and sulphatides (lower spot, 40 μg); (5) psychosine (20 μg); (6) mixture of 1–5. Abbreviations: O = origin; SF_1 = first solvent front; SF_2 = second solvent front. Detection: phosphoric acid–copper acetate spray.

form–acetone–methanol–acetic acid–water, 65:35:11:3:1.5), glucopsychosine was separated from galactopsychosine. Therefore, in the studies where separation of gluco- and galactolipids is important[21], system D may be substituted for system C.

In comparison with the similar TLC procedure for analysis of glycolipids[9] some noteworthy improvements are introduced in the present method: (1) separation of gluco- and galactocerebrosides may be performed simultaneously with other lipids; (2) no additional development is necessary to remove fatty acids which may interfere with the separation of some glycolipids; (3) the systems described provide a convenient separation of labelled glycolipids after the incorporation of radioactive substrates since the risk of contaminating lipid fractions with non-lipidic radioactive material is greatly reduced.

A shortcoming of the method described in this study is that the systems C and D, which separate cerebrosides, sulphatides and related sphingolipids (psychosine, sphingosine and ceramide), are less successful in tissues with an appreciable content of ceramide di-, tri-, and tetrahexosides.

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CHROM. 4669

DISPLACEMENT ELECTROPHORESIS

EXPERIMENTS WITH COUNTERFLOW OF ELECTROLYTE

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SUMMARY

Where ions with small differences in mobility need to be separated, the counterflow technique makes it possible to obtain a complete separation, even in short tubes. If an ion is present in a mixture in too great an amount it normally causes "mixed zones". These mixed zones can be resolved by use of counterflow. The voltages required are relatively low.

INTRODUCTION

Displacement electrophoresis has been shown to be of analytical value in many cases[1–10]. Only small amounts of sample are needed and the self-sharpening characteristic of displacement techniques, is of value wherever ions are present with small differences in mobility.

Because the inside diameter of the capillary tubes is very small and the concentration of the leading-electrolyte is small, high voltages are needed for the separation of ions with small mobilities. If the sample consists of ions with only small differences in mobility, long capillaries are used and hence still higher voltages are needed. A counterflow of electrolyte can be used to overcome these problems. Instead of voltages up to 30 kV, voltages of 2 kV can be used, because a capillary tube with a length of 30 cm can be taken and used irrespective of the mixture to be separated.

EXPERIMENTAL

The apparatus consists of a teflon capillary with an O.D. of 0.6 mm and an I.D. of 0.4 mm. This capillary is connected between two electrodes, the anode and cathode compartment, respectively. These electrodes are described in ref. 5.

If we consider a sample of acids injected into the capillary, these acids will move in the electric field and because of the difference in mobility, zones of the separate acids will be formed. These zones will all move at the same speed if the steady-

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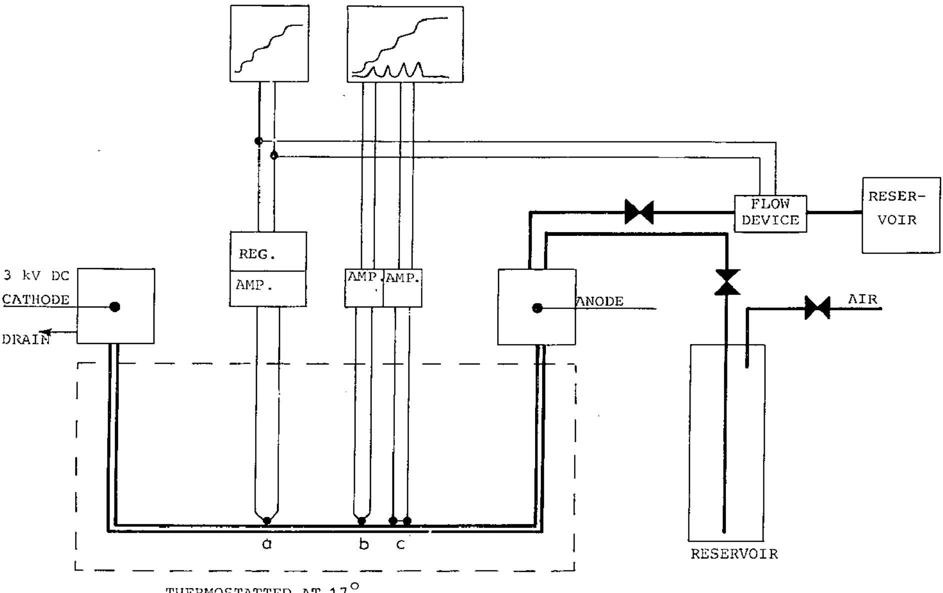

Fig. 1. Schematic diagram of the apparatus used for the analysis with counterflow. The teflon capillary, mounted between two electrode compartments, is thermostated at 17°. The capillary can be filled with electrolyte by a pressure system. The flow device and the reservoir is filled with the leading electrolyte.

state is reached. The ion with the highest mobility is at the front, while that with the least will be at the rear. As a result of this the electric resistivity increases in a stepwise fashion from the front zone to the rear zone. As the current is maintained constant, the rate of heat production correspondingly increases from the front to the rear. This makes it possible to detect the zones by means of three thermocouples, which are mounted around the capillary tube (Fig. 1).

The thermocouples a and b measure the temperature of the capillary tube. The reference junction is thermostated at 17°. Thermocouple c measures the difference in temperature between two points on the capillary tube. If a zone reaches *e.g.* thermocouple a, the signal from it is amplified and registered by a recorder. In addition the signal is, after amplification, led to a regulator which controls the counterflow device in such a way that the zone stands still at this thermocouple.

However, due to the potential drop which is still present, the separation ultimately reaches a steady state where the diffusion, the selfsharpening of the zones, the electroendosmosis and the disturbance of the zones by the counterflow of electrolyte come into equilibrium. The counterflow is then stopped. The zones start moving again and they are detected by a second set of thermocouples (b and c). Thermocouple b measures the temperature of the capillary, which is determined by the ion-species present in the zone just passing. Thermocouple c measures the temperature-drop along the capillary. The recorded curve allows the length of a zone in the capillary to be determined from the distance between two successive peaks and gives information about the amount of the given ion-species (ref. 1). The counterflow technique was tested with different systems.

A peristaltic pump giving a counterflow of 100 μl/h disturbed the zones too much; instead of a better separation mixed zones were formed where under normal conditions, without a counterflow of electrolyte, a full separation was obtained. The separation was better if the viscosity of the leading electrolyte, also used as the electrolyte for the counterflow, was increased up to 100 c.p.s. by dissolving a suitable polymer in it.

A syringe-pump gives much better results, the disturbance of the zones being less. In this case, addition of a polymer to the leading electrolyte did not give better separations.

The best results, however, were obtained where the counterflow of electrolyte was created by a higher level of electrolyte in the electrode compartment[9,10], towards which the sample is moving (Fig. 2). The rate of counterflow can be controlled by the signal from the leading electrolyte as in ref. 10. A change in this reference due to a hot zone means that this zone cannot pass the "control-thermocouple", because the disturbance of the reference-signal is eliminated by increasing the level in the anode compartment.

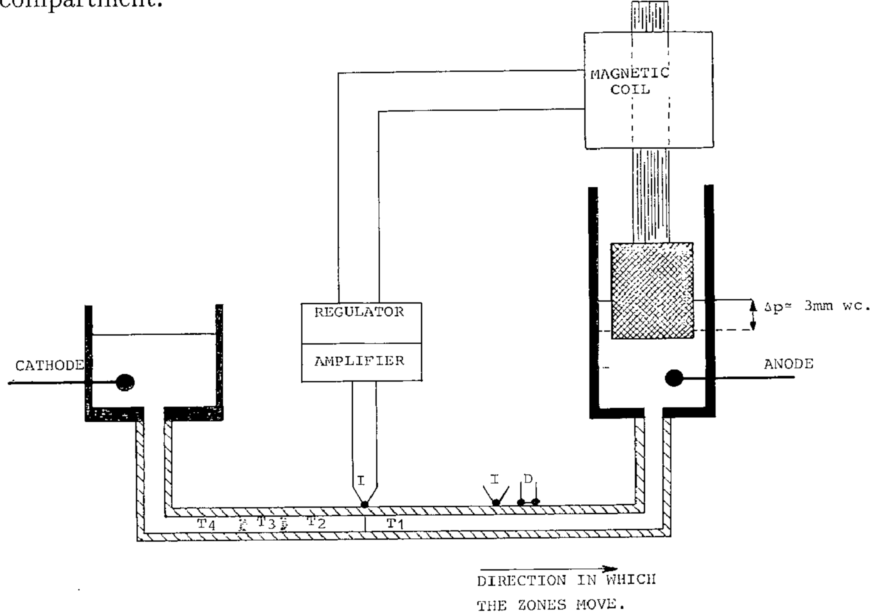

Fig. 2. Schematic diagram of the flow device, which gives the best results for experimental work with counterflow. A temperature change in the capillary tube is detected by the thermocouple mounted around the capillary. This signal is, after amplification and comparison with a reference signal, led to a regulator which increases the current in a magnetic coil, which in turn operates a plunger. This movement creates a higher level in the electrode compartment and gives an adequate counterflow.

RESULTS AND DISCUSSION

The experiments show that a counterflow technique can be used with success. Counterflow of electrolyte in a capillary with the dimensions described and a length of 30 cm gives, if a voltage of 2 kV is applied, the same separation as that in a capillary tube with a length of 100 cm, and the same inside and outside diameter and an applied

voltage of 15 kV, but with no counterflow. The analysis time will, however, be increased by 30%. The detection system, based on temperature measurements on the outside of the capillary, could perhaps be further developed. A minimum value for the difference in mobility, where it is possible to separate the ions in two different zones, cannot be given yet. At the present time, a difference in mobility of 0.5% is needed to separate ions into two successive zones. The step heights, characteristic for the ion-species, measured with and without counterflow were not the same. The distance between two successive peaks was changed if a counterflow technique was used. To elucidate this a series of experiments was performed. Fig. 3 shows the result. The —●— values were measured in a glass capillary with an I.D. of 0.6 mm and an O.D. of 0.8 mm (Fig. 3, A).

The values in section B of Fig. 3 are measured in a teflon capillary, 30 cm long and with the same diameter as before. As leading electrolyte a solution of histidine (0.01 *M*) and histidine HCl (0.01 *M*) in water was used in both cases. From Fig. 3 it is clear that use of a counterflow of electrolyte causes the step heights to be changed. The reason for this is as follows.

In our apparatus the anode compartment has a membrane in it, so that the capillary can be rinsed and filled again with electrolyte without disturbing the electrolyte in the anode compartment[5]. This membrane is made of cellulose acetate. The

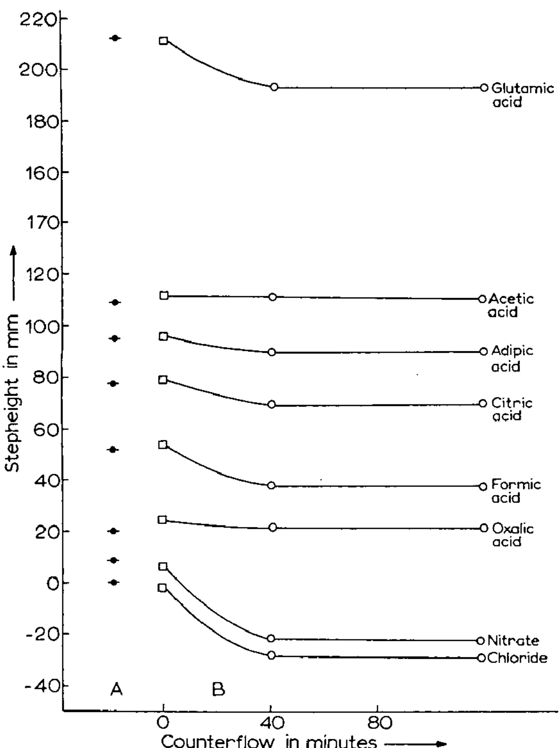

Fig. 3. This shows step heights of different materials. The —●— values were measured in a glass capillary without counterflow. The accuracy was about 2%. The values of section B are measured in a teflon capillary and counterflow was also used. For further explanations see text.

cellulose acetate is more permeable for H^+ than it is for the histH^+ used as counterion. During the analysis a pH change occurs as a result. Because the H^+ ion is very mobile, this pH shift influences the separation during the experiment. The more that is injected, the longer the analysis will be. The step height will be constant when twice the amount is injected. In the case of other acids being analysed, the analysis time is different and so is the step height. The bad reproducibility (2%) found in our early experiments in measuring step heights has the same cause.

In counterflow experiments the counterflow enters the system in the same way as water for rinsing or the electrolyte for filling the capillary. The counterflow will carry with it a pH disturbance and a new steady-state will be formed dependent on the current used. In our experiments this steady state was reached after 40 min. Due to the pH shift the "chloride-line" or base-line drops after a while (Fig. 3). The nitrate ion is also related to a strong acid and therefore the nitrate drops in a similar way to the "chloride-line". The reason for this is that the H^+ ions increase the conductivity of the electrolyte in the capillary in the presence of a strong acid. The current is maintained constant and so the heat generated is less.

The other acids, however, are weak. The velocity of the zones must be constant in spite of a lower pH. Since less of the weak acid is ionised a higher voltage is needed at this lower pH to give the zone the same velocity as before, hence the heat generated

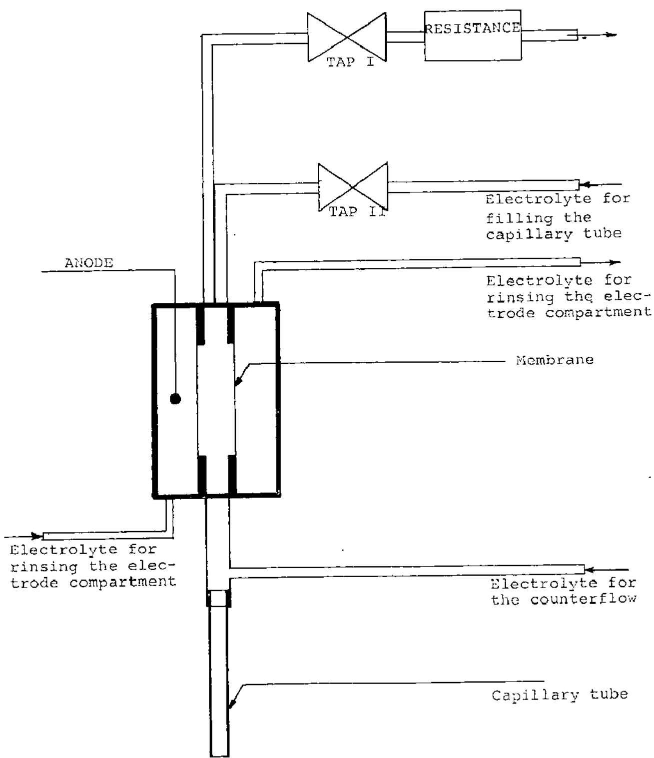

Fig. 4. The anode compartment, and the arrangement for counterflow are illustrated. Thus constructed, a counterflow can be given in the capillary tube as well as a flow in the anode compartment. This prevents disturbances to occur in the capillary tube, due to the use of a membrane.

is greater and the step height is enlarged with respect to the "chloride-line". To overcome these problems an electrode compartment was constructed as shown in Fig. 4. Experiments without counterflow now gave the same results as experiments with counterflow. Using counterflow, and tap I (Fig. 4) open, the right resistance of flow can be chosen so that a part of the electrolyte flows into the capillary tube and a part through tap I. Thus a pH-shift cannot enter into the capillary. Figs. 5 and 6 show a mixture of acids separated without and with counterflow. Both electropherograms were reproducible. For simplicity only two acids were separated. In Fig. 5 the separation was poor, too much was injected and the capillary was too short for a complete separation. Fig. 6 shows an electropherogram without the mixed zone consisting of the ions not yet separated. The analysis time was increased by 40% by using the counterflow technique. The conditions for these analyses were:

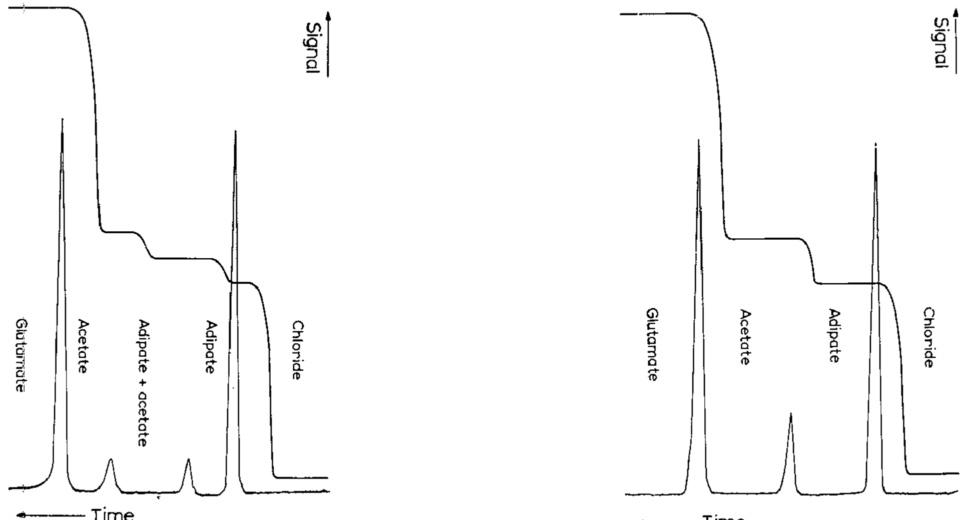

Fig. 5. Electropherogram of adipate and acetate. Injection of too much material results in a zone of unseparated material persisting. The capillary was too short for a complete separation.

Fig. 6. Electropherogram of the same mixture of adipate and acetate as shown in Fig. 5. By using counterflow the length of the capillary is effectively enlarged. The separation is complete.

The current was 60 μA.

The system was thermostated at 17°.

The capillary tube was filled with a solution of histidine (0.01 *M*) and histidine HCl (0.01 *M*) in water.

The displacer was glutamic acid (0.01 *M*).

The capillary had an effective length of 80 cm.

In both cases 4 μl of a solution containing adipic acid (0.02 *M*) and acetic acid (0.04 *M*) were injected.

ACKNOWLEDGEMENT

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CHROM. 4659

SÉPARATION DE LANTHANIDES PAR CHROMATOGRAPHIE SUR PAPIER ÉCHANGEUR D'IONS SA-2 EN MILIEU α-HYDROXYISOBUTYRIQUE

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SUMMARY

Separation of lanthanides by chromatography on ion-exchange paper SA-2 in α-hydroxy-isobutyrate medium

Quick separation of the rare earths La, Ce, Pr, Nd, Pm, Sm, Eu and Y was accomplished by chromatography on strongly acidic ion-exchange paper (SA-2) with ammonium α-hydroxyisobutyrate as the eluent. Calculations on complex formation show that the most selective distribution factor between these metals will be obtained when using 0.24 M α-hydroxyisobutyrate at pH 5.0. These conclusions have been confirmed experimentally. The optimal operation conditions are discussed and illustrative examples of the separation of rare earths from fission products are provided. The technique can be used for even a few tenths of micrograms of each element.

INTRODUCTION

La difficulté de séparation des lanthanides provient de la similitude de leurs propriétés physiques et chimiques. Tant à l'échelon analytique que préparatif, de nombreuses méthodes ont été expérimentées avec plus ou moins de succès. Il est possible de diviser en trois grandes parties les méthodes microanalytiques (quelques dizaines de microgrammes au plus de séparation) par chromatographie sur papier:

(1) Chromatographie de partage avec élution en milieu organique[1–8]. Les résultats montrent que la méthode n'aboutit pas à de bonnes résolutions rapides d'un grand nombre d'éléments.

(2) Chromatographie sur papiers préalablement traités au moyen d'échangeurs d'ions liquides, soit d'échangeur d'anions (la tri-n-octylamine)[9,10], soit d'échangeur de

cations (l'acide di(2-éthylhexyl) orthophosphorique)[11–13]. Les séparations ne sont satisfaisantes que pour des éléments non voisins car les taches sont assez étalées. En utilisant comme phase fixe le DNS (acide dinonylnaphtalène sulfonique) et comme éluant des solutions aqueuses mixtes d'acides complexants comme l'EDTA, l'acide lactique ou l'acide hydroxybutyrique dans $NaClO_4$, WERNER a obtenu de bons résultats[14,15]. Le système se comporte comme un échangeur de cations, mais le temps de développement est long (10 à 12 h).

(3) Chromatographie sur papiers échangeurs d'ions, soit dans des supports imprégnés d'échangeurs cationiques minéraux comme le phosphate de zirconium[16] (les facteurs de séparation sont faibles), soit d'échangeurs synthétiques (essais de LEDERER[17]), soit le plus souvent sur échangeurs d'anions ammonium quaternaire[18,19]. Une investigation systématique sur papiers échangeurs d'anions à base de cellulose chimiquement modifiée[20] par élution en milieu citrique n'a pas abouti à une séparation satisfaisante. On a en effet calculé un facteur de séparation de 2.6 pour Ce–Eu, ce qui est faible pour deux éléments éloignés. Sur papier SB-2, SAKODYNSKY[21] a obtenu de bons résultats en éluant par HNO_3 dans le méthanol, mais on a encore la présence d'un milieu organique qui augmente le temps de résolution. Signalons enfin un travail par chromatographie circulaire accélérée, sur papier SA-2 (réf. 22) pour de très faibles quantités.

On rencontre dans la littérature, un nombre considérable de travaux concernant la séparation à l'échelon analytique (de l'ordre du centigramme) sur colonnes d'échangeurs d'ions, en particulier en milieux citrique, lactique et α-hydroxyisobutyrique. C'est ce dernier complexant qui amène les résultats les plus probants dans de bonnes conditions expérimentales[23,24]. Les facteurs de séparation entre deux éléments voisins sont de l'ordre de 1.6 à 2.0. On retrouve de récents travaux développant le sujet[25–27]. Si en chromatographie sur papier échangeur l'emploi de ce complexant n'est pas cité, en électrophorèse à haute tension, il donne satisfaction[28,29].

Ce sont ces raisons qui nous ont incités à étudier un système papier SA-2 (qui contient des grains de résine à groupements échangeurs sulfoniques identiques à ceux utilisés en colonne), acide α-hydroxyisobutyrique (α-HIB).

Étude du système papier SA-2 /acide α-HIB/ Lanthanides

La complexion par l'acide α-HIB

Symbolisons par M^{3+} un cation lanthanide et AH l'acide. On sait que les complexes formés sont du type MA^{2+}, MA_2^+, MA_3 et MA_4^-, dont l'importance respective est fonction de la concentration en acide C_A et du pH. Les constantes d'instabilité de ces diverses espèces sont connues[30,31]. Le pouvoir complexant augmente avec le numéro atomique; on remarque que le lanthane ne donne que LaA^{2+} et LaA_2^+, le cérium ne conduit pas à CeA_4^-. L'affinité des échangeurs d'ions dépendant de la charge[32], il était intéressant de calculer à priori un système sélectif papier–complexant–lanthanide.

Calcul du système sélectif; rapport des complexions en solution

Définissons par R_3, R_2, R_1, R_0, R_{-1}, les quantités $[M^{3+}]/\Sigma M$, $[MA^{2+}]/\Sigma M$, $[MA_2^+]/\Sigma M$, $[MA_3]/\Sigma M$ et $[MA_4^-]/\Sigma M$. On a évidemment: $\Sigma M = [M^{3+}] + [MA^{2+}] + [MA_2^+] + [MA_3] + [MA_4^-]$.

Les équilibres de complexion s'écrivent:

$$M^{3+} + A^- \rightleftarrows MA^{2+} (K_1);\ MA^{2+} + A^- \rightleftarrows MA_2^+ (K_2)$$

$$MA_2^+ + A^- \rightleftarrows MA_3 (K_3);\ MA_3 + A^- \rightleftarrows MA_4^- (K_4)$$

$$[MA^{2+}] = \frac{[A^-][M^{3+}]}{K_1};\ [MA_2^+] = \frac{[A^-]^2[M^{3+}]}{K_1K_2}$$

$$[MA_3] = \frac{[A^-]^3[M^{3+}]}{K_1K_2K_3};\ [MA_4^-] = \frac{[A^-]^4[M^{3+}]}{K_1K_2K_3K_4}$$

On obtient donc:

$$\Sigma M = [M^{3+}]\left[1 + \frac{[A^-]}{K_1} + \frac{[A^-]^2}{K_1K_2} + \frac{[A^-]^3}{K_1K_2K_3} + \frac{[A^-]^4}{K_1K_2K_3K_4}\right]$$

ce qui conduit à:

$$R_3 = \frac{[M^{3+}]}{\Sigma M} = \frac{1}{1 + \frac{[A^-]}{K_1} + \frac{[A^-]^2}{K_1K_2} + \frac{[A^-]^3}{K_1K_2K_3} + \frac{[A^-]^4}{K_1K_2K_3K_4}}$$

Soit C_A la concentration en acide,

$C_A = [A^-] + [AH] + [MA^{2+}] + [MA_2^+] + [MA_3] + [MA_4^-]$.

On peut considérer qu'en conditions limites de partage, tous les termes en [MA] sont négligeables, comme c'est le cas en chromatographie sur papier. Si K_a est la constante de dissociation de l'acide:

$$[A^-] = \frac{C_A \cdot K_a}{(K_a) + [H^+]}$$

Connaissant C_A, K_a et $[H^+]$, on peut ainsi calculer R_3.

Or $$R_2 = \frac{[MA^{2+}]}{\Sigma M} = \frac{[A^-]}{K_1} \cdot \frac{[M^{3+}]}{\Sigma M} = R_3 \frac{[A^-]}{K_1}$$

De même $$R_1 = R_3 \frac{[A^-]^2}{K_1K_2};\ R_0 = R_3 \frac{[A^-]^3}{K_1K_2K_3};\ R_{-1} = R_3 \frac{[A^-]^4}{K_1K_2K_3K_4}$$

La mise en évidence d'un système sélectif (valeurs de R_F les mieux distribuées) est donc fonction de $[A^-]$. À l'aide d'une calculatrice Olivetti Programma 101, on a trouvé la valeur la plus favorable: $[A^-] = 0.12$. Il apparaît alors une infinité de solutions favorables, $[A^-]$ dépendant naturellement de la concentration totale en acide et du pH. Voir le Tableau I.

La et Ce toujours cations auront des R_F faibles, Sm, Eu et Y des R_F élevés. On constate un grand espace entre Nd et Sm qui correspond au Pm, non inclus dans le Tableau I, faute de données. Ce type de calcul permet de mieux comprendre le phénomène. Le choix des meilleures conditions afin de séparer simultanément un grand nombre d'éléments doit être tel que les éléments soient plus ou moins complexés, mais jamais entièrement ni nullement, ce qui permet d'éviter de faire appel à des techniques comme le gradient de concentration ou de pH pour obtenir une gamme étendue de R_F. Par contre, pour ne séparer que deux ou trois éléments, les conditions ne seront pas les mêmes, car on aura plus de latitude sur les valeurs de R_F.

TABLEAU I

VALEURS DE R_x POUR $[A^-] = 0.12$

	La	*Ce*	*Pr*	*Nd*	*Sm*	*Eu*	*Y*
R_3	—	—	—	—	—	—	—
R_2	0.13	0.02	0.02	0.02	0.01		
R_1	0.87	0.75	0.45	0.11	0.10	0.08	0.03
R_0	—	0.23	0.31	0.56	0.30	0.29	0.22
R_{-1}	—	—	0.22	0.29	0.59	0.62	0.75

Correction pour séquestration par la résine. Dans un travail précédent[33], nous avons déjà examiné l'effet qui résulte de l'extraction des ions de la solution par le papier SA-2. Dans le cas présent, il est plus simple d'admettre que les coefficients de distribution, en l'absence de complexant, sont sensiblement de même ordre de grandeur pour l'ensemble des terres rares entre solution et papier échangeur. La présence de ce dernier dans le système retardera d'environ une unité pH le pouvoir complexant de la solution. C'est donc pour $C_A = 0.24\,M$ et pH 5.0 que se situent les meilleures conditions.

RÉSULTATS

En faisant varier C_A et le pH, nous avons obtenu expérimentalement diverses valeurs de R_F. La chromatographie donnant des résultats dont la reproductibilité est assez médiocre, en valeurs absolues, mais satisfaisantes en valeurs relatives, ce sont des moyennes. Voir le Tableau II.

TABLEAU II

VALEURS DE R_F POUR DIVERSES CONCENTRATIONS C_A EN ACIDE ET DIVERS pH

T = trainée importante dans le sens du front.

	C_A/pH							
	0.1/5.0	*0.1/6.0*	*0.2/4.2*	*0.2/6.0*	*0.24/5.0*	*0.25/4.8*	*0.44/4.1*	*0.5/6.0*
La	0.01	0.01	0.01	0.09	0.08	0.11	0.16	~1
Ce	0.01	0.01	—	0.14	0.17	0.23	0.33	—
Pr	0.01	0.01	0.06	0.27	0.25	0.34	0.45	—
Nd	0.02	0.02T	0.09	0.30T	0.35	0.46	0.58	—
Sm	0.05	0.05T	0.22	—	0.63	0.79	—	—
Eu	0.10	0.10T	0.36	—	0.77	—	—	—
Y	—	—	0.59	—	0.93	—	—	—

Il est intéressant de calculer les facteurs de séparation pour les meilleures conditions expérimentales; pour deux ions 1 et 2, c'est le rapport: $[1/(R_F - 1)_1]/[1/(R_F - 1)_2]$.

Dans la séquence suivante, la valeur numérique de ce coefficient est relative aux deux éléments qui l'encadrent.

La | 2.3 | Ce | 1.6 | Pr | 1.6 | Nd | 3.2 | Sm | 2.0 | Eu | 4.1 | Y

Ce sont des valeurs élevées.

Dans la pratique, il n'est pas suffisant que les R_F soient différents pour obtenir de bonnes séparations; encore est-il nécessaire que les taches ne soient pas trop étalées comme il arrive souvent en chromatographie. Dans ce cas, le mode opératoire revêt une grande importance.

PARTIE EXPÉRIMENTALE

Mise en solution des terres rares

Nous avons constitué des solutions mères à 100 mg/ml en attaquant les oxydes par l'acide nitrique au $\frac{1}{3}$, puis un mélange des sept éléments à 15 mg/ml de chacun d'entre eux. On a parfois repris par la solution servant au développement, les résultats sont identiques dans les deux cas.

Conditionnement du papier SA-2

C'est un mélange à 40–45% en poids de grains de résine Amberlite IR-120 très fins noyés dans l'α-cellulose, livré sous forme Na^+; ses propriétés chimiques sont évidemment les mêmes que celles de la résine: grande affinité pour les ions trivalents, pas de sélectivité pour les terres rares. Il contient des impuretés dont Fe^{3+}; aussi lui avons nous fait subir le traitement suivant: HCl 4 M–NH_4SCN 4 M–NH_4OH 3 M–NH_4NO_3 3 M. Entre chaque opération, on rince abondamment à l'eau déionisée. Le support se trouve ainsi finalement sous forme NH_4^+.

Préparation du complexant

Nous avons utilisé un produit Fluka pur (>98%). Les solutions sont préparées par pesée et ajustage au pH voulu par l'ammoniaque du commerce. Il est préférable de les préparer juste avant l'emploi (comme pour l'acide lactique ou l'acide citrique).

Développement

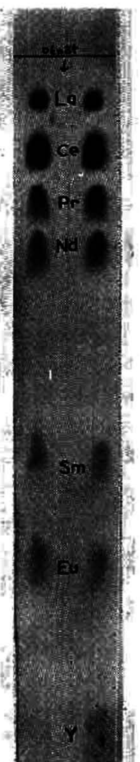
L'expérience nous a montré que la technique descendante était supérieure à la chromatographie ascendante: nécessité d'une quantité de développant moindre, et surtout avance du front plus régulière. Les conditions dans lesquelles les meilleurs résultats sont obtenus sont assez critiques; nous décrivons ci-après le mode opératoire adopté.

On effectue le dépot par une micropipette Pedersen de 2 μl (soit 30 μg de chaque élément) à 8 cm du bord supérieur de la bande sous forme de tache ou mieux de trait sur une largeur de 1 cm; dans ce cas, les traînées sont réduites. 5 cm de la bande (de longueur totale 55 cm) trempent dans le réservoir de solvant. L'expérience demande environ 3 h. Pour des papiers de lots différents, ce temps est variable. Nous avons par exemple constaté une durée de 3½ h pour un autre échantillon. Il n'est pas nécessaire de saturer préalablement la cuve (Chromatank Shandon Panglas 300) car le solvant utilisé est l'eau.

Certains auteurs préconisent d'effectuer le dépôt derrière le front de développant afin d'éviter des effets d'analyse frontale: dans notre cas, les résultats ont été moins satisfaisants.

Révélation

Après séchage, on vaporise une solution aqueuse d'alizarine sulfonate de sodium



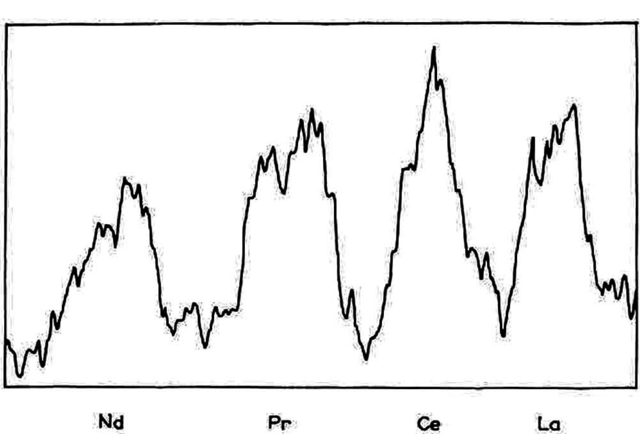


Fig. 1. Photographie d'une séparation après révélation. (La coloration des taches de Sm, Eu et Y s'estompe rapidement.)

Fig. 2. Enregistrement densitométrique des taches de La, Ce, Pr et Nd.

à 0.5% et on expose aux vapeurs d'ammoniaque. On obtient des taches rose violacé sur fond rose qui s'atténuent rapidement après disparition de l'effet de NH_4OH.

APPLICATIONS

La Fig. 1 réprésente une photographie, après séparation, des sept éléments, à partir d'un mélange synthétique. On remarque que les taches sont d'autant plus étalées que le R_F est élevé. On ne constate pas la formation de trainées importantes; il existe un trou entre Nd et Sm correspondant au Pm; de même entre Eu et Y se placerait le Tb.

La Fig. 2 montre des exemples d'enregistrement densitométrique des taches sur "Chromoscan" après révélation. On obtient les résultats sans filtre monochromateur. Les pics sont très nets. L'hétérogénéité du papier explique l'irrégularité de la ligne de base.

La Fig. 3 est une autoradiographie de séparation obtenue dans les conditions suivantes: le groupe des terres rares (et le baryum) ont été extraîts d'une solution après irradiation d'un copeau d'uranium; on a ensuite ajouté des éléments inactifs entraî-

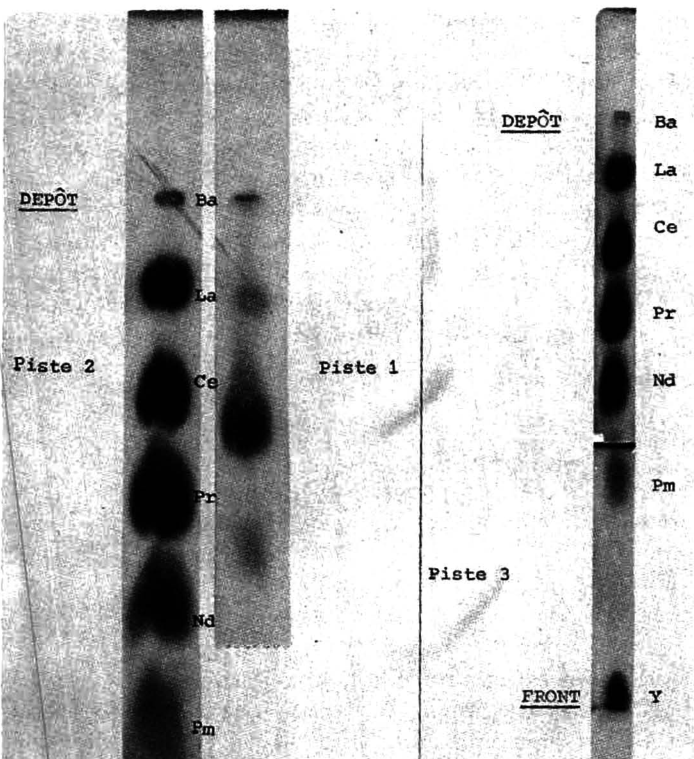


Fig. 3. Autoradiographie des terres rares après séparation. Piste 1: 5 µg de Ce entraîneur; piste 2: 30 µg de chaque élément entraîneur; piste 3: 5 µg de chaque élément entraîneur.

neurs. La première piste correspond à 5 µg de Ce entraîneur, contenant les autres éléments sous forme d'impuretés, la seconde 30 µg de chaque terre rare entraîneur, la troisième 5 µg. Sur la 3ème piste, les taches de Ba, La, Ce, Pr, Nd, Pm et Y dans le front apparaissant nettement.

La séparation s'est effectuée dans les mêmes conditions que pour le mélange synthétique, avec les précautions usuelles.

CONCLUSIONS

La mise au point de cette méthode simple par rapport aux techniques de chromatographie en milieux non aqueux, rapide (3 h), plus adaptée à la séparation de quelques dizaines de microgrammes que l'électrophorèse à haute tension (d'appareillage plus complexe) a montré l'analogie entre les phénomènes sur colonnes d'échangeurs et le papier contenant les mêmes résines. Le calcul préalable des meilleures conditions opératoires permet d'une part de mieux situer celles-ci, d'autre part, de comprendre le phénomène plus quantitativement.

D'intéressants développements sont à entrevoir à cette échelle microanalytique: tentative de dosage par densitométrie, d'un mélange après séparation. L'étude d'une standardisation des conditions de révélation reste à faire ainsi que des essais quantitatifs par voie radiochimique.

Est-il possible d'augmenter les quantités séparables? Dans les conditions expérimentales décrites, la limite semble atteinte. Pour des couples ou des triades d'éléments surtout non voisins, il est possible de séparer quelques centaines de microgrammes en les déposant en ligne. Par contre, pour les sept éléments, il est nécessaire d'augmenter la capacité; des essais sur couches minces de résines n'ont pas encore fourni les résultats escomptés du fait de l'hétérogénéité de ce type de support. Nous envisageons par contre, la fabrication de papiers échangeurs plus épais. En conclusion, nous pensons que l'utilisation des colonnes d'échangeurs d'ions pour résoudre un problème de séparation analytique de quelques centaines de microgrammes, voire de quelques milligrammes, peut être avantageusement remplacée par les techniques que nous venons d'énoncer. D'autres familles d'éléments peuvent être séparées par des techniques analogues.

REMERCIEMENTS

Nous tenons à remercier Mlle O. MUNO, pour sa contribution technique. L'ensemble de ces travaux a été exécuté pour le C.E.A. et avec sa collaboration, sous le contrat No. 2149/e.

RÉSUMÉ

On décrit une méthode de séparation rapide (3 h environ) des lanthanides La, Ce, Pr, Nd, Pm, Sm, Eu et Y par chromatographie sur papiers échangeurs d'ions SA-2, en milieu α-hydroxyisobutyrate d'ammonium (α-HIB). Un calcul de complexion, vérifié expérimentalement, a donné les meilleures conditions qui se situent en 0.24 M α-HIB à pH 5.0. Les conditions opératoires optimales sont discutées. Un exemple d'application à la séparation de terres rares produits de fission est montré. La méthode se révèle valable pour des quantités de quelques dizaines de microgrammes de chaque élément.

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THE CHROMATOGRAPHIC PROCESS IN A REVERSED-PHASE SYSTEM FOR THE SEPARATION OF SOME METAL IONS, BY ION-ASSOCIATION DISTRIBUTION

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SUMMARY

After a consideration on the distribution of metal ions in reversed-phase systems consisting of tri-*n*-butyl phosphate and 3–9 M hydrochloric acid, a general equation relating the partition coefficients of the metal ions in the system and their R_F values is proposed

$$\alpha^1 = A\,\{1/(R_F - 1)\} - B$$

where α^1 is the experimentally determined partition coefficient of a solute. A is a volume ratio term, not necessarily the ratio of the amount of reversed-phase material to the amount of solvent covering a particular area of the plate, and being concerned with the intermixing of the phases and the use of part of the stationary phase in binding to the cellulose layer. B is a term related to the interaction of the mobile and stationary phases. In systems where no interaction occurs and the stationary phase is all chromatographically active, the equation reduces to the ideal equation of chromatography, *viz.* the MARTIN relationship.

INTRODUCTION

The use of reversed-phase systems for the chromatographic separation of compounds is widespread, in both organic and inorganic fields. The mechanism of the separation is generally regarded as partition of a solute between two liquid phases, one a mobile phase and the other the stationary phase. These phases are generally regarded as independent and discrete and the various chromatographic equations such as that derived by MARTIN and co-workers[1], which have served as the basis of much of the present theoretical knowledge of chromatography, assume this to be self

evident. One result of this assumption is that the mechanism of chromatography is often assumed to be simple partition between two distinct well defined phases because the MARTIN or a similar relationship is valid for the system. This assumption is not necessarily correct, and it was thus decided to investigate systems in which the phases were not necessarily discrete and could have mutual interaction and thence to evolve a more general relationship than that indicated by MARTIN. However, we must first consider the processes by which distribution of solutes takes place in systems in which the MARTIN relationship is known to be valid. These usually involve a relatively non-polar organic phase and an aqueous phase and the solute to be distributed between these phases has no discrete charge, but may contain groups, usually the chromatographically functional group, which is polarised or is capable of being so. The molecules of the solvents of the two phases may exert a dipole-induced dipole attraction on each other, but this is not of sufficient magnitude to cause dissolution of any one phase in the other to such an extent that discrete phases are not discernible. Thus solution of an organic solute in a non-polar organic phase occurs because the solute units fit into the solvent molecular pattern and little or no electronic disturbance is thereby caused, and the induced dipole caused in the solute by the dipole of the molecules of the aqueous phase is insufficient to give a complex of the type (organic solute–aqueous solvent) sufficiently stable to exist in the aqueous phase as a solvated (aquated) species.

In the case of inorganic metal complexes, which may possess an electric charge and which are distributed between a stationary phase containing polar groupings and a mobile phase, which is generally ionic or has at least a relatively high dipole or dielectric constant, then solution occurs because of somewhat different mechanisms to those postulated above. In some systems it is possible to get partition of an inorganic species between a non-aqueous stationary phase and an aqueous mobile phase, by an ion-association mechanism[2] or by a somewhat related ion-exchange system[3]. In both cases, to a large extent there is a similarity of the various species involved; and the mechanisms are somewhat analogous. We considered that it is possible to have the solute species occurring in the two phases, in different chemical entities. These may have electrical properties differing only in degree but not in type. Furthermore the similarity in the electrical nature of the two phases may be such that there could be some doubt concerning the absolute chromatographic and physical boundaries of the two phases. It was thus decided to use some ion-association systems and to investigate the relationship between the partition coefficients of substances distributed between various chromatographic phases and the R_F values of these substances in the same chromatographic phases. Although CERRAI[3] had postulated an ion-exchange mechanism for the separation of metal chlorides on a reversed-phase system with a stationary phase of HDEHP (di-(2-ethylhexyl) orthophosphoric acid) supported on cellulose paper, using various molarities of hydrochloric acid as the eluent, in his derivation of working equations he used the same considerations regarding the separation of the phases as did MARTIN, except that he did not assume that the whole of the HDEHP was necessarily chromatographically involved, and related the quantity $1/(R_F - 1)$ to the effective concentration of HDEHP on the impregnated paper. He did not, however, explain the various deviations which occurred, nor did he explain why the effective concentration was not the sum total concentration of HDEHP. The support was assumed to have no chromatographically functional role.

It was thus decided to investigate systems having a stationary phase of tri-*n*-butyl phosphate (TBP) supported on a thin layer of cellulose powder and a mobile phase of hydrochloric acid of various molarities. The solutes were the appropriate ionic species containing cadmium, cobalt(II), copper(II), manganese(II), palladium(II), and zinc.

MUSIL *et al.*[4] had previously reported chromatographing some metal chlorides on paper impregnated with a TBP–methanol mixture using various molarities of hydrochloric acid as eluent, and had attempted to find a relationship between the R_F value of the metal chlorides and their partition between TBP and hydrochloric acid. No interpretation of their results was attempted. Before any systematic study can be made of the relationship between partition coefficients and chromatographic parameters for all kinds of systems it is necessary to examine the equation postulated by MARTIN, who was concerned only with relatively simple non-polar organic systems. Thus he postulated the relationship:

$$\alpha = \frac{A_L}{A_S} \cdot \left(\frac{1}{R_F} - 1\right)$$

where

α = the partition coefficient for the distribution of the solute between the mobile and stationary phases (of the chromatographic system).

A_L = the cross-sectional area of the mobile phase (of the chromatographic system)

A_S = the cross-sectional area of the stationary phase (of the chromatographic system)

R_F is defined as the ratio of the distance moved by the solute and that moved by the mobile phase.

(It is generally assumed that these terms refer to the whole system.)

Each of these terms must be examined in conditions practically identical with those appertaining to the actual chromatographic system. In addition, it is considered necessary to examine physically the phases during the development of the chromatogram so that any changes occurring may provide further data.

Many of the previous workers on such relationships have either assumed values for either A_L or A_S or determined them using simple non-polar organic reversed-phase systems. These approaches are exemplified respectively by the work of MULVANEY *et al.*[5] and COPIUS PEEREBOOM[6]. Any slight deviations from the MARTIN relationship have generally been attributed to experimental error.

The purpose of the work was to establish experimentally a more general equation which would be valid for both polar and non-polar systems.

EXPERIMENTAL

Chromatography

The TBP was purified by a method reported previously[5,7] using a batch process. All the batches were recombined to give a homogenous material.

The method and apparatus used for the chromatography of the metal ions was reported previously[2], and is summarised as follows: Purified and sieved cellulose powder was mixed with a solution of TBP in carbon tetrachloride and used to coat

glass plates. The carbon tetrachloride was caused to evaporate and to the pure TBP layers solutions of metal chlorides were applied. The plates were placed in a small-volume double saturation chamber[8], pure hydrochloric acid of known molarity (3.0; 4.0; 4.5; 5.0; 6.0; 7.0; 7.5; 8.0; 9.0 M) was used as the mobile phase, and the chromatograms were developed by an ascending technique under controlled temperature conditions (25 $\pm$ 0.5°). The eluent was allowed to move a fixed distance (12.5 $\pm$ 0.25 cm) from the point of application of the metal ions. The development times ranged from 1 to 3 h, depending on the molarity of the acid. There was an increase in time of development with the increase in the acid molarity.

After very rapid removal of most of the TBP and the acid (achieved by using temperatures in excess of 120°), the plates were sprayed with various chromogenic reagents[2], and the R_F values were determined.

Each R_F value reported is the mean of at least four values obtained on different plates, and differing by not more than $\pm$ 0.01 R_F units from the arithmetic mean. (This precision was possible only since the chromatography was done under strictly standardised conditions. Very slight variations in the purity of the TBP gave variations greater than $\pm$ 0.01 R_F units—see DISCUSSION OF THE RESULTS.)

The molarity of each of the hydrochloric acid solutions was determined titrimetrically.

Determination of the cross-sectional area of the stationary phase

This was done at only two different molarities of hydrochloric acid (6 M and 8 M). Coated plates were placed in a saturation chamber, the atmosphere of which was saturated with the vapours of either 6 M or 8 M acid, and the plates were allowed to stand for approximately 2 h in the appropriate atmosphere. (This time was chosen as being a median between the times taken for plates to run under normal conditions and as being sufficient to allow for any gravitational drainage of the stationary phase to occur.) After standing, the plates were removed from the saturation chamber, and were divided into bands parallel to the upper edge of the plate, *viz.* what would be the solvent front under development conditions. For the distribution of the phosphate on the layers the bands were transferred to dry tared sintered glass crucibles, and the crucibles and contents were weighed. Carbon tetrachloride was then passed through the cellulose to remove the TBP. The crucibles were then dried in an air oven, to constant weight. The process was repeated until constant weight was obtained between two successive washings with carbon tetrachloride. Control experiments using non-impregnated cellulose layers were done using the same total volume of carbon tetrachloride. This enabled corrections to be made for any material other than the phosphate which may have been washed out of the cellulose by the carbon tetrachloride. The results are shown in Table I.

The values quoted for the weight of TBP found were obtained from plates taken at random from alternate batches of plates made and used in subsequent experiments. By this method it was thought possible to eliminate errors caused by the operator during the spreading of the plates.

To see if the TBP was moved up the plate by the flow of HCl, a small series of plates was developed at 6 M HCl and the TBP was measured in strips parallel to the solvent front as before (except that the bands were only measured at 4–6 cm, 8–10 cm, and 12–14 cm from the point of application). The results obtained were within the

range of results obtained for the above experiments. It was thus concluded that although there would be dissolution of the TBP by the HCl, saturation conditions were established and thus no TBP was effectively transported by the HCl at this molarity and under these experimental conditions. Thus the amount of TBP covering a particular area of the plate remains experimentally constant at this molarity of HCl.

Distribution of hydrochloric acid on the eluted layers

The plates, already impregnated with the TBP, were eluted with two different concentrations of HCl (6 *M* and 8 *M*) to a distance of 14 cm from the point of application of the solutes. The chromatoplate was removed from the saturation chamber, and the layer was removed in bands (2 cm wide) using a specially designed scraper. The acid was leached from the cellulose with water and the amount of acid was determined titrimetrically using NaOH (0.5 *M*) as the titrant and screened Methyl Orange as a visual end point indicator.

TABLE I

A_L/A_S RATIOS AT 6 *M* AND 8 *M* HCl

Distance from line of application (cm)	*Amount of TBP (mg)*[a]	A_L/A_S, *6 M*[b]	A_L/A_S, *8 M*[b]
0–2	109.6		
2–4			
4–6	108.0	9.21	6.38
6–8		8.85	6.16
8–10	108.5	7.16	6.06
10–12		6.79	5.81
12–14	117.9	6.31	5.05
14–16		4.51	4.02
16–18	117.7	—	—
18–20		—	—

[a] Amount of TBP in a band of 18 × 2 cm.

[b] The A_L/A_S ratio for a given band was calculated from the measured volumes of stationary phase and mobile phase in that band, corrected for the acid extracted by the TBP and the increase in volume of the stationary phase as a result of this extraction. The values quoted for this ratio are the mean of at least four determinations.

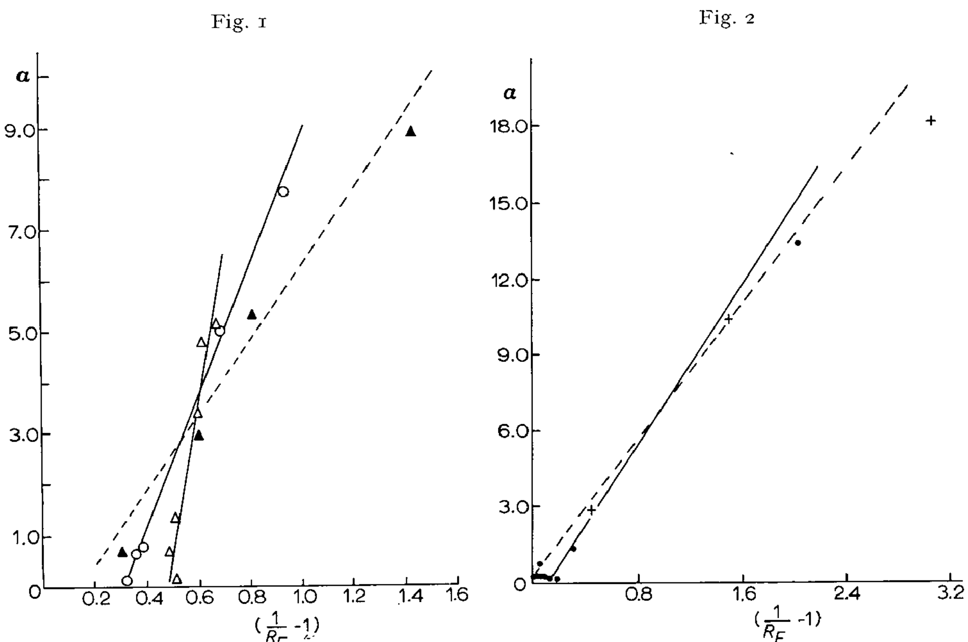
The cellulose above the apparent solvent front was also treated in the above manner to determine its acid content. The means of at least four determinations for each acid concentration were used to calculate the A_L/A_S ratios given in Table I.

In separate experiments the total amounts of acid left after developing the plates were determined by titrating not only the acid on the layer (over the whole length of the plate) but also that remaining in the bottom of the saturation chamber and on the walls of the vessel. The differences in the amounts of acid introduced into the chamber and the amounts recovered were not sufficiently great (less than 2%) to indicate that any significant hydrolysis of the TBP by the HCl had occurred and we thus concluded that the layers were substantially chemically unchanged during the development of the chromatogram.

Determination of the partition coefficients of the metal ions

The amounts of HCl and TBP having been determined, the same ratios of metal ion, HCl and TBP were shaken together in a separating funnel (approximately 20–30 ml portions were used). After allowing equilibrium to be established, the funnel was placed in an air oven at 25 ± 0.5° for 30 min and the two phases were allowed to separate. When separate, the lower aqueous phase was removed and the metal concentration determined either by atomic absorption or titrimetrically. In the case of cadmium, no hollow cathode lamp was available and the concentration of the metal ion remaining in the aqueous phase was generally too small to give a good visual end point in the titration with EDTA. Thus the metal ion was re-extracted from the non-aqueous phase by shaking the phase with distilled water (two aliquots of 15 ml were used). The combined extracts were then titrated at pH 5.0 using EDTA and pyridylazonaphthol (0.1% w/v ethanolic solution) as the visual indicator.

The metals which had been determined by means of atomic absorption methods were assayed on various commercial instruments, the choice of instrument being governed by the availability of the various hollow cathode lamps required. In all the determinations using atomic absorption methods, it was necessary to prepare standard calibration graphs using hydrochloric acid solutions of the same molarity as that used in each particular determination. It was also necessary to shake this acid with TBP



Plot of the variation of $1/(R_F - 1)$ *vs.* the partition coefficients for the various molarities of acids used. Fig. 1. △—△, 9 *M*; ○—○, 8 *M*; ▲— — —▲, 7.5 *M*. Fig. 2. — — + — —, 5 *M* HCl; — — · — —, 6 *M* HCl.

Equations to lines:

$9\,M;\ \alpha = 28.80\ \{1/(R_F - 1)\} - 14.12$

$8\,M;\ \alpha = 12.37\ \{1/(R_F - 1)\} - 3.80$

$7.5\,M;\ \alpha = 7.45\ \{1/(R_F - 1)\} - 2.42$

$6\,M;\ \alpha = 6.81\ \{1/(R_F - 1)\} - 0.97$

$5\,M;\ \alpha = 5.97\ \{1/(R_F - 1)\} - 0.08$

TABLE II

PARTITION COEFFICIENTS AND CHROMATOGRAPHIC PARAMETERS

Metal	*Acid molarity*	*R_F value*	*$1/(R_F - 1)$*	*a*
Cadmium	3	0.18	4.56	22.40
	4	0.22	4.45	19.75
	5	0.24	3.17	18.05
	6	0.32	2.13	13.16
	7.5	0.41	1.44	8.83
	8	0.52	0.94	7.74
	9	0.59	0.68	5.12
Cobalt	3	0.96	0.04	0.00
	4	0.95	0.05	0.00
	5	0.95	0.05	0.00
	6	0.85	0.18	0.03
	7	0.83	0.20	0.38
	8	0.72	0.38	0.77
	9	0.65	0.52	1.27
Copper	3	0.96	0.04	0.075
	4.5	0.95	0.05	0.14
	5	0.91	0.10	0.37
	6	0.82	0.22	0.51
	7.5	0.76	0.32	0.635
	8	0.74	0.35	0.64
	9	0.67	0.49	0.67
Manganese	4	0.95	0.05	0.03
	5	0.93	0.08	0.04
	6	0.89	0.13	0.05
	7	0.87	0.15	0.075
	8	0.76	0.315	0.11
	9	0.66	0.52	0.16
Palladium	5	0.70	0.43	2.6
	6	0.67	0.49	2.8
	7.5	0.63	0.59	2.9
	8	0.63	0.59	3.5
	9	0.63	0.59	3.2
Zinc	4.5	0.36	1.78	11.80
	5	0.40	1.50	10.40
	6	0.47	1.13	8.33
	7.5	0.55	0.82	5.27
	8	0.59	0.69	5.02
	9	0.62	0.62	4.83

before adding the appropriate metal salt. (This is necessitated by the enhancement of the absorption caused by some chloride complexes, and the suppressive effects of the pyrolysis products of TBP contained in the aqueous phases.)

The results are reported in Table II with the appropriate R_F values.

Graphical relationships

The relationships between the partition coefficients and the R_F values were obtained by plotting the variation of $1/(R_F-1)$ against the partition coefficients for the various molarities of acids used. The method of least mean squares was used to obtain the best fit to the experimental values.

The lines obtained are shown as Figs. 1 and 2.

DISCUSSION OF THE RESULTS

The R_F values and partition coefficients

The values quoted here are those obtained using an "homogenised" sample of TBP. We have used on other occasions single batches of TBP, purified by a method similar to that quoted here, and have obtained R_F values and partition coefficients which gave relationships as linear as those reported here but with noticeable and significant variations. This lack of agreement in the values for a particular ion in the supposedly same "TBP/HCl" systems is evident throughout the literature[9–11].

It has been shown[12] that these differences are caused by the varying amounts of the lower butyl phosphates (and in some cases *n*-butanol) which were not completely removed by the purification processes. These lower butyl phosphate esters are reported to cause synergistic effects with TBP[13,14]. Thus to obviate any variations in the particular coefficient measurements and in the R_F values, it is necessary to use a completely homogenous sample of purified TBP throughout. As mentioned above, the values obtained are only strictly comparable when *inter alia*. However, the same overall conclusions may be deduced from any such system; the shapes of the graphs relating the chromatographic parameters and the distribution parameters are comparable. The order of R_F values is in good agreement with those values previously reported with analogous systems[4,15,16] and the partition coefficients follow the same patterns as those previously reported[12,16,17], although the variations throughout the literature make it impossible to have direct and strict comparisons.

The partition coefficients cannot be determined *in situ* on the plate because of the practical difficulties involved, and the exact conditions cannot be duplicated in bulk, since the amount of cellulose needed to represent the support would render impossible the mixing of the phases by agitation.

The relationship between the R_F values and the experimentally determined partition coefficients

Although various workers have investigated the distribution process in partition chromatography from various aspects, for example as a discontinuous system[1] or kinetic equilibria[3,18–20], or involving steady state phenomena[21], all the equations derived are almost identical and reduce effectively to the MARTIN equation. Only CERRAI[3] has indicated that the volume of the organic phase which should be considered is not necessarily the total volume of the substance present. He reported that he could find no direct proportionality between the molarity of the impregnant (HDEHP) used to treat the paper and the effective concentration of this taking part in the chromatographic process. However, his method of estimating that amount which is present in the partition process is based on the absorption of one metal into one system.

From considerations of the plots of the experimentally determined partition coefficients of the metals studied here, and the term $1/(R_F-1)$ (Figs. 1 and 2), it appears that there is a linear relationship of the following type

$$\alpha^1 = A\left(\frac{1}{R_F} - 1\right) - B$$

where

α^1 is the experimentally determined partition coefficient of the solute between

TABLE III

EQUATIONS FOR THE GENERAL EXPRESSION $a' = A\{1/(R_F - 1)\} - B$

Molarity (M)	A term	B term
5	5.97	0.08
6	6.91	0.97
7.5	7.45	2.42
8	12.37	3.80
9	28.80	14.12

the two phases "in bulk", which need not necessarily be the same thermodynamic function as envisaged by MARTIN and others;

A is a function for a particular system involving the ratio of the actual volumes of the phases concerned in the partition of the solute on the chromatogram; and

B is a term related to the interaction of the two phases in a particular system.

We regard this as the general equation of any chromatographic process, and suggest that where there is no interaction between the phases, which can then be considered to be discrete, $B = 0$, and the equation becomes essentially of the same form as the MARTIN equation. If we assume that the volumes of the two phases measured experimentally are identical with those taking part in the distribution process, then the equation becomes identical with the MARTIN equation.

However, in the systems considered here, there are not ideal conditions. This is made evident if we consider Table III, which collects the equations calculated from the results given in Table II and shown graphically in Figs. 1 and 2. (Although R_F values greater than 0.85 units and less than 0.1 units are not sufficiently precise to

TABLE IV

COMPARISON OF A_L/A_S RATIOS

Ion	R_F value[a]	A_L/A_S (exptl.)[b]	A_L/A_S (calc.)[c]	A term[d]
Using 6 M HCl as the eluent				
Cd	0.32	9.6	6.2	6.81
Co	0.85	6.6	0.2	
Cu	0.82	6.8	2.3	
Mn	0.89	6.4	0.4	
Pd	0.67	7.8	5.7	
Zn	0.47	8.9	7.4	
Using 8 M HCl as the eluent				
Cd	0.52	5.8	8.3	12.37
Co	0.72	6.0	2.1	
Cu	0.74	6.0	1.8	
Mn	0.76	5.9	0.35	
Pd	0.63	6.1	6.0	
Zn	0.59	6.1	7.3	

[a] Values are the mean of at least four results.
[b] Obtained by graphical interpretation of results in Table I.
[c] Calculated from $A_L/A_S = a/\{1/(R_F - 1)\}$.
[d] Obtained from slopes of graphs given in Figs. 1 and 2.

be used in calculations, they are given in Table II to indicate the orders of separation of the various ions; they have not been used for the calculation of the equations to the experimental values.) Thus we can see that the A terms vary from approximately 6 to 30 and the B term from 0.08 to approximately 14. Within a particular system the variation between theory (*i.e.*, no interaction of phases) and practice (*i.e.*, probable interaction between phases) is well illustrated by Table IV. The lack of correlation between the observed and the calculated values for the A terms leads us to consider whether or not there were definable chromatographic layers in the systems studied.

The discreteness of the two phases

In the course of the investigation we observed that as the concentration of the acidic phase increased the developed plates appeared progressively drier, although the volume of acid used as the solvent was kept constant, and the same length of chromatographic run was allowed. This apparent lack of mobile phase we attributed to increased dissolution of the mobile phase into the stationary phase. Previous workers on TBP/HCl systems have studied the mutual solubility of the two substances. HARDY[22] reported that if equal volumes of TBP and water (or dilute acid solutions) are equilibrated, the resulting ratio of organic phase to inorganic phase will be 1.13. This ratio progressively increases with increase in acid concentration, until at 11 M HCl the ratio is 1.90, thus corresponding to an increase of 33% in the organic phase. In the systems reported here (TBP/5 M–9 M HCl) the value of B increases from 0.08 to 14.12. This we regard as an indication of the amount of the HCl entering the TBP phase.

To some extent, this will account for the discrepancy between the volume ratio (A) calculated from the graph and that calculated from the experimentally determined amounts of TBP and HCl present on a plate, since in the latter case it has been tacitly assumed that each substance was contained in a separate layer.

Other factors, such as the mechanisms of the mutual dissolution of hydrochloric acid and TBP, and of the partitioning of the metal species, must also be considered when seeking an explanation of the variations in the volume ratio term.

In a review[23] of the use of TBP in the solvent extraction of metal ions from mineral acids, MARCUS discussed the extraction of HCl by TBP and indicated the probable composition of some of the extracted species existing in the organic phase. The species are mainly partly dissociated ion pairs of the type $[(BuO)_3 \cdot POH]^+$ $[(H_2O)_n \cdot Cl^-]^-$, which co-exist in the TBP phase with other neutral species containing TBP, *viz.* $[TBP(H_2O)_x]$ and $[TBP(HCl)_y]$, the former being more prevalent in low acidity conditions, the latter being found mainly at high molarities. Various solvation ratios have been reported[24–26] and the most probable composition of the complex has a 1:3 ratio of TBP: H_2O. The consequent transfer of both water and acid into the organic phase will inevitably cause relatively large volume changes in both phases, and the differences in the chemical natures of the two phases will also be reduced.

Other factors play a part in this alteration in the phases. It has been reported[27,28] that the amount of HCl extracted into TBP, when metal ions are present, is greater than that extracted from solutions not containing metal ions. This synergistic increase is to some extent a result of the composition of the extracted, metal-containing species. If we consider the extraction of cobalt from hydrochloric acid solutions, we find that in the aqueous phase the cobalt(II)-containing species is an ion-association system

of the type $(H_3^+O)_2[(H_2O)_2 \cdot CoCl_4]^{2-}$. When some dissociation takes place there can be some replacement of the water by the TBP, to give an ion pair of the type $[(BuO)_3POH]^+{}_2 \cdot [H_2O \cdot TBP \cdot CoCl_4]^{2-}$, which is preferentially extracted into the TBP layer.

The degree of dissociation of an ion pair depends on the dielectric constant of the solvent medium, and hence, since the electrical conductance of hydrochloric acid solutions rapidly decreases with increase in the molarity of the HCl[29], there will be a decrease in the dielectric constant of the aqueous phase. This means that there will be a very little tendency for the cobalt(II) ions to be pulled from the TBP layer, and hence the R_F values will decrease with increase in molarity of the HCl layer.

The role of the cellulose

The cellulose substrate is a relatively polar part of the system and its role must be considered. As we have previously reported[30], some of the TBP will be held in the amorphous regions of the cellulose and it is possible that some or all of these TBP molecules are not available for protonation or for forming complexes with the metal species. The TBP is held to the cellulose by hydrogen bonds formed between the polar hydrogen atoms of the hydroxyl group of the cellulose molecule and the oxygen of the phosphorus ester. This bond must be relatively strong since it is through such bonds that the TBP is held to the so-called inert support. Although there is a possibility that the polar groups of the cellulose could take part in the formation of the complexes, we consider this to be unlikely since the amount of TBP present will probably cover all the available polar sites. It is thus probable that we can regard the cellulose as playing no part in the partition process involving the metal-containing species.

CONCLUSION

In systems such as those investigated here, it is considered that the mechanism of the partition of the solute between the TBP and the HCl, and the mutual solubility by extraction or otherwise of these two latter substances, make it probable that no definable phase interface exists, where there is only one or other of the two substances. However, from the linearity of the relationship between the observed partition coefficients and the term $1/(R_F - 1)$ for such systems it is apparent that there exists an equation of the type

$$\alpha^1 = A\left(\frac{1}{R_F} - 1\right) - B,$$

similar to the MARTIN equation and related equations. However, the volume ratio term (A) cannot be determined experimentally because of the impossibility of exactly defining the phases or their boundaries. Although the total amount of the impregnant is not used in the partition process, there is no chemical distinction between the impregnant so used and that used in the partition process. The deviation of the A term from the experimentally determined ratio of the impregnant and solvent is probably a measure of both the interaction of the two phases and the bonding of the stationary phase to the cellulose support.

It is suggested that the MARTIN equation is essentially the ideal form of the

more general equation and that the smaller is the interaction between the two chromatographically active layers for any system, the nearer will be the experimental equation to the ideal MARTIN equation.

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Short Communication

CHROM. 4763

Separation of transuranium elements by gas chromatography of their chlorides

Recently we have shown[1] the possibility of separation of chlorides of lanthanide elements by gas chromatography at relatively low temperature when using the vapours of aluminium trichloride as a component of the carrier gas. The starting point was the work of GRUEN AND ØYE[2,3] who have found that Al_2Cl_6 vapours interact with solid $NdCl_3$ to form gaseous complexes containing neodymium. We have verified[1] that this is the common property of lanthanide elements. By adding Al_2Cl_6 vapours into the carrier gas in the chromatographic experiments we were able to synthesise the complexes at the moment of introduction of the sample of lanthanide chlorides into the column. The excess of Al_2Cl_6 also suppresses the dissociation of the unstable complex molecules. The experiments were carried out in glass capillary columns (2.5 m × 1 mm I.D.) at 250° by the method of gas–solid chromatography. In this case the vapours of aluminium trichloride also serve to modify dynamically the surface of the glass[4,5].

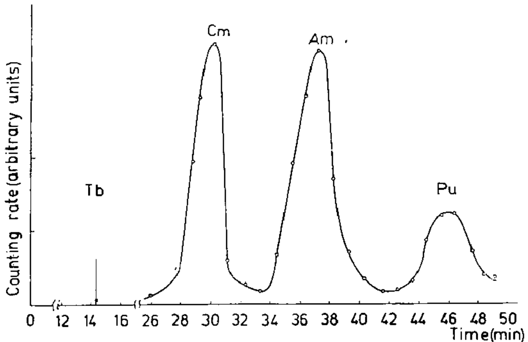
Here we report experiments on the separation of the transuranium elements by an analogous technique. We also investigated the behaviour of protactinium and uranium. Use was made of isotopes ^{231}Pa, ^{233}U, ^{237}Np, ^{239}Pu, ^{241}Am and ^{244}Cm in amounts not in excess of 1 µg.

The details of the experimental procedure were described in the previous report[1]. In the present work we used a longer column (10 m × 1 mm I.D.). It should be noted that the chromatogram was measured by collecting fractions of the Al_2Cl_6 condensate at the exit of the column and by measuring their radioactivity. For the identification of the alpha active elements a fraction was dissolved in hydrochloric acid, about 100 µg of lanthanum carrier were added and then precipitated by an excess of the sodium hydroxide to dissolve aluminium hydroxide. The precipitate was painted onto a metallic disc and the alpha spectrum measured with a surface-barrier detector and multichannel pulse-height analyser.

Some examples of the separation of various mixtures are shown in Figs. 1–3. Beta active ^{160}Tb was added to all samples as a monitor.

It can be seen from Fig. 1 that curium and americium are eluted from the column in the order of decreasing atomic number and are found on the chromatogram at the position of lighter lanthanide elements. The same situation arises when separating tervalent ions of Am and Cm by ion-exchange chromatography[6]. This provides the first experimental evidence that trichlorides of transuranium elements form with Al_2Cl_6 complexes of the same type as trichlorides of lanthanide elements.

The elution of plutonium just after the peaks of americium and curium (Fig. 1) with similar separation factors of the adjacent elements indicates without any doubt that under the conditions of our experiments plutonium exists in the tervalent state. This is in agreement with the fact that the only higher plutonium chloride known, $PuCl_4$, is very unstable and may exist only in the gas phase in an excess of chlorine[7].



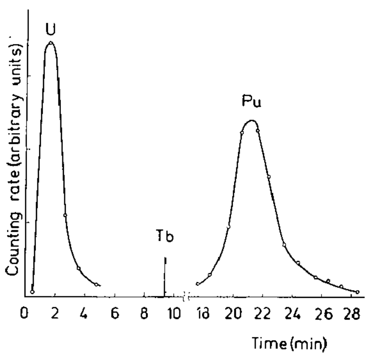


Fig. 1. Separation of a plutonium–americium–curium mixture. Column temperature, 250°; Al_2Cl_6 partial vapour pressure, ~ 100 mm Hg; helium flow rate, 8 ml/min.

Fig. 2. Separation of a uranium–plutonium mixture. Column temperature, 255°; Al_2Cl_6 partial vapour pressure, ~ 100 mm Hg; helium flow rate, 8 ml/min.

The elution of plutonium in such a position relative to Am and Cm as that shown in Fig. 1 is uncommon in chromatography of transuranium elements as, for example, in ion-exchange and extraction chromatography separations plutonium as a rule is found in solution in a higher oxidation state than 3 +.

It can be seen from Figs. 2 and 3 that the retention times of chlorides of protactinium, uranium and neptunium are very small. One of the possible explanations is that these elements are chlorinated, under the conditions of the experiment, to their higher chlorides, which themselves are rather volatile. However, on the basis of the available data one would expect that at 500°, which is the temperature at introduction of the sample, these higher chlorides will dissociate to form tetrachlorides. So it seems more probable that the short retention time, in any case for Np and U, is due to the high effective volatility of complexes of aluminium trichloride with

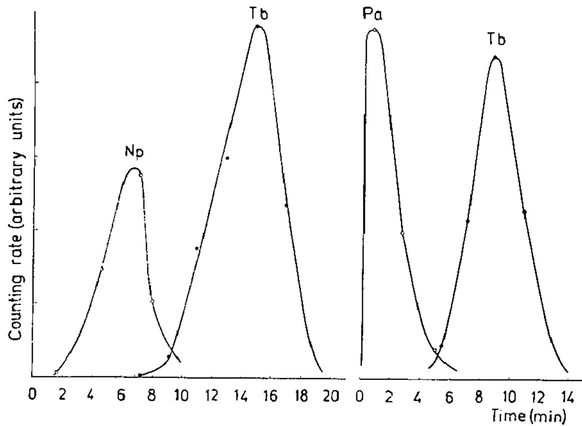


Fig. 3. Chromatograms of chlorides of protactinium and neptunium. Column temperature, 250°; Al_2Cl_6 partial vapour pressure, ~ 100 mm Hg; helium flow rate, 8 ml/min.

tetrachlorides of the above elements. GRUEN AND McBETH[8] proved the existence of such complexes of UCl_4.

It should be noted that at the effectiveness of 100–150 theoretical plates the column provides quite a good separation of the pair americium–curium. The peaks are almost symmetrical, thus providing evidence that Al_2Cl_6 vapours are very efficient in modifying the surface of the glass.

The suggested method of separation of transuranium elements can be used for solving various problems of radiochemical analysis.

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Notes

CHROM. 4660

Gas chromatography: a method of solid injection

In gas chromatography, test materials are usually dissolved in a suitable solvent and an aliquot applied to the column by means of a microsyringe. Even when the concentration of the solute is high, the solvent is usually the largest amount of material introduced into the column. The recorder tracing may show a large solvent peak with a long tailing edge which often interferes with early peaks. To minimize this problem it is general practice to limit the volume injected to 1–5 µl, and to avoid working at high sensitivity. Many methods have been described for reducing or eliminating the troublesome solvent peak. These include the use of an inlet splitter as described by CLARKE[1] and solid injection by means of a needle[2], platinum spiral[3] or gauze[4]. A different system consists of a precolumn inlet having both a cool and hot zone[5] and this enables the solvent peak to be well separated from the first sample peak. Occasionally it is possible to dissolve the sample in a non-volatile solvent such as silicone oil[6].

The method described here is a development of earlier work when it was shown that trifluoroacetylated amino acid methyl esters could be concentrated without loss[7]. It relies on the application of a liquid sample to a precolumn and the solvent being evaporated without loss of solutes. The precolumn is then placed in the heater zone of the apparatus for gas chromatography. Up to 95% of the final test solution can be injected on to the column, thus making better use of the potential sensitivity offered by gas chromatography[8,9].

Apparatus

A gas chromatograph Pye Series 104, Model 24, fitted with two flame ionization detectors (W. G. Pye Ltd., Cambridge, Great Britain) was used in conjunction with a Honeywell 10 mV 1 sec strip chart recorder (Honeywell Controls Ltd., 411 Taunton Road, Greenford, Middx., Great Britain). Integration of peak areas was carried out with the Kent Chromalog 2 digital integrator (Kent Instruments Ltd., Luton, Beds., Great Britain). Nitrogen (99.9% "white spot" from British Oxygen Co. Ltd., and "high purity oxygen-free" from Air Products Ltd.) was used as carrier gas.

Modification of the instrument. The hole below the septum (B in Fig. 1) in the injection head (C) was enlarged by drilling to a diameter of 3.18 mm. When fitting the chromatography column into the injection head (C) by means of the usual O-ring (E) and back-nut (F) an additional silicone rubber O-ring (D) with 6.3 mm O.D. and 2.7 mm I.D. (Griffin & George Ltd., Alperton, Wembley, Middx., Great Britain) was inserted into the recess as shown.

Precolumn. The precolumn (see Fig. 1) consisted of an 8 cm long glass tube with a 3.1 mm O.D. and wall thickness 0.8 mm. A disc of Whatman glass fibre paper (H. Reeve Angel and Co. Ltd, London, Great Britain) was fused to the bottom end of the tube. This was done by heating the tip of the tube to red heat and pressing the tip against the filter paper placed on an asbestos sheet. This tube was filled loosely to a

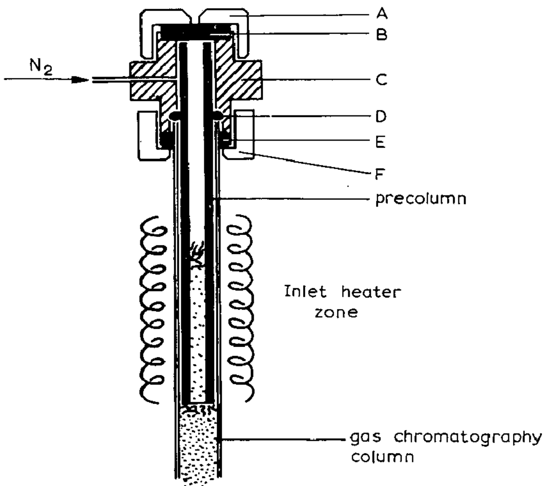

Fig. 1. Injection head of gas chromatograph showing precolumn in position. A = knurled nut; B = septum; C = inlet injection block; D, E = silicone O-rings; F = back-nut.

depth of about 3 cm with the same deactivated support material used in the gas chromatography column and a little pretreated glass yarn[10] was inserted on top. When the precolumn was in position the packing was in the inlet heater zone and the top end close to the septum. No differences were observed whether our support material was coated with stationary phase or not. The sizes of the precolumn and the O-ring (D) were selected so that a push fit was obtained and during gas chromatography of the sample all the carrier gas passed through the precolumn.

Method of use. The liquid sample was applied to the packing in the precolumn and the solvent removed by evaporation (see below). The knurled nut (A) was slowly unscrewed to release the carrier gas pressure in the column. The precolumn was pushed into place and the knurled nut with its underlying septum was immediately screwed tightly back into position. The carrier gas flow controls were not altered. There was no observable blow-back of the packing when the pressure was slowly released at the inlet end of the column.

Experimental

An attempt was made to reduce the size of the solvent peak by injecting small volumes of various test solutions on to the column with a Hamilton microsyringe. But as the volume decreased from 0.5 to 0.1 μl so the HETP[11] values of the peaks increased. Furthermore, amounts of less than 1 μl were not easily reproducible. A needle-type micro-solids injector[2] and a gauze[4] were tried as methods of injection. Even when 1 μl of methylene chloride solvent was evaporated prior to injection considerable losses occurred of such test materials as bibenzyl, pyrene and trifluoroacetylated amino acid methyl esters, and low efficiencies were recorded.

A 1 μl aliquot of a standard mixture (dodecanoic, tridecanoic, tetradecanoic and hexadecanoic acid methyl esters in methylene chloride) was transferred by microsyringe to a precolumn, which was then immediately placed in position in the apparatus for gas chromatography. The HETP values and the peak areas obtained gave

comparable results with those obtained by direct injection of 1 μl on to the column thus showing that this new method of injection was satisfactory.

The same standard solution was diluted × 30 with methylene chloride and 30 μl of this diluted solution was applied by means of a syringe to the precolumn. Most of the solvent was removed by placing the precolumn in a B14 test tube, which was immersed in an ice-water bath at 0° whilst rotary evaporation was carried out, first with a water pump for 1 min and then with an oil pump for 0.5 min. The vacuum measured during the evaporation was 10 torr. Initial use of the oil pump caused bumping. Preliminary experiments are advisable[7]. The sample was then chromatographed.

A total of 100 μl of the original solution diluted × 100 was then applied to the precolumn for chromatography. Since the total capacity of the precolumn was 30–40 μl the sample was applied by means of three separate applications followed by solvent evaporation each time.

TABLE I

PEAK AREAS FOR DODECANOIC, TRIDECANOIC AND TETRADECANOIC ACID METHYL ESTERS RELATIVE TO HEXADECANOIC ACID METHYL ESTER TAKEN AS 1.0

Gas chromatography conditions as in Fig. 2. A = 6 replicates, 1 μl test solution injected through septum with Hamilton microsyringe (as in Fig. 2a); B = 6 replicates, 30 μl test solution diluted × 30 applied to precolumn and solvent evaporated (as in Fig. 2c); C = 4 replicates, 100 μl test solution diluted × 100 applied to precolumn and solvent evaporated.

Method of injection	*Fatty acid methyl ester*	*Mean peak area*	*% coefficient of variation*
A	Dodecanoic	0.366	2.13
	Tridecanoic	0.454	1.56
	Tetradecanoic	0.675	1.45
B	Dodecanoic	0.348	1.22
	Tridecanoic	0.441	0.82
	Tetradecanoic	0.658	0.40
C	Dodecanoic	0.355	2.70
	Tridecanoic	0.448	0.84
	Tetradecanoic	0.664	0.67

Table I presents the mean peak areas obtained for dodecanoic, tridecanoic and tetradecanoic acid methyl esters relative to hexadecanoic acid methyl ester. A comparison can be made of the results obtained after injecting 1 μl, 30 μl and 100 μl of test solution. In each case the percentage coefficient of variation for each injection decreases with increased retention time, but the quantitative results are similar.

Instead of applying the sample by syringe an alternative method was tried. This consisted of lowering the precolumn into the test solution and allowing the solution to be drawn up by capillary attraction. This method was satisfactory provided that the total liquid in the tube was less than the amount needed to saturate the packing. Approximately 90% of the total sample was taken up but the exact amount taken up could not be determined.

The advantages of a method, whereby the solvent peak is greatly reduced or even eliminated entirely from the chromatogram and a large volume of test solution may be used, are seen by comparison of Figs. 2a, b and c. In Fig. 2a 1 μl of a solution

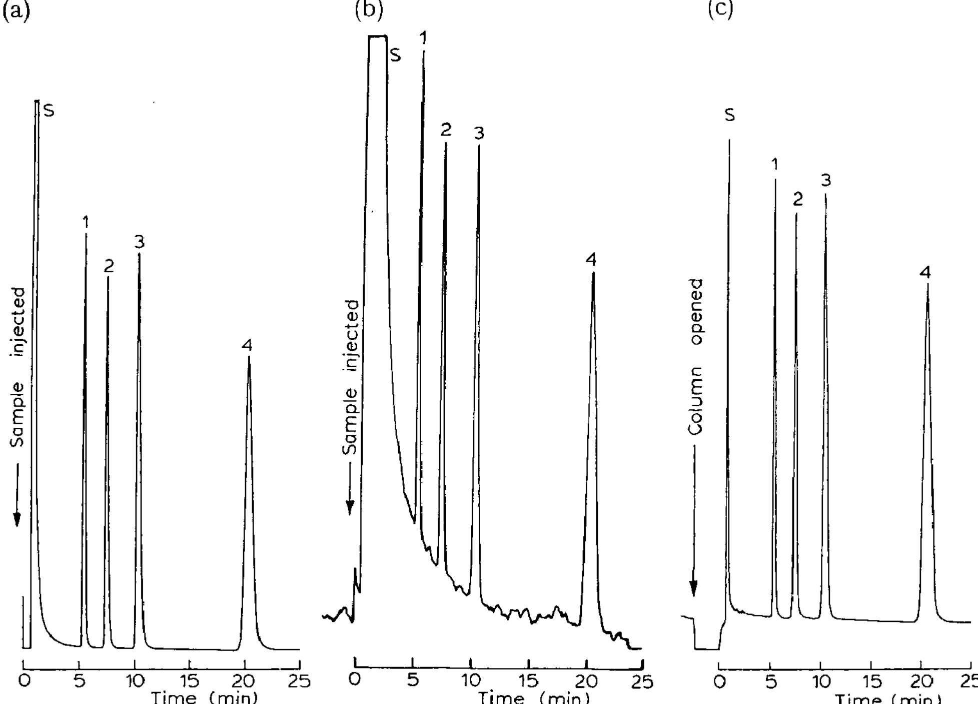

Fig. 2. (a) Gas chromatogram of a mixture of dodecanoic, tridecanoic, tetradecanoic and hexadecanoic acid methyl esters in methylene chloride. Conditions: glass column, at inlet end 10 cm × 3.2 mm I.D. joined to remainder of column 2 m × 2.5 mm I.D. packed with 10% (w/w) diethylene glycol succinate on Diatoport S 80–100 mesh; inlet heater block temperature, 190°; oven temperature, 150°; nitrogen gas flow, 30 ml/min; amplifier setting 5×10^{-10}A for f.s.d. Method of sample injection: 1 μl by Hamilton microsyringe through septum. 1 = methyl dodecanoate; 2 = methyl tridecanoate; 3 = methyl tetradecanoate; 4 = methyl hexadecanoate; S = solvent.
(b) Gas chromatogram of the same solution as in (a), except that it was diluted × 30 with methylene chloride. Conditions as in (a) with amplifier setting 20×10^{-12}A for f.s.d. Method of sample injection: 1 μl by Hamilton microsyringe through septum.
(c) Gas chromatogram of the same diluted solution as in (b). Conditions as in (a) and (b). Amplifier setting 5×10^{-10}A for f.s.d. Method of sample injection: 30 μl applied to a precolumn and the solvent removed before chromatography.

of fatty acid methyl esters in methylene chloride was injected on to the column with a microsyringe by injection through the septum. The sensitivity of the amplifier setting was attenuated to 5×10^{-10}A for full scale deflection and the ester peaks are well resolved clear of the solvent peak. In Fig. 2b 1 μl of the same sample diluted × 30 with methylene chloride was injected in the same manner with amplifier setting at 20×10^{-12}A for f.s.d. This illustrates the general problem of gas chromatography at high sensitivity. The baseline is "noisy", the solvent peak is large and interferes with the early peaks although the retention times remain the same. In Fig. 2c, 30 μl of the sample (as in Fig. 2b) was transferred to a precolumn and the solvent evaporated before chromatography. Note that the residual solvent gives a narrow peak, the size of which varies from injection to injection but does not interfere with the sample peaks. The drop in the baseline before the solvent peak is due to a temporary decrease

of nitrogen flow to the detector when the precolumn is inserted into position, but this does not interfere with the subsequent separation.

A methylene chloride solution of the lower fatty acid (octanoic, nonanoic, decanoic and dodecanoic) methyl esters was evaporated from a precolumn using temperatures from 0° to −20° with evaporation times of 1 to 4 min. Some differential losses usually occurred, ranging from about 6% octanoate down to 0–1% in the case of decanoate, so that our method cannot be recommended for these lower fatty acid methyl esters. It is successful with all the trifluoroacetylated amino acid methyl esters[7]. Although there are serious limitations to the use of the method, we believe that it could be useful in many cases where the solvent is very volatile and the test materials relatively non-volatile.

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CHROM. 4667

Preparation of volatile derivatives of amino acids on a solid support followed by direct injection into the gas chromatography column

In gas chromatography the test material is usually dissolved in a suitable solvent and an aliquot injected on to the column by means of a microsyringe. Because of the problems encountered, which become greater as the volume of the liquid injected increases, only a small portion of the total sample is usually used. The dilution of the test material by solvent often necessitates working at a high sensitivity with a high detector "noise" level. Furthermore, the solvent often interferes with those sample peaks having short retention times. In order to avoid these drawbacks, we have tried to find a system whereby a derivatization reaction can take place on a solid support, and is followed by direct injection on to the column. SAROFF[1] first proposed such a method for amino acids (see BLAU[2]). In this paper we present a method for preparing the N-trifluoroacetyl amino acid methyl ester derivatives[3,4] on a platinum support.

Apparatus

Gas chromatograph, Pye Series 104, Model 24, fitted with two flame ionization detectors (W. G. Pye Ltd., P.O. Box 60, Cambridge, Great Britain). Speedomax W, 1 mV recorder (Leeds and Northrup Ltd., Birmingham, Great Britain). Glass column, 3.2 m $\times$ 2.5 mm I.D., packed with Diatoport S, 80–100 mesh coated with 2.5% (w/w) of stationary phase (XE60–QF–1-MS200, 100 cSt in the proportions 46:27:27 (w/w) respectively[3]).

The apparatus for carrying out the derivatization was made from Rotaflo stopcock units (Scientific Supplies Co. Ltd., Vine Hill, London, Great Britain) as shown in Fig. 1.

An aqueous solution containing 2.5 mμmole of each amino acid was evaporated on to a small ball of platinum wire. This was made by winding (50 cm $\times$ 0.05 mm diameter) loosely on to a 0.5 cm length of thick platinum wire. Depending on the method of winding the liquid-holding capacity of the ball was 10–20 μl. To prevent the ball from contacting surrounding surfaces the protruding thick wire was inserted into a small block of teflon, A in Fig. 1.

Derivatization procedure

About 50 μl of dry methanolic HCl (4 mmole/ml) was placed in vessel B (see Fig. 1) and the stopcock closed. About 50 μl of freshly distilled trifluoroacetic (TFA) anhydride was placed in C and the stopcock closed. The piece of teflon with the projecting platinum was introduced into the body of the apparatus (D) by unscrewing the stopcock E. The outlet F was connected to an oil pump and the reaction vessel evacuated (1–10 torr.). Stopcock E was then closed to the side arm F.

Esterification. Stopcock B was opened and the apparatus was placed in an oven at 70° for 1 h. B was then closed and excess methanolic HCl removed by the pump through the side arm F with stopcock E open. After 10 min E was again closed with vacuum conditions in D.

Trifluoroacetylation. Stopcock C was opened and the apparatus kept at 30° for

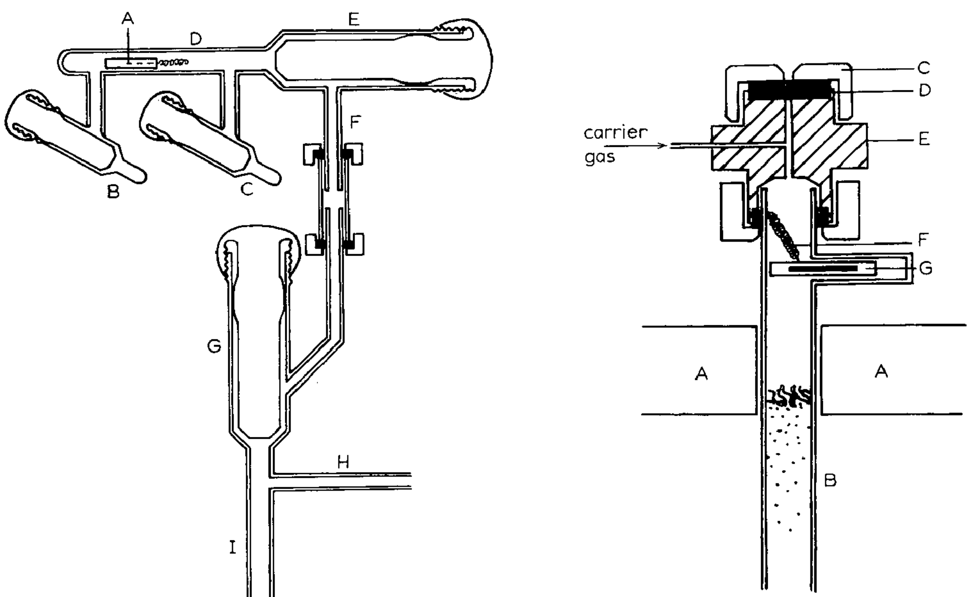

Fig. 1. Apparatus for derivatization of amino acids on a solid support. A = block of teflon supporting platinum wire; B and C = Rotaflo stopcock TF2/13 or TF2/C1/13; D = reaction vessel; E = Rotaflo stopcock TF6/18; F = side tube; G = Rotaflo stopcock TF6/18; H = carrier gas inlet; I = tube leading to inlet heater zone of gas chromatography column.

Fig. 2. Diagram of the injection head for solid injection. A = inlet heater zone; B = glass column; C = knurled nut; D = septum for conventional injection by syringe; E = inlet injection block with carrier gas inlet; F = platinum ball; G = glass covered magnet.

10 min. These conditions were satisfactory for all the amino acids, alanine to phenylalanine[3]. It was, however, necessary to maintain the apparatus at 80° for 60 min in order to obtain good derivatization of lysine, tyrosine and arginine. At the end of the reaction period stopcock C was closed.

Sample injection

Method 1. The apparatus was connected to the arm of the Rotaflo unit (G) shown in Fig. 1 by means of two back-nuts tightening on to a centre-piece at F. After opening stopcock E, the platinum ball was made to fall into tube F by tapping slightly; E was then closed. When stopcock G was unscrewed momentarily the platinum fell into the inlet heater zone of the gas chromatography column. The carrier gas flow through tube H was unaffected during this process. The Rotaflo unit (G) may be attached to the chromatography column by fittings as shown at F, or a permanent glass-to-glass junction can be made. About 8 platinum balls may be dropped in before it becomes necessary to open the column at G to extract these.

Method 2. This simpler method is shown in Fig. 2. The gas flow through the column was interrupted when the knurled nut (C) was unscrewed and the platinum was allowed to fall on to the glass-covered soft steel magnet (G). When the baseline on the recorder was re-established, sideways movement of the magnet allowed the platinum to drop into the inlet heater zone (A) of the column (B). It took about 10–15 sec to open, drop in the platinum, and reclose. One criticism of this method is that the

carrier gas is flowing past the sample in the cold and thus some of the more volatile compounds may be carried on to the column before the platinum is allowed to drop into the heated zone. In practice we found that peak shapes and HETP values were equivalent to those obtained by the conventional method of injection by syringe.

Discussion

The method of preparing the derivatives gave good peaks for all the amino acids shown in the separation, alanine to phenylalanine[3]. Methionine gave a peak height lower than expected and this may be due to an oxidative effect catalyzed by platinum. The temperature of trifluoroacetylation could not be greater than 30°, otherwise losses of the more volatile derivatives occurred due to evaporation in the reaction compartment. Some of the less volatile amino acids[3] presented some problems because of the need for more drastic trifluoroacetylating conditions. Arginine gave a high yield only when a mixture of equal volumes of TFA acid and TFA anhydride were used and some condensation was allowed to occur on the platinum before standing at 80° for 1 h. Under these conditions tryptophan gave a low yield.

The system is of use for other compounds where it is possible to carry out the derivatization in the vapour phase[5,6]. Theoretically, the total sample applied to the platinum need only be that which produces a sufficient quantity of derivative to give a response with the detector.

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Dünnschichtchromatographische Trennung von Aflatoxinen auf Kieselgel-Fertigplatten

Bei den üblichen Nachweisverfahren werden die Aflatoxine (toxische Stoffwechselprodukte von *Aspergillus flavus* und verwandten Schimmelpilzen[1]) auf Kieselgel-Platten dünnschichtchromatographisch getrennt. Für Routineuntersuchungen in kleinen Laboratorien ist die Herstellung der erforderlichen Kieselgel-Platten zu zeitraubend und kostspielig. Es wurde daher die Brauchbarkeit von fünf gebrauchsfertigen Kieselgel-Platten bei verschiedenen Steigmitteln vergleichend untersucht. Über die Verwendung solcher Platten wurde bereits von anderen Autoren[2,3] berichtet.

Material und Methodik

Verwendete Kieselgel-Fertigplatten. Die Tabelle I gibt eine kurze Beschreibung der untersuchten Fertigplatten. Die Dicke der Kieselgel-Schicht beträgt 0.2 mm (Platten 1, 2, 3, 5) und 0.25 mm (Platte 4).

Steigmittel. Folgende Steigmittel wurden verwendet: (a) Methanol–Chloroform (1.1:98.9)[2], (b) Methanol–Chloroform (1.5:98.5)[4], (c) Methanol–Chloroform (3:97)[3,5,6], (d) Methanol–Chloroform (5:95)[7,8], (e) Methanol–Chloroform (7:93)[8,9], (f) Methanol–Chloroform (9:91)[10,11], (g) Aceton–Chloroform (1:9)[12–14], (h) Benzol–Äthanol–Wasser (Oberphase) (46:35:19)[15,16], (i) Benzol–Methanol–Eisessig (48:4:2)[17], (k) Methanol–Eisessig–Benzol (5:5:90)[15], (l) Toluol–Äthylacetat–Ameisensäure (5:4:1)[17], (m) Benzol–Äthylacetat–Ameisensäure (5:4:1)[18] und (n) Äthylacetat–10% NH_4OH (Oberphase) (1:1)[18].

Durchführung der Vergleichsuntersuchungen. Auf entsprechende Zuschnitte der einzelnen Streifen wurden 0.001 ml einer Standard-Aflatoxin-Lösung (0.02‰ B_1 und 0.0038‰ G_1 in Chloroform; C. Roth, Karlsruhe, B.R.D.) aufgetragen. Die Entwicklung wurde bei 20–22° in Tanks durchgeführt, die mit Filtrierpapier ausgekleidet waren und an jedem Tag mit neuem Steigmittel beschickt wurden. Die fluoreszieren-

TABELLE I

BESCHREIBUNG DER UNTERSUCHTEN FERTIGPLATTEN

Nr.	*Hersteller*	*Bezeichnung der Platten*	*Trägermaterial*
1	Macherey, Nagel & Co., Düren (B.R.D.)	Polygram Sil N-HR	Polyterephthalsäureester
2	Eastman Kodak Co., Rochester, N.Y. (U.S.A.)	Eastman Chromagram K 301 V	Polyäthylenterephthalat
3	Riedel-de Haën A.G., Seelze (B.R.D.)	DC-Karte SI	Aluminium
4	E. Merck A.G., Darmstadt (B.R.D.)	DC-Alufolie Kieselgel (Artikel-Nr. 5553)	Aluminium
5	M. Woelm, Eschwege (B.R.D.)	DC-Folie Woelm Kieselgel	Aluminium

den Flecke wurden unter langwelligem UV-Licht (Blak-Ray, UVL-21; Hormuth-Vetter, Heidelberg, B.R.D.) lokalisiert.

Beobachtungen

Aktivierte (30 min/105°) und nicht aktivierte Platten reagierten gleichartig, weswegen im folgenden die Beobachtungen an den nicht aktivierten Platten beschrieben werden sollen.

Platten 1 und 2. Die Aflatoxine werden getrennt durch die Steigmittel (a), (b), (c), (d), (e), (f), (g) und (n), nur unzulänglich durch (h), (i), (l) und (m), überhaupt nicht durch (k). Innerhalb der Reihe (a)–(f) steigen die R_F-Werte mit zunehmendem Methanol-Gehalt.

Platten 3 und 4. Bei Einsatz der Steigmittel (c), (d), (e), (f), (h) und (n) wandern die Aflatoxine vom Startpunkt weg, erreichen allerdings nur geringe R_F-Werte und können nur als kompakte Flecke lokalisiert werden, d.h. B_1 und G_1 trennen sich nicht voneinander. Bei (a), (b), (g), (k), (l) und (m) bleiben die Aflatoxine am Auftragungsort. Das Verhalten bei (i) wurde nicht untersucht.

Platte 5. Lediglich bei Steigmittel (n) legen die Aflatoxine einen geringen Weg zurück, werden jedoch nicht aufgetrennt. Bei Verwendung der übrigen Fliessmittel bleiben die Substanzen am Startpunkt.

Diskussion

Das Trägermaterial der Kieselgel-Schicht sowie das Steigmittel beeinflussen Wanderung und Auftrennung des Aflatoxin-Gemisches. Während bei Kunststoff-Folien als Unterlage (Platten 1 und 2) die Aflatoxine wandern, bleiben sie bei Aluminiumfolien-Unterlage (Platten 3–5) meist ganz am Startpunkt zurück. Als Ursache ist hierfür wohl eine spezifische Beeinflussung des Kieselgels durch das Metallgitter des Aluminiums zu nennen, wodurch die Wanderung der Aflatoxine verhindert wird. Platte 1 besitzt einen stärkeren Trenneffekt als Platte 2, ausserdem ist auf der ersten Platte im UV-Licht der Kontrast zwischen Kieselgel und schwachen Aflatoxin-Flecken deutlicher als bei Platte 2, was wohl auf Unterschiede in den einzelnen Kieselgel-Typen beruht. Die Polygram Sil N-HR-Fertigplatte (Macherey, Nagel & Co.) kann daher zur Auftrennung von Aflatoxinen empfohlen werden.

Als Steigmittel können alle untersuchten Kombinationen von Methanol und Chloroform (a–f) verwendet werden; wegen eines gering besseren Trenneffektes ist Methanol–Chloroform (3:97) vorzuziehen. Mit diesem Gemisch konnten auf Platte 1 für Aflatoxin B_1 ein R_F-Wert von 0.49, für G_1 ein solcher von 0.40 ermittelt werden. Die ursprünglich für die Identifizierung von Ochratoxinen entwickelten Steigmittel (i), (k) und (l) sind für die Trennung von Aflatoxinen unter den vorliegenden Bedingungen unbrauchbar.

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CHROM. 4663

Specific gas chromatographic determination of amitriptyline in human urine following therapeutic doses

The tricyclic antidepressant amitriptyline is widely prescribed in the treatment of mental disease. Spectrophotometric methods for the analysis of amitriptyline in blood and urine have been described[1–3], but these do not differentiate the parent drug from its demethylated metabolite, nortriptyline. Other spectrophotometric procedures, involving prior separation of these compounds by thin-layer chromatography[4,5] are prohibitively time-consuming. Further, these methods are insensitive in the concentration range encountered after therapeutic dosage.

HUCKER AND MILLER[6] have reported the gas chromatographic separation of amitriptyline and several related tertiary amines after exhaustive methylation to the corresponding olefinic derivative. The formation of a common product by amitriptyline and nortriptyline mitigates against the application of such a procedure to biological studies.

The present paper describes a gas–liquid chromatographic procedure for the separation of several tricyclic antidepressants and its application to the determination of therapeutic levels of amitriptyline in human urine.

Experimental

Reagents. The following reagents were used: Analar petroleum spirit (40–60°) (Hopkin & Williams Ltd., Chadwell Heath, Essex) purified by re-distillation; 1 *N* sulphuric acid and 2 *N* sodium hydroxide washed with re-distilled petroleum spirit; anhydrous sodium sulphate (Analar). The internal standard was a 0.15 mg% solution of triphenylamine (Koch-Light Laboratories Ltd., Colnbrook, Bucks.) in re-distilled petroleum spirit.

Gas chromatography. A Pye 104 Model 24 dual column gas chromatograph,

equipped with a flame ionisation detector and a 1-mV Honeywell recorder, was used. The column was a 7 ft. × ¼ in. I.D. coiled glass tube, which had been silanised with a 5% solution of dimethyldichlorosilane in benzene over a period of 24 h. Glass wool was silanised in the same solution. After drying at 100°, the column was packed with 1% polyvinyl pyrrolidinone (PVP) (Varian Aerograph, Fife, Scotland) and 3% Versamid 900 (Perkin-Elmer Ltd., Beaconsfield, Bucks.) on 80–100 mesh high-performance Chromosorb W (Perkin-Elmer Ltd.). This packing was prepared as follows: 0.2 g of PVP were dissolved in 200 ml of methanol. 19.8 g of the support material were gradually added to the flask with constant swirling. The solvent was removed under vacuum in a rotary evaporator over a period of 3–4 h. 0.56 g of Versamid 900 were dissolved in 200 ml of a mixture of chloroform and methanol (87:13) and 18.1 g of the PVP-coated support gently added with occasional swirling. Again, the solvent was removed *in vacuo* over a period of 3–4 h at 40°. The prepared column was then packed with the coated support by closing one end with silanised glass-wool and applying a vacuum. After filling, the other end was closed with silanised glass-wool and the packed column conditioned at 245° for 48 h with a nitrogen flow rate of 60 ml/min. This column deteriorates if cooled in the absence of nitrogen, and the maintenance of a constant supply of the carrier gas when not in use is a wise precaution. The instrument settings were as follows: column temp., 215°; injection port temp., max; detector temp., 240°; carrier gas flow rate, 60 ml/min.

Extraction procedure. A 10-ml sample of urine was acidified by the addition of 2.0 ml of 1 *N* sulphuric acid and extracted with 10 ml petroleum spirit by shaking for 10 min in a 30-ml centrifuge tube. After centrifugation for 15 min at 3,000 r.p.m., the organic layer was discarded and the aqueous residue made alkaline (pH 11–12) with 2.0 ml of 2 *N* sodium hydroxide. The extraction was repeated using a further 10 ml of petroleum spirit and after centrifugation, 8 ml of the top organic layer was transferred to a second tube containing approximately 3 g of anhydrous sodium sulphate. On carrying out a second extraction with 10 ml of petroleum spirit, the organic fractions were bulked, thoroughly shaken with the anhydrous sodium sulphate and left to stand for 10 min. The dried extract was transferred to a third tube, the anhydrous sodium sulphate being washed with 5 ml petroleum spirit and the washings added to the extract. Evaporation was carried out under a stream of nitrogen in a 10-ml conical centrifuge tube to which 1.0 ml of the triphenylamine internal standard solution had been added, the tube being immersed in a water bath at 60°. The residue was taken up in 100 μl of dried chloroform and 5 μl of this were injected on to the gas chromatograph.

Results and discussion

Quantitation. A range of standard solutions each containing 15 μg/ml of triphenylamine and from 2 μg/ml to 20 μg/ml of amitriptyline free base were made in petroleum spirit. A standard curve was prepared by injecting 5-μl aliquots of these solutions just prior to measuring test samples. The ratio of the peak height of amitriptyline to triphenylamine was linear over the range 0.01 to 0.1 μg of amitriptyline on injection. The relative retention time of amitriptyline with respect to triphenylamine was 1.80 (Fig. 1).

Recovery studies. Amounts ranging from 0.5 μg to 2.0 μg of amitriptyline as the hydrochloride were added to 10 ml samples of blank urine to examine the efficiency of

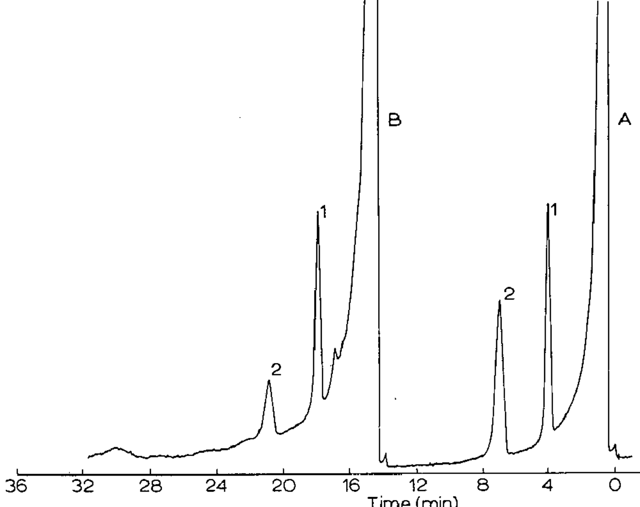

Fig. 1. (A) Chromatogram of a standard amitriptyline solution showing the separation of the internal standard triphenylamine (1) and amitriptyline (2). Attenuation, 10^2. (B) Chromatogram of a urine extract containing amitriptyline (2) with triphenylamine added (1). Attenuation, 10^2.

the extraction procedure. The mean recovery achieved was $90 \pm 7\%$.

Specificity. Amitriptyline was well separated from the other principal tricyclic antidepressants—nortriptyline, imipramine, desipramine, protriptyline and prothiadene (Fig. 2)—though trimipramine (less frequently prescribed) had the same retention time as amitriptyline on this system.

Application. Single oral doses of 50 mg of amitriptyline hydrochloride were

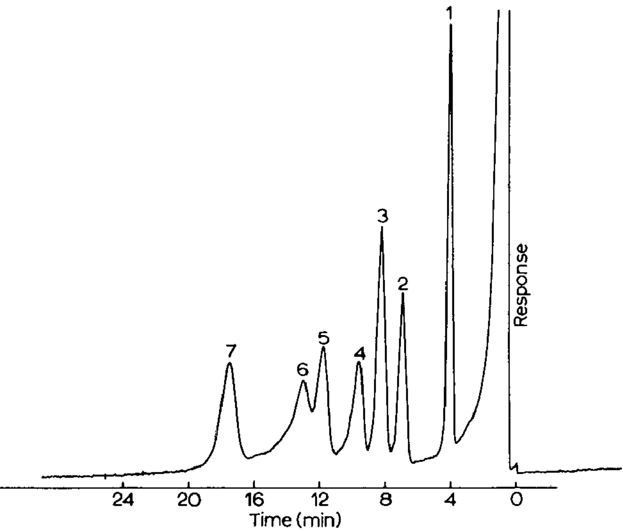

Fig. 2. Separation of triphenylamine (1), amitriptyline (2), imipramine (3), nortriptyline (4), desipramine (5), protriptyline (6), and prothiadene (7).

administered to five subjects in tablet form. Urine samples were collected at regular intervals over a period of 24 h. After measurement of the volume, the samples were stored at 4° prior to analysis.

Results and discussion. The results from five experiments are tabulated in Table I. In the subjects tested, only about 0.15% of the dose ingested appeared in the urine as free amitriptyline during the first 24 h. Amitriptyline could not be detected in the urine after this period. Previous radioactive studies[7,8] have indicated that 10 to 20% of the dose is excreted in the first 24-h urine, as a mixture of amitriptyline, nortriptyline and five other metabolites. No data on the relative amounts of the compounds excreted has been given. Other workers[4,9], using spectrophotometric analysis, have stated that approximately 1.0% of the dose is excreted as unchanged amitriptyline during the first 24 h. HUCKER[10] reported the two major metabolic reactions of amitriptyline in man to be hydroxylation and N-demethylation to nortriptyline. Working with

TABLE I

THE EXCRETION OF AMITRIPTYLINE IN THE URINE OF FIVE SUBJECTS FOLLOWING ORAL DOSES OF 50 mg AMITRIPTYLINE HYDROCHLORIDE

Subject	*Time (h)*	*Volume (ml)*	*Amitriptyline concentration (μg/ml)*	*Excreted amitriptyline (μg)*	*Total excreted in 24 h (μg)*	*% of dose excreted as unchanged amitriptyline*
M1	0	0	0.0	0	74.6	0.17
	4	764	0.028	21.4		
	8	511	0.059	30.2		
	12	113	0.099	11.2		
	16	152	0.029	4.4		
	24	295	0.025	7.4		
M2	0	0	0.0	0	23.0	0.052
	4	742	0.013	9.6		
	8	617	0.009	5.6		
	12	338	0.023	7.8		
	16	240	0.0	0		
	24	505	0.0	0		
M3	0	0	0.0	0	75.5	0.17
	4	1158	0.027	31.3		
	8	418	0.023	9.6		
	12	230	0.060	13.8		
	16	182	0.074	13.5		
	24	302	0.024	7.3		
F1	0	0	0.0	0	89.5	0.20
	4	870	0.035	30.5		
	8	253	0.091	23.0		
	12	263	0.045	11.8		
	16	176	0.063	11.1		
	24	278	0.047	13.1		
F2	0	0	0.0	0	64.4	0.15
	4	497	0.013	6.5		
	8	607	0.073	44.3		
	12	280	0.037	10.4		
	16	112	0.028	3.2		
	24	154	0.0	0		

nortriptyline, AMUNDSON AND MANTHEY[11] found that this drug appeared in very small quantities in the urine, being excreted mainly in the form of hydroxylated derivatives. The present work, in which only a small fraction of the ingested dose of amitriptyline was found in the urine as unchanged drug, was in agreement with these previous findings.

Nortriptyline was not detected in any of the samples analysed, although it should be stated that the gas chromatographic system was approximately ten times less sensitive towards this drug than towards amitriptyline. In cases of amitriptyline overdosage, however, both compounds have been readily detected and measured by this procedure.

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The quantitative separation of 3',5'-cyclic adenosine monophosphate, adenosine-5'-monophosphate, adenosine-5'-diphosphate, and adenosine-5'-triphosphate by ion-exchange chromatography on diethylaminoethyl Sephadex

During the course of an investigation into the degradation of adenine nucleotides by mammalian skin we needed a method for the quantitative separation of the various adenine nucleotides from each other that would involve a minimum of time and manipulation with a maximum of ease. The following paper describes the procedures that we have developed which partially fulfill some of these requirements.

Separations of adenine nucleotides by means of column chromatography on various ion exchangers have been previously described by BRADHAM, ÖCKERMAN, COHN[1–3] and many others. The theory of systematic variation of elution gradients has been described by BOCK and others[4,5]. Our latest methods involve an integration and modification of the above procedures. In brief, we are able to separate quantitatively, with good recoveries, 3'5'-cyclic adenosine monophosphate (CAMP), adenosine-5'-monophosphate (AMP), adenosine-5'-diphosphate (ADP), and adenosine-5'-triphosphate (ATP), by use of a concave gradient of ammonium bicarbonate (0–0.4 M), on a column of diethylaminoethyl Sephadex (DEAE-Sephadex).

Experimental

Materials. DEAE-Sephadex A-25 (medium) was obtained from the Pharmacia Company and prepared as recommended by them in the chloride form with the following exception: Fines were removed by repeated decantation of the gel after swelling but prior to the alkaline and acid wash.

The number of decantations varied but was generally greater than ten. This was done in order to prevent excessive back pressure on the column while the eluants were being pumped through at high rates of speed. The various nucleotides were obtained from Sigma Company. All solutions were made up in deionized glass distilled water.

Apparatus. The chromatography columns used were 10.5 × 250 mm glass columns fitted with a coarse sintered glass disk at the bottom. The top of the column was plugged with a No. 00 rubber stopper pierced by No. 19 stainless-steel hypodermic needle. All connections were made with intramedic polyethylene tubing (PE-100 obtained from Clay Adams Co.) attached to standard luer or luer lock connectors, with the exception of the connection between the gradient vessels, which was 0.25-in. rubber tubing. The gradient vessels were prepared by boring $\frac{1}{4}$-in. holes at the sides of the bases of nalgene graduated cylinders. The larger vessel was a 1-l cylinder with an I.D. of 62 mm. The smaller cylinder had a capacity of 500 ml and an I.D. of 46 mm. Disposable plastic polyethylene syringes (1.0 ml) were cut at the 0.6-ml mark and forced into the $\frac{1}{4}$-in. holes bored in the cylinders. The edges were sealed by use of a Sears-Roebuck hot melt glue gun. This gave inexpensive luer connectors at the base of the gradient vessels (Fig. 1). The pump used was a Milton Roy Model 196-47 metering pump, which has a maximum pumping capacity of 240 ml/h. Various fraction collectors were used but it was found that those fitted with drop counting were the

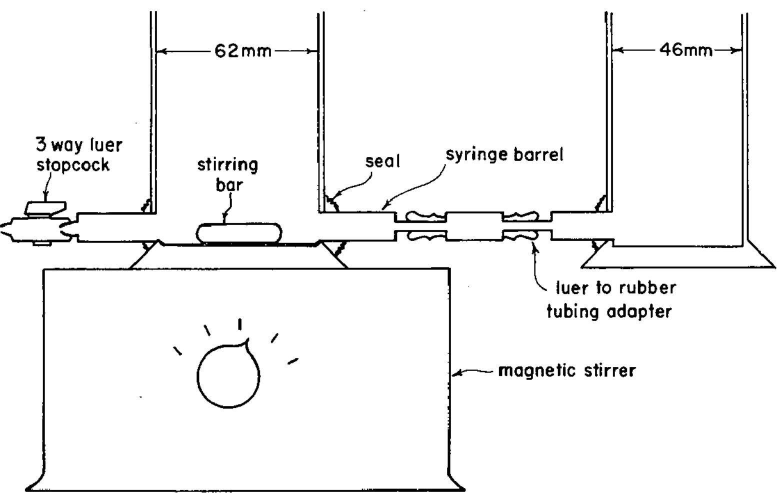

Fig. 1. A schematic representation of the gradient apparatus.

most efficient since changes in the ionic strength of the elution medium caused shrinking of the gel and this in turn varied the back pressure on the pump.

Separation procedures. Separation of the four nucleotides was accomplished as follows: Mixtures of known amounts of known nucleotides were titrated to pH 7.0 and then diluted to 5 ml. This volume was then pumped through the column four successive times, the original container being washed into the column each time. The material emerging from the column after the four successive passes essentially had no optical density at 257 mμ. The column was then washed with 100 ml of distilled water and the gradient commenced by removing the clamp from the rubber tubing connecting the two cylinders. The gradient consisted of 660 ml of distilled water in the wide cylinder and 340 ml of 0.4 M ammonium bicarbonate in the smaller cylinder. This gives an approximately 0.25-in. difference in heights of liquid, which is sufficient to offset the greater density of the ammonium bicarbonate solutions. A magnetic stirrer was placed in the larger cylinder and adjusted to constant speed. The fraction collector was set to collect 100 10-ml fractions. The pump was set at its maximum speed of 240 ml/h. The total separation procedure generally takes 4.5 h. Peaks are detected by reading optical density at 257 mμ of aliquots from each tube (generally 0.5 ml added to 2.5 ml of water in a 1.0-cm 3-ml quartz cuvette).

Determination of recoveries. Where recoveries were being determined, the same amounts of known nucleotides were added first to the titration vessel and then to a volumetric flask (250 ml) using the same pipette in the same manner. To each of the volumetric flasks 25 ml of 0.4 M ammonium bicarbonate were added and then they were made up to volume. After mixing, optical density was determined on a portion of each of the volumetric flasks and then the amounts added were calculated. After collection of fractions and determinations of peaks, each peak was carefully washed into a flask, made up to volume and the optical density of an aliquot read. The optical densities of the portions of each peak removed from the fraction collector tubes to determine the position of these peaks were added up and their sum added to the

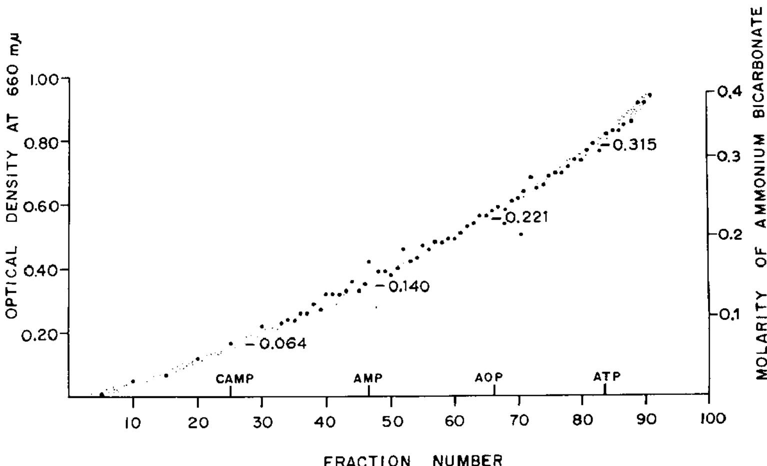

Fig. 2. The shape of the gradient, determined by the use of Methylene Blue. The average center of each peak is indicated along with the predicted ammonium bicarbonate concentration.

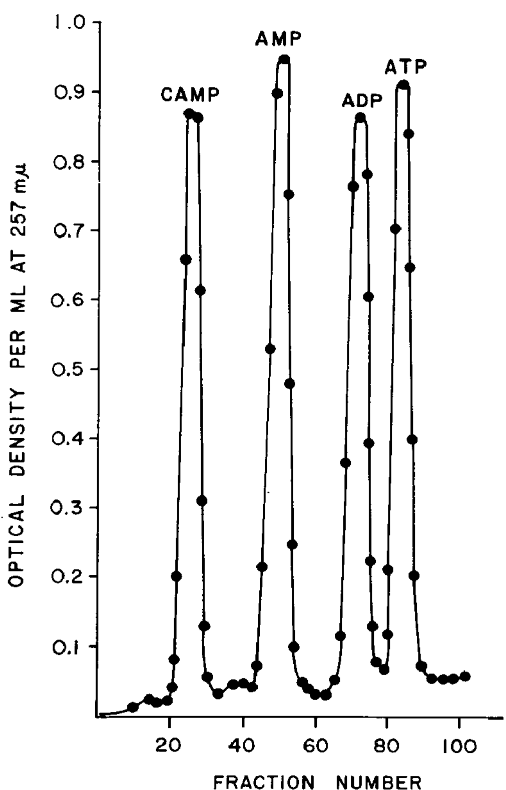

Fig. 3. A typical separation of four knowns added to the column.

recovered material, after subtraction of the column blank. The column blank was measured by doing a complete run without added nucleotides and measuring the optical density of the 100 fractions collected.

Determination of the gradient. Even though the shape of the gradient may be conveniently calculated[3,4], there is generally a deviation from the ideal due to variations in the shape of containers and volumes of connections. Therefore, we checked the shape of the gradient as follows: The gradient vessels were connected as in an experiment but the 0.4 *M* ammonium bicarbonate solution was replaced by a solution of Methylene Blue in 0.4 *M* ammonium bicarbonate having an optical density of a 1.0 at 660 mμ. The optical density of each of the recovered fractions was then read at 660 mμ in a Coleman junior spectrophotometer and the results charted as shown in Fig. 2.

Results

The results of a typical run where known amounts of known nucleotides were added to the column are shown plotted in Fig. 3. The nucleotides are eluted from the column in order of increasing acidity. It can be seen that there is a very clear separation between each of the knowns added, most especially CAMP. In the procedures that we have used the CAMP peak emerges completely in less than 1 h from the start. The columns described in the preceding section have accommodated up to 40 μmoles of each of the four nucleotides and still managed a complete separation. We have also had very little difficulty in detecting a 2-μmole peak next to a 40-μmole peak by these procedures. In general, recoveries are very good, averaging 100 $\pm$ 1%. Typical recoveries are shown in Table I. In additional experiments we added varying amounts of standard solutions of each of the four nucleotides together and determined recoveries on each individual. There was a variation of as much as 15% in these procedures with ADP and ATP and sometimes cyclic AMP being low while AMP was generally high. Our suspicions that hydrolysis of the three more labile nucleotides was

TABLE I

RECOVERY OF KNOWN ADDITIONS TO COLUMNS

The figures in the first two columns represent total absorbancy at 257 mμ.

	Amount added	*Recovery*	%
CAMP	118.0	117.0	99.1
	225.8	229.6	101.6
	88.8	88.0	99.0
	87.5	88.9	101.6
AMP	117.3	117.0	99.7
ADP	67.5	66.5	98.5
ATP	109.5	110.0	99.5
	203.3	204.7	100.6
	97.8	90.9	97.9
	95.5	95.5	100.1
	103.5	101.3	97.8

causing this situation initially was substantiated by the fact that although individual recoveries might vary, the total recovery from total material added to the column was very close to 100%.

When we added freshly made standards one at a time to the column and determined the recoveries of the various peaks these suspicions were indeed verified.

Discussion

In general, methods for the separation of CAMP involve the use of more than one column whereas methods that separate the various adenine nucleotides by a single-column procedure make no provision for the isolation of CAMP. The procedures described in the preceding pages have been used by a number of persons with varying skills and training with equally good results. Indeed, it would seem that if this procedure were used in conjunction with a 260-mμ UV analyzer then it might be possible to obtain quantitative results directly from the optical density readings of the recorder.

It is necessary to repack a column before each analysis. However, since the Sephadex is fully expanded and stored in the chloride form with all fines removed it takes less than 15 min to empty out the used material, wash, and repack completely. We also used a number of variations in the shape, concentration, and quantities of the gradient. None of them were as satisfactory as the one shown in the preceding pages. There is also the possibility that faster pumping speed might considerably shorten the time for this separation. Although we have not tried this, there is no indication that this would not be a successful variation.

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Ternary mobile phases in adsorption thin-layer chromatography

In thin-layer chromatography multi-component solvent systems are generally used as mobile phases due to their adsorptive capacity for the substances being separated. Establishing an optimal chromatography system and the separation of substances is easiest if mono- or di-component solvent systems are used. The results of investigations for both systems can be easily presented on simple diagrams, *i.e.* chromatographic spectra or curves giving the relationship R_F = f(composition of the solvent)[1-8].

The task is much more difficult with three-component solvent systems; few reports have so far dealt with this problem. They have mostly shown the relationship R_F = f(the composition of a three-component solvent system) on spatial diagrams[9]. However, diagrams of this type serve only theoretical considerations and have never been used in analytical practice. A lack of general rules does not make the choice of solvents for the system easy.

The aim of the present paper is to study the behaviour of some substances of different chemical structure during chromatography in three-component solvent systems, in addition to find an easy way of choosing optimal three-component solvent systems and presenting them on diagrams which would be available for practical use.

Experimental

Investigations were carried out by ascending TLC, using silica gel (Merck) as adsorbent. Layers of the adsorbent, 0.3 mm thick, were heated for 2 h at 135°. The following components were used as model substances: quinoline (class B according to PIMENTEL AND MCCLELLAN's classification[10]; acridine (class B); acridine orange (class B); carbazole (class AB); and fluoranthene (class N).

The spots of acridine, acridine orange and fluoranthene were detected in UV light, and those of carbazole and quinoline by spraying with H_2SO_4–$KMnO_4$ and Dragendorff's reagent, respectively[6].

The chromatograms were developed to a distance of 16 cm, using a ternary mobile phase made up of the following solvents:

1. Water (class AB)
2. Methanol (class AB)
3. Dioxane (class B)
4. Chloroform (class A)
5. Carbon tetrachloride (class N)

The organic solvents were dehydrated[8,11]. In each three-component mixture the concentration of two of the components changed steadily, the amount of the third component added being kept constant. However, the composition of the whole three-component mixture changed, as seen on the diagrams.

Measurements were made in a thermostated room at 25°, to ensure the reproducibility of the R_F values which were determined with an accuracy of ± 0.02 R_F[12-14].

Results

The results are presented on diagrams as a two-coordinate system. R_F values of

the substances studied are put on the ordinates, the concentration of three-component mobile phase on the abscissa. The concentration values of this phase (volume fractions) were put on Gibbs' triangles in which the angles correspond to the single solvents. The straight line drawn across the Gibbs' triangle shows the composition of the

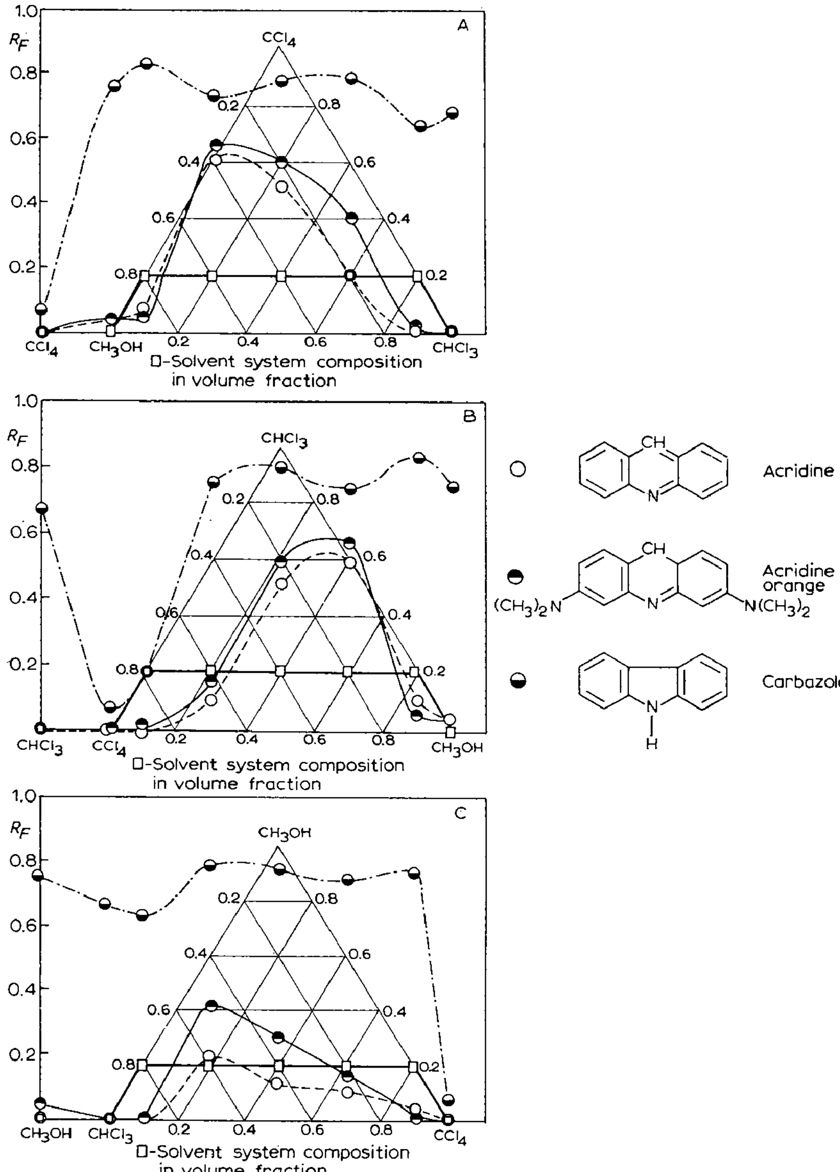

Fig. 1. R_F values of acridine, acridine orange and carbazole obtained by using methanol, chloroform and carbon tetrachloride as a mobile phase. (A) at constant amount of carbon tetrachloride in the mixture; (B) at constant amount of chloroform in the mixture; (C) at constant amount of methanol in the mixture.

three-component mobile phase. The R_F values of the substances separated were placed at points corresponding to the projection of this straight line on the ordinates.

In order to make this diagram clearer, the R_F values were put on the ordinates next to the triangle, in the case of a single solvent—on the top of the triangle.

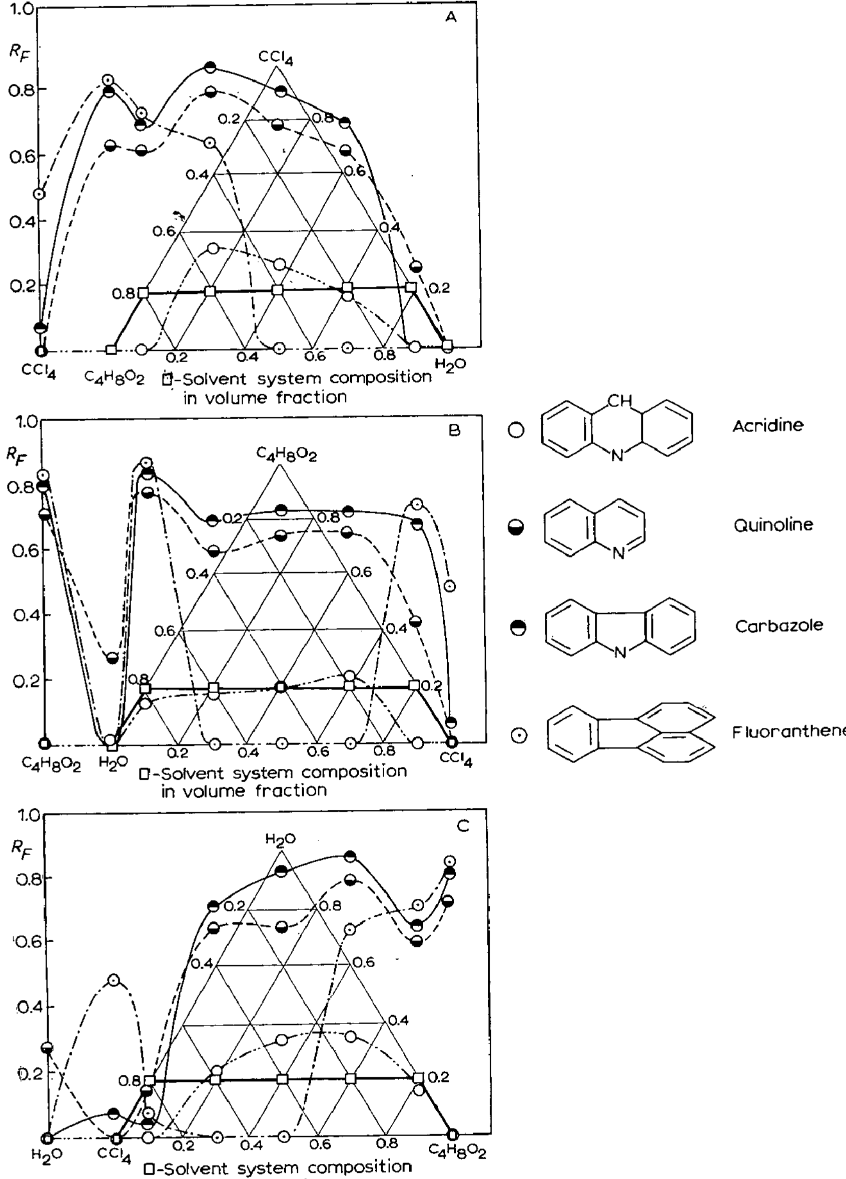

Fig. 2. R_F values of acridine, quinoline, carbazole and fluoranthene obtained by using water, ethyl acetate and carbon tetrachloride as a mobile phase. (A) at constant amount of carbon tetrachloride in the mixture; (B) at constant amount of dioxane in the mixture; (C) at constant amount of water in the mixture.

This diagram permits the determination of the relationship of R_F = f(composition of the three-component mobile phase). Diagrams of this type permit one to plan in advance the study of the conditions for the optimal separation of a mixture of substances by means of a three-component mobile phase. The above knowledge also allows the advance determination of the changes in the character of the electron-donor–acceptor properties of the three-component mobile phase, and thus their separation ability, which is necessary for finding an optimal chromatographic system.

Fig. 1 shows curves of function R_F = f(composition of three-component mobile phase carbon tetrachloride–methanol–chloroform) obtained for acridine, acridine orange and carbazole.

The diagrams show that good separation of this mixture is obtained at definite ratio of the three components making up mobile phase: chloroform–methanol–carbon tetrachloride = 0.6:0.2:0.2 (Fig. 1A) and 0.4:0.2:0.4 (Fig. 1C).

It can be seen that the separation of the components of this mixture is hardly possible in the case of a one-component mobile phase and difficult for a two-component mobile phase[8].

Fig. 2 shows to what degree the separation of acridine, quinoline, carbazole and fluoranthene is possible if systematic studies of the separation ability of three-component mobile phase are planned in advance.

The results show that in this case, also, the best separation of all the substances examined only occurred for the following composition of mobile phase: water–carbon tetrachloride–dioxane = 0.2:0.4:0.4 (Fig. 2C).

As seen in the diagram, the separation is impossible if one- or two-component mobile phases are used.

The above considerations show an easy way of presenting the results of the chromatographic separation of mixtures of substances with a three-component mobile phase, as well as of making a choice of the three-component mobile phase if its electron-donor–acceptor properties are known in advance.

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Ein Reagenz zum Nachweis von Acetylen-Derivaten auf der Dünnschichtplatte

Für den dünnschichtchromatographischen Nachweis von Acetylen-Derivaten, die in grosser Zahl aus Pflanzen isoliert wurden[1-5], aber auch als Arzneistoffe Verwendung finden[5], fehlt bisher ein Reagenz mit hoher Empfindlichkeit und hinreichender Spezifität. Von den Metallcarbonylen genügt das Dicobaltoctacarbonyl diesen Bedingungen[6]. Das nicht leicht zugängliche Reagenz ist aber nicht haltbar; zudem ist die Durchführung dieser Nachweisreaktion aufwendig.

Eine weitere Verbindung, die mit Acetylen-Verbindungen eine Farbreaktion ergibt[7], ist das 4-(4'-Nitrobenzyl)-pyridin ($O_2N-C_6H_4-CH_2-C_5H_4N$) (I), das schon seit längerer Zeit zur qualitativen und quantitativen Bestimmung von alkylierenden Stoffen verwendet wird[8-11]. Eine Lösung von I in Aceton (5%) (Kurzbezeichnung: NBP-Reagenz) gibt mit geringen Mengen eines Acetylen-Derivates nach kurzem Erwärmen auf dem Wasserbad (10 min) eine rote bis violette Färbung (λ_{max} 445–518 nm; Tabelle II)*, die nach Zugabe geringer Mengen Natronlauge (bis pH 9) eine Vertiefung erfährt. I gibt bei Abwesenheit eines Acetylen-Derivates und höherer OH-Ionen-Konzentration auch eine Rotfärbung (λ_{max} 655; 600 nm).

Die Reaktion, die auch zur quantitativen Bestimmung von Acetylen-Derivaten herangezogen werden kann[7], lässt sich auch auf der Dünnschichtplatte durchführen. Von den in die Untersuchungen einbezogenen Verbindungen (Tabelle I) geben einige diese Reaktion nicht. Für Polyacetylene, die in Arzneipflanzen vorkommen, liegen die Nachweisgrenzen bei der dünnschichtchromatographischen Analyse bei 0.1–8 γ (Tabelle II). Die Anwendbarkeit des NBP-Reagenzes wurde an mehreren Extrakten aus Pflanzen, von welchen bekannt ist, dass sie Acetylen-Verbindungen enthalten, untersucht (Tabelle III). Es liessen sich nicht alle Polyacetylene in den Dünnschichtchromatogrammen dieser Extrakte nachweisen. Darüber hinaus gaben noch einige Inhaltsstoffe eine Färbung, die sich UV-spektrometrisch nicht als Acetylen-Verbindungen identifizieren liessen. So finden sich z.B. im Dünnschichtchromatogramm eines Extraktes aus *Valeriana officinalis*, in der das Tridecen-(1)-pentain-(3,5,7,9,11) in geringen Mengen nachgewiesen wurde[12], drei Flecken, die kein für Polyacetylene charakteristisches UV-Spektrum besitzen. Extrakte aus den Handelsdrogen *Radix Pimpinellae*[13] bzw. *Herba Millefolii*[14,15] gaben Flecken, deren UV-Absorptionskurven nicht mit den der in diesen Pflanzen aufgefundenen Polyacetylene identisch sind. In *Aegopodium podagraria*[16,17] und *Anthriscus sylvestris*[18] kommen Acetylen-Derivate in so geringer Menge vor, dass sie sich im Dünnschichtchromatogramm der Extrakte nicht mit dem NBP-Reagenz nachweisen liessen; dagegen geben andere Inhaltsstoffe eine positive Reaktion.

Neben Acetylen-Derivaten wurden auch Vertreter anderer Stoffgruppen in die Untersuchungen einbezogen. Eine positive Reaktion mit dem NBP-Reagenz gaben unter anderen 4-Hydroxycumarin und Anthrachinon. Von Verbindungen, die in Pflanzen vorkommen können, zeigten die folgenden keine Farbreaktion mit dem NBP-Reagenz: Ölsäure, Chlorogensäure, Kaffeesäure, Benzaldehyd, Campfer, Thujon,

* Untersuchungen über die Natur der Farbstoffe sind noch nicht abgeschlossen.

TABELLE I

DIE MIT DEM NBP-REAGENZ UMGESETZTEN SYNTHETISCHEN UND NATÜRLICHEN ACETYLEN-VERBINDUNGEN

Verbindung	*Reaktion*	*Verbindung*	*Reaktion*
$CH_3-(C\equiv C)_5-CH=CH_2$	+	$CH_3OOC-C\equiv C-COOCH_3$	+
$H_2C=CH-(C\equiv C)_4-CH_2-CH_2-CHO$	+	$H_2N-CO-C\equiv C-CO-NH_2$	+
$CH_3-(C\equiv C)_3-(CH=CH)_2-(CH_2)_2-OOCCH_3$	+	$C_6H_5-C\equiv C-C(CH_3)(COOC_2H_5)_2$	+
$CH_3-(C\equiv C)_3-CH=CH-(CH_2)_2-CO-C_2H_5$	+	$C_6H_5-C\equiv C-J$	+
$CH_3-CH=CH-(C\equiv C)_2-CH=CH-CH(OH)-C_3H_7-n$	+	$HC\equiv C-(CH_2)_5-CH_3$	+
$CH_3-CH=CH-(C\equiv C)_2-CH(OH)-CH_2-CO-C_3H_7-n$	+	$HC\equiv C-CH_2OH$	+
$CH_3-CH=CH-(C\equiv C)_2-CH=CH-CHO$	+	$HC\equiv C-CH_2-O-$(Tetrahydropyranyl)	+
$H_3COOC-CH=CH-(C\equiv C)_2-CH=CH-COOCH_3$	+	$(CH_3)_2C(OH)-(C\equiv C)_2-C(OH)(CH_3)_2$	−
$C_6H_5-(C\equiv C)_2-C_6H_5$	+	(Cyclohexyl)(OH)$-(C\equiv C)_2-$(HO)(Cyclohexyl)	−
(Thienyl)$-(C\equiv C)_2-$(Thienyl)	+	$HOOC-C\equiv C-COOH$	−
$C_6H_5-CH(Cl)-(C\equiv C)_2-CH(Cl)-C_6H_5$	+	$HC\equiv C-(CH_2)_9-CH_3$	−
$C_6H_5-C\equiv C-CH_3$	+	$HC\equiv C-C(CH_3)_3$	−
$C_6H_5-C\equiv C-CH_2-N$(Piperidino)	+	$HC\equiv C-CH(OH)CH_3$	−
$C_6H_5-C\equiv C-CO-CH_2-CH_2-COOH$	+	$HC\equiv C-C(OH)(CH_3)_2$	−
$C_6H_5-C\equiv C-CO-C_6H_4-2-COOH$	+		

Menthol, Pulegon, β-Jonon, β-Carotin, Pulegonoxid, Santonin, Nardosinon, Cumarin, Aesculetin, Umbelliferon, Herniarin, Daphnetin, Collinin, Xanthotoxin, Imperatorin, Scopoletin, Scopolin, Carvacrol, Thymol, Flavon, Luteolin, Quercetin, Anthron, Dihydroquercetin, Rutin, Apigenin-7-glucosid, Kämpferol. Zusammenfassend ergibt sich, dass die NBP-Reaktion nur in Verbindung mit der UV-Spektroskopie für das Vorliegen eines Acetylen-Derivates beweisend ist.

Material und Methoden

Extraktion des Pflanzenmaterials. 100 g getrocknetes (bzw. 250 g frisches) Pflanzenmaterial wurden bei Raumtemperatur zweimal je zwei Tage mit Äther–Petroläther (Merck, Sdp. bis 40°) (1:1) ausgezogen. Die vereinigten Extrakte wurden filtriert und anschliessend im Wasserbad (40–50°) auf 25 ml eingeengt. Die über

TABELLE II

DIE NACHWEISGRENZEN FÜR EINIGE POLYACETYLEN-VERBINDUNGEN

Verbindung	*Isoliert aus*	*Laufmittel*	*R_F-Wert*	*Färbung*	*Nachweisgrenze bei (γ)*	*λ_{max} des Farbstoffes in vitro (nm)*
$CH_3-(C\equiv C)_5-CH=CH_2$	*Arnica chamissonis*[19]	I	0.62	rotviolett	0.1	518
$CH_3-(C\equiv C)_3-(CH=CH)_2-(CH_2)_2-OOCCH_3$	*Carduus marianus*[20]	I + II	0.13	dunkelrosa	0.3	445
$CH_3-(C\equiv C)_3-CH=CH-(CH_2)_2-CO-C_2H_5$	*Tanacetum vulgare*[21]	Bzl.-Pae.-Chlf. (4:5:1)	0.26	rot	8	445
$CH_3-CH=CH-(C\equiv C)_4-CH=CH-CH(OH)-C_3H_7$-*n*	*Pimpinella magna*[13]	I	0.2	dunkelrot	4	500
$H_3COOC-CH=CH-(C\equiv C)_2-CH=CH-COOCH_3$	*Fomes officinalis*[22]	I	0.31	violett	6	500

TABELLE III

ACETYLEN-VERBINDUNGEN, DIE SICH IN DEN PETROLÄTHER-ÄTHER-EXTRAKTEN EINIGER ARZNEIPFLANZEN MIT DEM NBP-REAGENZ NACHWEISEN LASSEN

Pflanzenmaterial	*Lauf-mittel*	*R_F-Wert*	*Farbe*	*UV-Maxima des Äther-Eluats (nm)*	*Polyacetylen*[a] *bzw. Chromophor*	*Literatur*
Wurzel von Petro-selinum-Arten (Handelsdroge)	II	0.20	hellrosa	284: 267; 251; 246; 239	Falcarinol	23
		0.72	hellrosa	285; 269; 256; 241	$-CH=CH-CO-(C\equiv C)_2-CO-CH=CH-$	
Petroselinum hortense Hoffm. (nichtgetrocknete unterirdische Teile)	III	0.76	blauviolett	292; 275; 260	$-(C\equiv C)_2-CO-CH=CH-$	23
		0.92	blauviolett	292; 275; 260		
Hydrocotyle asiatica L. (getrocknete unterirdische Teile)	II	0.15	violett	284; 267; 259; 245	$-(C\equiv C)_2-CH=CH-$	24
		0.37	violett	286; 270; 231; 211		
Pimpinella anisum L. (getrocknete unterirdische Teile)	I	0.30	rosa	336; 314; 296; 280; 266; 250	Aethusin	13
Apium graveolens L. (getrocknete unterirdische Teile)	III	0.9	violett	290; 274; 260; 217	Falcarinon	23
Heracleum sphondylium L. (nichtgetrocknete unterirdische Teile)	III	0.23	violett	284; 268; 254; 240; 217	$-CH=CH-CO-(C\equiv C)_2-CO-CH=CH-$	18
		0.42	blauviolett			
		0.73	violett	284; 269; 254; 219		
Angelica archangelica L. (Handelsdroge)	III	0.33	violett			
		0.62	blauviolett	291; 275; 260	$-(C\equiv C)_2-CO-CH=CH-$	18
		0.79	violett	284; 269; 254; 219	$-CH=CH-CO-(C\equiv C)_2-CO-CH=CH-$	

Wurzel von *Carlina acaulis* L. (Handelsdroge)	II	0.63	blauviolett	256; 242; 231	Carlinaoxid	25
Wurzel von *Arnica montana* L. (Handelsdroge)	II	0.67	blauviolett	407; 377; 348; 285; 269	$-CH=CH-(C\equiv C)_5-$	26
Blütenstände von *Matricaria chamomilla* L. (Handelsdroge)	I	0.49	blau	321; 313; 268; 251; 236	$CH_3-(C\equiv C)_2-CH=$ *cis*	27
		0.60	dunkelblau	318; 264; 249; 327	*trans*	
Carduus marianus L. (getrocknete unterirdische Teile)	I + II	0.08	dunkelrot	375; 349; 325; 308	$-CH=CH-(C\equiv C)_4-$	20
		0.13	dunkelrot	348; 324; (305); 280; 268	$-(CH=CH)_3-(C\equiv C)_2-$ oder $-(CH=CH)_2-(C\equiv C)_2-CH=CH-$	
		0.20	dunkelrot	280; 266; 251	$-CH=CH-(C\equiv C)_2-$	
		0.70	rotviolett	409; 377; 352; 323; 307	$-CH=CH-(C\equiv C)_5-$	
		0.75	dunkelrot	382; 355; 334; 308; 257	$-(CH=CH)-(C\equiv C)_4-$	
		0.80	dunkelrot	390; 360; 337; 315; 287	$-CH=CH-(C\equiv C)_4-CH=CH-$	
Tanacetum vulgare L. (getrocknete unterirdische Teile)	III	0.80	violett	334; 311; 292; 249; 242	Ponticaepoxid	21

[a] Für die Zuordnung der Verbindung sind weitere Eigenschaften heranzuziehen.

Na_2SO_4 getrocknete Lösung wurde zur dünnschichtchromatographischen Analyse verwendet.

Dünnschichtchromatographie. Die Schicht bestand aus Kieselgel HF_{254} (Merck), Schichtdicke 0.25 mm. Als Laufmittel wurden (I) Petroläther (Merck, Sdp. bis 40°), (II) Petroläther–Äther (5:1) und (III) Petroläther–Essigester (3:1) verwendet. Das Reagenz war 5% NBP in Aceton.

Nach Abdecken der einen Hälfte der Platte (20 × 20 cm) wurde mit 10 ml NBP-Reagenz besprüht, unter dem Heissluftföhn getrocknet und erneut 10 ml Reagenz aufgesprüht. Die auftretende Färbung kann durch Besprühen mit NaOH (*ca.* 10^{-5} N; pH 8.5–9) vertieft werden. Auf dem nicht besprühten Teil der Platte wurde das Sorptionsmittel in Höhe der Anfärbungen mit Äther eluiert und das UV-Spektrum gemessen (Spektralphotometer DMR 21, Zeiss), das beim Vorliegen eines Polyacetylens eine charakteristische Feinstruktur besitzt.

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CHROM. 4678

New thin-layer chromatographic solvent systems for glucosinolates (mustard oil glucosides)*

WAGNER *et al.*[1] separated six glucosinolates by thin-layer chromatography (TLC) on Silica Gel G using a solvent system: *n*-butanol–*n*-propanol–acetic acid–water (3:1:1:1). However, several glucosinolates (benzyl- and 2-hydroxy-2-phenylethyl-glucosinolate, benzyl- and 2-phenylethylglucosinolate, *p*-hydroxybenzyl- and 3-methylthiopropylglucosinolate etc.) are incompletely separated by their solvent and the development time is long.

The author has developed new solvent systems which afforded a rapid and improved resolution of a number of glucosinolates. The removal of allylglucosinolate and benzylglucosinolate from developed TLC plates was also studied to determine whether glucosinolates may be recovered without decomposition.

Methods

The glucosinolates used in this investigation were isolated from plants, except 2-phenylethylglucosinolate which was synthesized in our laboratory, and *p*-hydroxy-benzylglucosinolate which was obtained from Calbiochem (Los Angeles, U.S.A.). Plates 0.25 mm (analytical) or 0.40 mm (preparative) thick were prepared using a suspension of 30 g Silica Gel G (E. Merck, Darmstadt, G.F.R.) containing 0.3 g green fluorescent indicator (M. Woelm, Eschwege, G.F.R.) in 65 ml distilled water and activated at 120° for 1 h. A 2 µl aliquot of a 0.25% (w/v) glucosinolate solution was applied to the starting line 2 cm above the bottom of the plate. The plate was developed until the solvent front was 10 cm from the origin. The developed plates were allowed to stand for 30 min at room temperature, and, in addition, the plates developed by solvent systems IV to VII were dryed for 10 min at 110°. The glucosinolates were observed as absorbing areas under UV light of short wave length (254 nm) or as yellow spots after treatment with iodine vapor.

The stability of allyl- and benzylglucosinolate during TLC and the subsequent recovery of these glucosinolates from developed plates was examined as follows. A 100 µl aliquot of a 1% (w/v) glucosinolate solution was applied to a 20 × 20 cm plate, 0.40 mm thick. After developing and drying, the silica gel area containing the glucosinolate was removed and the glucosinolates eluted with 80% ethanol. The ethanol eluate was concentrated *in vacuo* and an aliquot was analyzed by TLC (analytical plate, 0.2 mm) using solvent system I.

Results and discussion

The R_F values obtained with the solvent systems are shown in Table I. Solvent system VII was a slight modification of the system of WAGNER *et al.* Compared to their original solvent system, system VII produced a similar pattern of separation of the glucosinolates although with higher R_F values. The glucosinolates appeared as compact, well defined spots when all seven solvent systems were employed. It was possible to resolve completely glucosinolates 1, 2, 3, 4 and 9, plus any one of 5, 6 or 7

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TABLE I

R_F VALUES OF GLUCOSINOLATES

Figures show R_F values multiplied by 100 and figures in parentheses show relative R_F values to that of allylglucosinolate. Solvent systems: (I) methyl ethyl ketone–ethanol–water (9:1:2); (II) acetone–chloroform–ethanol–water (6:1:1:1); (III) acetone–chloroform–ethanol–water (6:3:3.4:3); (IV) *n*-propanol-ethyl acetate-water (7:1:2); (V) *n*-butanol–benzene–ethanol–28% ammmonium hydroxide (4:1:2:3); (VI) *n*-butanol–*n*-propanol–pyridine–water (6:1:1:2); (VII) *n*-butanol–*n*-propanol–acetic acid–water (3:1:1:2).

No.	*Glucosinolate*	*Solvent system*						
		I	*II*	*III*	*IV*	*V*	*VI*	*VII*
1	3-Methylsulfinylpropyl-	16 (0.38)	16 (0.32)	23 (0.50)	30 (0.48)	36 (0.78)	11 (0.27)	28 (0.58)
2	Methyl-	27 (0.66)	38 (0.76)	32 (0.70)	50 (0.81)	36 (0.78)	22 (0.54)	36 (0.75)
3	2-Hydroxyisopropyl-	34 (0.83)	45 (0.90)	40 (0.87)	57 (0.92)	47 (1.02)	36 (0.88)	44 (0.90)
4	Allyl-	41 (1.00)	50 (1.00)	46 (1.00)	62 (1.00)	46 (1.00)	41 (1.00)	48 (1.00)
5	3-Methylthiopropyl-	50 (1.22)	59 (1.18)	54 (1.17)	65 (1.05)	53 (1.15)	50 (1.22)	55 (1.15)
6	*p*-Hydroxybenzyl-	52 (1.27)	59 (1.18)	50 (1.09)	69 (1.11)	44 (0.96)	51 (1.24)	53 (1.10)
7	Benzyl-	53 (1.29)	59 (1.18)	54 (1.17)	69 (1.11)	54 (1.17)	52 (1.27)	58 (1.21)
8	2-Hydroxy-2-phenylethyl-	54 (1.32)	61 (1.22)	54 (1.17)	73 (1.18)	56 (1.22)	56 (1.37)	57 (1.19)
9	2-Phenylethyl-	57 (1.39)	64 (1.28)	58 (1.26)	73 (1.18)	56 (1.22)	58 (1.41)	60 (1.25)
Development time[a]		30	30	40	90	90	120	120

[a] Time required to ascend 10 cm from origin.

using solvent systems I, II, or III. Glucosinolates 5 and 6, 6 and 7, as well as 6 and 8 were separated from each other using solvent system V, whereas glucosinolates 5 and 8 were resolved using solvent system IV. Glucosinolates 5 and 7 (with solvents IV and VII) and 7 and 8 (with solvents IV and VI) were at best incompletely separated. The development time was 3–4 times shorter using solvents I–III than with solvents IV–VII. Recoveries of allyl- and benzylglucosinolates from developed TLC plates showed no decomposition of these compounds in all solvent systems except VI. Partial decomposition of both glucosinolates was observed when solvent system VI was employed as evidenced by the appearance of additional spots on the analytical chromatographic plate. The decomposition may be due to the pyridine in the system, since it has been observed that several glucosinolates react with pyridine[2]. On the other hand, the glucosinolates were not influenced by solvent system VII containing acetic acid.

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CHROM. 4642

A modified enzymatic detection method for thin-layer chromatograms of pesticides

Enzymatic detection of pesticides on thin-layer chromatograms has become a useful tool in analysis of these compounds. The work done by MENDOZA *et al.*[1,2], WINTERLIN *et al.*[3] and ACKERMANN[4-6] are examples of the evolution of this method. We wish to report a modification which has the advantages of simplicity, sensitivity and easy detectability for a great number of pesticides.

Materials and methods

Honey bees are caught and immediately frozen on solid carbon dioxide. The frozen bees keep their enzymic activity in a refrigerator at − 20° for at least one year. A number of bee heads (25–40) are macerated (Ultra Turrax or Waring Blendor) with 75 ml of iced water and filtered through a G1 glass filter. The "bee enzyme solution" obtained rapidly loses its activity and has to be used immediately after preparation.

A chromatogram of pesticides or crop/fruit extracts is run according to one of the known methods[1-7]. The pesticides on the chromatogram are oxidized with bromine vapour in the following way: A wide beaker with water in it is placed in a closed jar of *ca.* 25 × 25 × 25 cm. After half an hour, 0.1 ml of bromine is pipetted into the

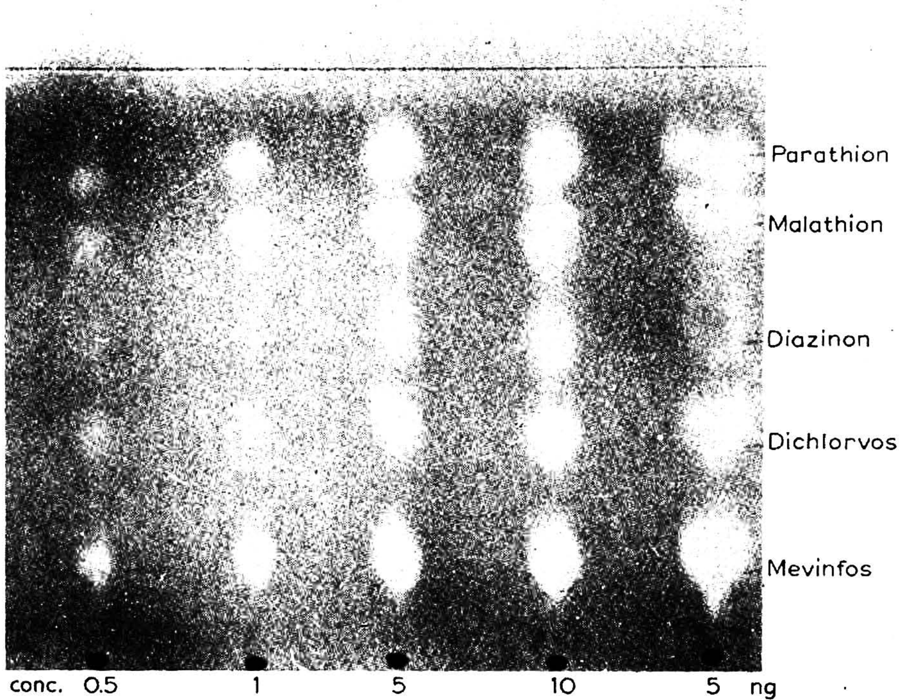

Fig. 1. Chromatogram of a mixture of five different pesticide concentrations (0.5–10 μg). Silica Gel G plate; thickness 500 μ; solvent, chloroform–ether (96:4). 5 μl of sample used except for the sample on the right hand side of the plate where 25 μl was used.

TABLE I

DETECTION OF PESTICIDES

— not detectable; ± just detectable; + detectable; ++ very easily detected.

Pesticide; common and alternative name(s)	*Chemical name*	*Sensitivity (ng)*										
		<0.1	*0.1*	*0.2*	*0.5*	*1.0*	*5.0*	*10.0*	*25.0*	*50.0*	*100.0*	*1000.0*
Azinfos-ethyl	O,O-Diethyl-S-(4-oxobenzotriazino-3-methyl) phosphorodithioate	++	++	++	++	++	++	++				
Azinfos-methyl, Guthion	O,O-Dimethyl-S-(4-oxobenzotriazino-3-methyl) phosphorodithioate	++	++	++	++	++	++	++				
Bromofos-methyl	O,O-Dimethyl-O-(2,5-dichloro-4-bromophenyl) phosphorodithioate		—	—	±	+	++	++				
Bromofos-ethyl	O,O-Diethyl-O-(2,5-dichloro-4-bromophenyl) phosphorodithioate		—	—	±	+	++	++				
Carbaryl, Sevin	N-Methyl-1-naphthyl carbamate		±	+	++	++	++	++				
Chlorphenvinfos	O,O-Diethyl-2-chloro-1-(2,4-dichlorophenyl) vinylphosphate		—	—	—	—	±	+	++			
Cidial, Phenthoat	O,O-Dimethyl-S-(1-carbethoxy-1-phenylmethyl) phosphorothioate	±	+	++	++	++	++	++				
Demeton	O,O-Diethyl-O-(2-ethylthio)ethyl phosphorothioate					—	±	+	+	++		
Demeton-methyl	O,O-Dimethyl-O-(2-ethylthio)ethyl phosphorothioate					—	±	+	++	++		
Demeton-methyl-sulphoxide	O,O-Dimethyl-S-(2-ethylsulphinyl)ethyl phosphorothioate					—	±	+	++	++		
Diazinon	O,O-Diethyl-O-(2-isopropyl-4-methyl-6-pyrimidinyl) phosphorothioate		—	—	±	+	++	++				
Dichlorvos	O,O-Dimethyl-O-2,2-dichlorovinyl phosphate		—	±	+	++	++	++				
Dimethoate, Rogor	O,O-Dimethyl-S-(N-methylcarbamoylmethyl) phosphorodithioate						—	—	—	—	—	+
Dioxathion, Delnax	2,3-*p*-Dioxanedithiol-S,S-bis-(O,O-diethylphosphorodithioate)					—	±	+	++	++		
Dithion, Dithione	O,O-Diethyl-O-7-hydroxy-3,4-teramethylene-coumarinyl phosphorothioate							—	—	—	±	+
EPN	O-Ethyl-O-(*p*-nitrophenyl)phenyl phosphorothioate	—	±	++	++	++	++	++				
Ethion, Nialate	O,O,O'O'-Tetraethyl-S,S'-methylene bisphosphorodithioate					—	—	±	+	++	++	
Folithion, Sumithion	O,O-Dimethyl-O-(3-methyl-4-nitrophenyl) phosphorothioate			—	±	+	++	++				
Hoe 2873	O,O-Diethyl-O-(6-ethoxy-carboxyl-5-methyl-pyrazolo(2,3a)pyrimidin-2-yl) phosphorothioate	—	±	++	++	++	++	++				

H 14503, Torak	O,O-Diethyl-S-(2-chloro-1-phthalimidoethyl) phosphorothioate	+	++	++	++	++	++	++				
Malathion	O,O-Dimethyl-S-(1,2-diethoxy carbamyl ethyl) phosphorodithioate		−	±	++	++	++	++				
Menazon	O,O-Dimethyl-S-(4,6-diamino-1,3,5-triazin-2-yl-methyl) phosphorodithioate		−	−	±	+	++	++				
Mevinphos, Phosdrin	O,O-Dimethyl-O-2-methoxycarbonyl-1-methylvinyl phosphate		±	+	++	++	++	++				
Nemafos, Zinophos	O,O-Diethyl-O-2-pyrazinyl phosphorothioate		±	++	++	++	++	++				
Parathion-ethyl	O,O-Diethyl-O-*p*-nitrophenyl phosphorothioate		−	±	++	++	++	++				
Parathion-methyl	O,O-Dimethyl-O-*p*-nitrophenyl phosphorothioate		−	±	++	++	++	++				
Phenkapton	O,O-Diethyl-S-(2,5-dichlorophenylthiomethyl) phosphorothiolothionate		−	−	±	+	++	++				
Phosalone	O,O-Diethyl-S-(6-chloro-2-oxo-3-benzoxazolylmethyl) phosphorodithionate		−	−	±	+	++	++				
Phosphamidon, Dimecron	O,O-Dimethyl-O-2-chloro-2-diethylcarbamoyl-1-methylvinyl phosphate						−	−	−	−	±	+
Prothoaat, Fac 20	O,O-Diethyl-S-(N-isopropylcarbamoylmethyl) phosphorodithioate						−	−	−	−	−	±
Ronnel, Fenchlorvos	O,O-Dimethyl-O-2,4,5-trichlorophenyl phosphorothioate		−	−	±	+	++	++				
Sulfotep	O,O,O′,O′-tetraethyl diphosphorothioate						−	−	±	+	++	
Thimet, Phorate	O,O-Diethyl-S-(ethylthiomethyl) phosphorodithioate		−	−	−	−	±	+	++	++		
Thiometon	O,O-Diethyl-S-(2-ethylthio-ethyl) phosphorodithioate			−	−	−	±	+	+	++		
Trichlorphon, Dipterex	O,O-Dimethyl-(1-hydroxy-2,2,2-trichloroethyl phosphonate)			±	+	+	++	++				
Trithion, Carbophenothion	O,O-Diethyl-S-*p*-chlorophenylthiomethyl phosphorodithioate					−	±	+	++	++		
Ultracid, Supracid	O,O-Dimethyl-5-methoxy-(3H)-1,3,4-thiadiazol-2-onyl-(3)-methyl phosphorodithioate		±	++	++	++	++	++				
Vamidothion	O,O-Dimethyl-S-{2-1-(N-methylcarbamyl)-ethyl} phosphorothioate							−	−	−	−	+
V.C. 13 Nemacide, Dichlofenthion	O,O-Diethyl-O-2,4-dichlorophenyl phosphorothioate							−	−	−	±	+
Formothion	O,O-Dimethyl-(N-methyl-N-formyl-carbamoyl) phosphorodithioate						−	±	+	++	++	
Ortho Dibrom, Naled	O,O-Dimethyl-O-1,2-dibromo-2,2-dichloroethyl phosphate	±	+	++	++	++	++	++				

bottom of the tank, which is then closed to allow the bromine vapour to distribute itself evenly throughout the tank. The thin-layer chromatogram is then exposed to the bromine vapour in the tank for 30 sec, removed from the tank, and allowed to stand in air until all traces of bromine smell have vanished (30 min). The plate is next sprayed with the "bee enzyme solution" (15–20 ml for a 20 × 20 cm plate, thickness 500 μ), then kept in a moist atmosphere at 37° for half an hour. After this incubation period, the plate is sprayed again with a solution consisting of 20 mg of 2-naphthyl acetate in 8 ml of ethanol mixed immediately before spraying with a solution containing 50 mg Fast Blue B in 32 ml of water (8–10 ml of the spray reagent is sufficient for one plate, as described above). The plate is allowed to stand in a moist atmosphere at 37° for a further 15 min. The pesticides appear as white spots on a magenta coloured background.

Results

Table I shows the approximate sensitivities of the method for a number of pesticides.

Fig. 1 shows a chromatogram with different concentrations of five pesticides. The plate consists of Silica Gel G (thickness 500 μ). The solvent system is chloroform–ether (96:4, v/v). Chromatospots were made with 5 μl of liquid; the spot on the right hand side is made with 25 μl.

Remarks

The sensitivities were determined with chromatospots consisting of 10 μl of sample. Trichlorphon was isomerized into DDVP prior to detection[4]. The use of buffer solutions instead of iced water did not influence the sensitivity.

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Dünnschichtchromatographische Trennungen und Detektionsmethoden in der Gruppe der Carbamat- und Harnstoffherbizide

Zur Trennung von Gemischen herbizider N-Phenylcarbamate und Phenylharnstoffe wurden schon mehrfach dünnschichtchromatographische Systeme empfohlen[1-6]. Es ist aber häufig erforderlich, die Wirkstoffe auch von solchen Verbindungen zu trennen, die als Nebenprodukte in den technischen Wirkstoffen enthalten sind bzw. als Abbauprodukte bei der hydrolytischen oder thermischen Spaltung entstehen. Die bisher beschriebenen Verfahren zeigten bei unseren Versuchen keine befriedigenden Resultate. Dagegen hat sich die von uns erprobte Methode der zweifachen eindimensionalen Entwicklung des Chromatogramms mit dem gleichen Fliessmittel bei einer Reihe von Trennungen in der Gruppe der Carbamat- und Harnstoffherbizide und ihrer Metaboliten ausgezeichnet bewährt.

Beim Nachweis von Spuren der Wirkstoffe oder ihrer Umwandlungsprodukte spielt ausserdem die Nachweisempfindlichkeit der Detektionsmethoden häufig eine entscheidende Rolle. Deshalb wurden die Nachweisgrenzen bei der Anwendung verschiedener Detektionsreagenzien anhand einiger ausgewählter Wirkstoffe ermittelt und verglichen.

Trennsystem

Schicht. 0.25 mm Kieselgel G (Merck), 30 min bei 120° aktiviert und über Blaugel aufbewahrt.

Fliessmittel. Benzol–Aceton (95:5).

Trennvorgang. Nach dem Auftragen der Substanzen wird die Schicht 30 min bei Kammersättigung äquilibriert. Anschliessend wird bei 20 ± 1° zweimal aufsteigend —mit einer Zwischentrocknung—über 10 cm chromatographiert.

Die Tabelle I zeigt eine Auswahl der erzielten Trennergebnisse.

TABELLE I

R_F-WERTE VON CARBAMAT- UND HARNSTOFFHERBIZIDEN UND EINIGEN IHRER ABBAUPRODUKTE

Herbizid oder Metabolit	$R_F \times 100$
Propham	85
Anilin	68
Methoxyfenuron [N-Phenyl-N′-methoxy-N′-methylharnstoff]	47
Proximpham [O-(N-Phenylcarbamoyl)-propanonoxim]	41
N,N′-Diphenylharnstoff	23
Fenuron	6
Phenylharnstoff	0
Chlorpropham	78
3-Chloranilin	60
3,3′-Dichlor-N,N′-diphenylharnstoff	32
3,4-Dichloranilin	58
Linuron	45
3,3′,4,4′-Tetrachlor-N,N′-diphenylharnstoff	27
Diuron	12
Metobromuron	49
Chloroxuron	13

Der Nachweis von Hydrolyseprodukten der Carbamat- und Harnstoffherbizide spielt vor allem bei der Untersuchung von Wirkstoffrückständen im Boden und im Erntegut eine Rolle, wobei man dem toxikologisch bedenklichen Anilin bzw. den substituierten Anilinen besondere Aufmerksamkeit widmen muss.

Im Zusammenhang mit der gaschromatographischen Analyse der Wirkstoffe dieser Stoffklassen sind auch thermische Spaltprodukte von Bedeutung, weil es in vielen Fällen möglich ist, die schwerflüchtigen oder thermisch labilen Wirkstoffe in der gaschromatographischen Apparatur quantitativ zu zersetzen und ihre chromatographischen Spaltprodukte zu bestimmen. Dabei können als gaschromatographische Fraktionen Aniline oder die entsprechenden Phenylisocyanate auftreten[7]. Ihre dünnschichtchromatographische Unterscheidung bereitet Schwierigkeiten, weil die einander entsprechenden Aniline und Phenylisocyanate in dem oben beschriebenen sowie in anderen erprobten Trennsystemen gleiche R_F-Werte aufweisen und sich auch gegenüber den üblichen Detektionsmitteln völlig gleich verhalten.

Die Identifizierung gelingt jedoch, wenn man die Dünnschichtplatten vor oder nach der Trennung in eine Entwicklungskammer stellt, die mit konzentrierter wässriger Ammoniaklösung beschickt ist. Dabei setzen sich die Phenylisocyanate zu den Monophenylharnstoffen um, die sich von den Anilinen sowohl im Retentionsverhalten wie auch in der Reaktionsfähigkeit mit verschiedenen Detektionsmitteln deutlich unterscheiden.

Nachweismethoden

Die bisher empfohlenen Verfahren zur Detektion herbizider N-Phenylcarbamate und Phenylharnstoffe lassen sich in vier Gruppen zusammenfassen:

(1) Thermische Zersetzung der Wirkstoffe in der Dünnschicht zum Anilin und Bildung der gelben Schiffschen Basen durch Besprühen mit *p*-Dimethylaminobenzaldehyd (Verfahren nach HENKEL[1]).

(2) Hydrolytische Spaltung der Wirkstoffe zum Anilin und Bildung eines roten Azofarbstoffes mit 1-Naphthol. Die Empfindlichkeit dieses zuerst von FONO *et al.*[8] beschriebenen Verfahrens wurde von ASKEW *et al.*[3] durch die Anwendung von Jodwasserstoffsäure bei der hydrolytischen Zersetzung gesteigert.

(3) Besprühen mit Ninhydrinlösung und anschliessendes Erhitzen. Für dieses Verfahren werden von KATZ[2] und ASKEW *et al.*[3] unterschiedliche Varianten vorgeschlagen.

(4) Nachweis halogenhaltiger Verbindungen mit $AgNO_3$-Sprühreagens nach MITCHELL[9] und Bestrahlen mit UV-Licht. FINOCCHIARO UND BENSON[5] zeigten, dass sich auch die kernhalogenierten N-Phenylcarbamat- und Phenylharnstoff-Herbizide auf diese Weise sehr empfindlich detektieren lassen. Die Nachweisgrenzen liegen bei 0.1 μg.

Die drei erstgenannten Verfahren wurden an Hand einiger ausgewählter Beispiele vergleichend untersucht, indem die Nachweisgrenzen nach vorheriger Dünnschichtchromatographie in 0.25 mm-Kieselgelschichten festgestellt wurden. Als Nachweisgrenze bezeichnen wir in der Dünnschichtchromatographie die kleinste Menge der chromatographierten Verbindung, die nach der Detektion von mehreren Personen mit Sicherheit erkannt wird. Das in der Pestizid-Analytik viel gebräuchliche $AgNO_3$-Sprühreagens wurde in den Vergleich nicht mit einbezogen, weil es als Gruppen-

reagens nur für halogenhaltige Carbamat- und Harnstoffherbizide geeignet ist.

Als Detektionsmittel von universellem Nachweisvermögen für organische Verbindungen wurden Jod–Schwefelsäure und eine extrem verdünnte Lösung von Rhodamin B (Lit. 10) mit herangezogen, die den gleichzeitigen Nachweis von Wirkstoffen aus anderen Verbindungsklassen ermöglichen.

Reagenzien und ihre Anwendung

p-Dimethylaminobenzaldehyd. 1 g des Reagens wird in einem Gemisch aus 30 ml Äthanol, 3 ml konzentrierter Salzsäure und 180 ml *n*-Butanol gelöst. Die Platten werden nach der chromatographischen Trennung besprüht. Eventuell vorhandene Aniline oder Phenylisocyanate erscheinen sofort als gelbe Flecke. Anschliessend wird im Trockenschrank 30 min auf 120° erhitzt. Dabei treten die Flecke der Carbamat- und Harnstoffverbindungen hervor.

Jodwasserstoffsäure. 25 ml Jodwasserstoffsäure, Dichte 1.7, werden mit 25 ml Eisessig versetzt und mit 50 ml Wasser verdünnt.

Natriumnitrit. 5 g Natriumnitrit werden in 100 ml 0.2 *N* Salzsäure gelöst. Die Lösung wird täglich frisch bereitet.

1-*Naphthol.* 5 g 1-Naphthol werden in 100 ml Methanol gelöst. Die Lösung kann einige Tage im Kühlschrank aufbewahrt werden. Die Schicht wird mit wenig Jodwasserstoffsäure besprüht, eine Deckplatte aufgelegt und 30 min auf 120° erwärmt. Nach dem Abkühlen wird mit Natriumnitritlösung besprüht und bei mässiger Wärme getrocknet. Beim anschliessenden Aufsprühen von 1-Naphthollösung erscheinen rötliche Flecke.

Ninhydrin. 0.5 g Reagens werden in 95 ml *n*-Butanol gelöst und mit 10%iger Essigsäure auf 100 ml aufgefüllt. Nach dem Besprühen wird 10 min auf 140° erhitzt (Variante nach KATZ[2]).

Jod–Schwefelsäure. Gemisch aus gleichen Teilen einer 0.5 *N* Lösung von Jod in Aceton und einer 20%igen Schwefelsäure.

Rhodamin B. 0.005%ige wässrige Lösung.

Ergebnisse und Diskussion

Die Nachweisgrenzen von sechs ausgewählten Herbiziden in 0.25 mm Kieselgelschichten sind in der Tabelle II angegeben. Der Vergleich der Nachweisgrenzen zeigt, dass die von HENKEL[1] empfohlene Methode der thermischen Zersetzung und Kondensation zur Schiffschen Base (I) einen empfindlichen Gruppennachweis ermöglicht. Da die Durchführung einfach ist und Unsicherheiten beim Nachweis nie auftraten, wird diese Methode von uns bevorzugt.

Bei der Untersuchung von Boden- oder Pflanzenextrakten kann der Nachweis als gelbe Schiffsche Base durch gelb oder bräunlich gefärbte Coextraktivstoffe gestört werden. In solchen Fällen wendet man vorteilhaft das etwas aufwendigere Verfahren des Nachweises als Azofarbstoff an (II), bei dem rote bis rotviolette Flecke entstehen. Das Verfahren ist ebenfalls sehr empfindlich, erfordert aber für seine sichere Anwendung einige Sorgfalt. Vor dem Diazotieren sind vor allem Reste von Jod, die aus der Jodwasserstoffsäure stammen, gründlich zu entfernen.

In der Reihe III wurden die Schichten vor dem Diazotieren lediglich wie beim Verfahren I 30 min auf 120° erwärmt. Es zeigt sich, dass der Verzicht auf die Anwen-

TABELLE II

NACHWEISGRENZEN AUF 0.25 mm KIESELGELSCHICHTEN

Nachweisverfahren	*Nachweisgrenze (µg)*					
	Propham	*Chlorpropham*	*Proximpham*	*Fenuron*	*Diuron*	*Metobromuron*
I *p*-Dimethylaminobenzaldehyd; Thermische Zersetzung	0.2	0.2	0.4	0.3	0.3	0.3
II Hydrolyse mit HI; $NaNO_2$/1-Naphthol	0.1	0.1	0.2	0.2	0.4	0.2
III Thermische Zersetzung; $NaNO_2$/1-Naphthol	0.7	0.4	0.5	2.0	0.7	0.4
IV Ninhydrin	—[a]	—[a]	—[a]	0.4	1.0	1.5
V Jod–Schwefelsäure	2.0	1.0	1.0	0.2	0.3	1.0
VI Rhodamin B	0.5	0.3	3.0	4.0	2.0	1.5

[a] Mengen unter 5 µg nicht nachweisbar.

dung der Jodwasserstoffsäure die Nachweisempfindlichkeit vermindert. Ausserdem entstehen in der Nähe der Nachweisgrenze nicht immer deutliche Flecke.

Das Ninhydrin-Sprühreagens (Verfahren IV) ist den Verfahren I und II deutlich unterlegen. Hinzu kommt, dass dieses Reagens durch seine positive Reaktion mit Aminen, Aminosäuren und Aminozuckern für den Nachweis von Herbizid-Rückständen zu wenig spezifisch ist.

Bei Verwendung von Aluminiumoxid als Schichtmaterial lassen sich die Wirkstoffe nicht in allen Fällen mit der gleichen Empfindlichkeit nachweisen. So gelingt die Bildung der Azofarbstoffe nur in Verbindung mit der vorangehenden thermischen Zersetzung der Wirkstoffe, wobei die Nachweisgrenzen im Vergleich zu Kieselgelschichten etwa den zehnfachen Wert annehmen. Nach der Anwendung von Jodwasserstoffsäure versagt der Nachweis gänzlich, weil ein Teil des Jods in der Aluminiumoxidschicht so fest gebunden wird, dass es sich auch im Warmluftstrom nicht verflüchtigt und die nachfolgende Farbreaktion verhindert. Überraschenderweise führt auch die Reduktion des Jods durch Besprühen mit Thiosulfatlösung nicht zum Erfolg.

Unbefriedigende Ergebnisse werden auch mit dem Ninhydrin-Sprühreagens auf Aluminiumoxidschichten erhalten. Zum Beispiel sind bei Tageslicht erst Mengen zwischen 10 und 50 µg Fenuron als undeutliche Flecke zu erkennen. Bei Betrachtung im UV-Licht liegt die Nachweisgrenze von Fenuron bei 5 µg.

Dagegen gelingt die Detektion als Schiffsche Base auf Aluminiumoxid-Dünnschichten ebenso zuverlässig und empfindlich wie auf Kieselgel, wenn man die Schicht erst nach der thermischen Zersetzung mit dem Reagens besprüht.

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Quantitative determination of 3,4-benzopyrene in the air near gas-works retorts

In two earlier papers extracts of industrial air particulates were purified by two-dimensional thin-layer chromatography prior to examination by gas chromatography[1] or UV-absorption spectroscopy[2]. The thin-layer system described by KÖHLER *et al.*[3] was used with some minor modifications[2]. In the earlier work[1], a quantitative analysis of the 3,4-benzopyrene fraction extracted from the thin layer was attempted with the aid of an electron capture detector attached to the gas chromatograph. However, it was found that the recovery was not reproducible and varied between 60 and 95%. It was found later that losses occurred during both the purification procedure and the gas chromatographic analysis. Fluctuations were also found in the sensitivity of the electron capture detector, very likely due to imperfect purification.

In this study smaller thin-layer plates (10 × 10 cm) have been used, and this is found to reduce the losses in the purification procedure. In order to improve the reproducibility of the detector response, the heating system of the detector of the commercial gas chromatograph was modified so that a constant temperature (mostly 185°) could be maintained despite a draught in the laboratory. Glass liners inserted in the ends of the glass columns minimized decomposition due to contact with metal surfaces. Since the benzopyrene fractions extracted from the thin layers were also analyzed by absorption spectroscopy errors occurring at some steps of the analysis were easily found.

Experimental

Reagents. The following reagents were used: benzene p.a. (Merck); toluene p.a. (Merck); *n*-hexane puriss min. 99% (Kebo); methanol p.a. (Merck); anhydrous ether A.R. (Mallinckrodt); Silica Gel H according to Stahl (Merck); Cellulose Powder MN 300 Ac., acetyl content approx. 40% (Macherey, Nagel & Co.); and ethyl alcohol, 95%.

The benzene was redistilled before use; the first and last 10% of the distillate was discarded. All reagents used were frequently checked by blanks.

Apparatus. The following apparatus was used: Desaga–Stahl kit for the preparation of thin layers on 20 × 20 cm glass plates; Varian-Aerograph Model 204 gas chromatograph with 250 mCi tritium source electron capture detectors; Leeds and Northrup Speedomax H 1 mV recorders; Beckman DBG UV–visual spectrophotometer equipped with recorder S.03507H, Spectrosil semimicro cells of 1 cm path length and variable beam attenuators.

Thin-layer chromatography. An air particulate extract, as used in previous qualitative work[2], was diluted to give a 3,4-benzopyrene concentration of about 20 ng/μl.

Sufficient slurry to cover five 20 × 20 cm glass plates with a thin layer 0.25 mm thick was prepared from 25 g silica gel, 12 g cellulose acetate and 65 g 95% alcohol by stirring mechanically at about 1000 r.p.m. for 5 min. After drying in air for 10 min, the plates were heated in an oven at 120° for 30 min. The 20 × 20 cm adsorbent layers were then divided into four 10 × 10 cm sections by scraping with a stylus. Four plates with sixteen 10 × 10 cm layer sections were now treated as follows.

1.0 μl of the diluted benzene extract was applied to the outer corner of 15 of the layer sections, 15 mm from each side of the plate, to give a spot diameter of not more than 3 mm. Standard additions of 50 ng of 3,4-benzopyrene were then made to 12 of the 15 starting points. The 16th layer section was used as a blank. The four plates were now placed in a tank to run eight thin-layer sections in the first direction. After turning the plates, the remaining eight sections were run in the same tank. The second direction runs were performed in the corresponding way. The mobile phases were *n*-hexane–toluene (9:1) first direction, and ether–methanol–water (4:4:1) second direction. After completion of the runs, the thin layers were dried in air. On examination under a 254-nm UV-lamp, the fluorescent B*a*P-spots could be outlined with a stylus.

The adsorbent in the outlined areas was collected in suction tubes[2] from which the benzopyrene fractions were extracted by five successive 0.2-ml quantities of benzene[2]. The solvent was then evaporated in a stream of nitrogen and the fraction dissolved in 200 μl of benzene.

Using the same procedure three more series of thin layers were run with double the amount of particulate extract and a standard addition of 100, 200 and 400 ng of 3,4-benzopyrene, respectively. In the 400 ng series the thickness of the thin layers was increased to 0.40 mm and the fraction was dissolved in 300 μl of benzene.

Spectrophotometry. After the addition of the solvent, the test tube was gently shaken and the solution immediately transferred to the sample cell with a pipette. The scanning range was 430–350 nm and pure benzene was used as reference since no background absorption appeared when scanning the blank sample. The absorption peaks were enlarged up to five times with a scale expander. After the scan, two 5 to 7 μl portions were removed from the sample cell with two microsyringes for examination by gas chromatography. After injection, a second scan was made and then another two portions were removed.

Gas chromatography. The columns were silanized pyrex glass tubes, 1 m length, with an O.D. of 3 mm and an I.D. of 1.8 mm, and the coil has a diameter of 100 mm. The column packing was silanized Gas-Chrom P (100–120 mesh) coated with 6% QF-1. The columns were conditioned for 48 h at 225° with an inlet nitrogen pressure of 0.5 atm.

For the GLC separation of the 3,4-benzopyrene in the TLC fraction, the column temperature was set to 195° and inlet nitrogen pressure to 2.0 atm, giving a flow of about 30 ml/min. Injection and detector temperatures were 220° and 185°, respectively.

In order to prevent thermal decomposition, the steel capillaries normally joining the columns to the detectors were disconnected and the columns were connected directly to the detector sockets. For the same reason, the front ends of the columns and the injector glass liners were joined with teflon tubes.

To reduce the response variation errors, an injection of a standard was made after four sample injections. The two channels of the gas chromatograph were in this respect considered as two individual chromatographs, marked as ECD A and ECD B, respectively, *cf.* Tables I and II.

Results and discussion

When silica gel was used instead of aluminium oxide in order to reduce tailing in the first direction[1] the retention value of 2,3-(*o*-phenylene)pyrene, present in the particulate extract, coincided with that of 3,4-benzopyrene[2]. Consequently, the 3,4-

benzopyrene fraction, *i.e.* the TLC containing all the visible 3,4-benzopyrene contained also small quantities of both 3,4-benzofluoranthene and 2,3-(*o*-phenylene)-pyrene. These two contaminants are easily separated from 3,4-benzopyrene in the GLC column but must be corrected for in spectrophotometry. This is done by measuring the absorbancy of the 3,4-benzopyrene fraction at three wavelengths. If BP, BF and PP denote 3,4-benzopyrene, 3,4-benzofluoranthene and 2,3-(*o*-phenylene)pyrene and A, C and ε denote absorbance, concentration and absorptivity, the following equations are valid, according to Beer's law, for mixtures of the three polyaromatic hydrocarbons.

$$A_{\lambda n} = \varepsilon_{\lambda n}^{\mathrm{BP}} C^{\mathrm{BP}} + \varepsilon_{\lambda n}^{\mathrm{BF}} C^{\mathrm{BF}} + \varepsilon_{\lambda n}^{\mathrm{PP}} C^{\mathrm{PP}} \qquad n = 1, 2, 3.$$

Elimination of C^{BF} and C^{PP} gives

$$C^{\mathrm{BP}} = k_1 A_{\lambda 1} + k_2 A_{\lambda 2} + k_3 A_{\lambda 3}$$

TABLE I

GAS CHROMATOGRAPHIC ANALYSIS OF B*a*P; CHANNEL A

Amounts of B*a*P in the TLC fractions and deviations from mean values found by analysis by GC, ECD channel A.

TLC fraction	*Standard addition*							
	400 ng		*200 ng*		*100 ng*		*50 ng*	
	x_i *(BaP found)*	$x_i - \bar{x}$	x_i *(BaP found)*	$x_i - \bar{x}$	x_i *(BaP found)*	$x_i - \bar{x}$	x_i *(BaP found)*	$x_i - \bar{x}$
1	423	19	213	4	113	−1	60	1
	406	2	214	5	109	−5	61	2
2	410	6	208	−1	109	−5	64	5
	410	6	211	2	114	0	64	5
3	411	7	212	3	113	−1	58	−1
	402	−2	209	0	113	−1	59	0
4	403	−1	195	−14	113	−1	59	0
	375	−29	202	−7	113	−1	64	5
5	400	−4	217	8	110	−4	60	1
	409	5	216	7	113	−1	60	1
6	405	1	227	18	111	−3	56	−3
	414	10	214	5	107	−7	60	1
7	395	−9	212	3	108	−6	60	1
	406	2	202	−7	106	−8	55	−4
8	392	−12	218	9	113	−1	57	−2
	384	−20	216	7	113	−1	54	−5
9	379	−25	205	−4	130	26	58	−1
	410	6	205	−4	123	9	55	−4
10	399	−5	214	5	125	11	56	−3
	409	5	210	1	124	10	58	−1
11	401	−3	208	−1	—	—	56	−3
	405	1	207	−2	—	—	54	−5
12	418	14	192	−17	—	—	63	4
	428	24	197	−12	—	—	65	6
01	31	—	26	—	33	—	16	—
	31	—	28	—	29	—	16	—
02	29	—	28	—	31	—	15	—
	31	—	27	—	28	—	15	—
03	29	—	28	—	29	—	14	—
	26	—	28	—	27	—	14	—

where k_1, k_2 and k_3 can be calculated from ε-values. The absorptivities for 3,4-benzopyrene were determined by scanning six standard solutions, varying from 200 to 1200 ng per 200 μl. The plot of absorbance *versus* concentration showed that Beer's law was obeyed. Absorptivities for 3,4-benzofluoranthene and 2,3-(*o*-phenylene)pyrene were taken from the spectrum of each.

Thus, it was found that the amount of 3,4-benzopyrene in the TLC fraction, when dissolved in 200 μl of benzene, was given by the expression

$$1940\,(A_{\lambda 1} + 1.09\,A_{\lambda 2} - 2.21\,A_{\lambda 3})\text{ ng}$$

The wavelengths chosen were two maxima and one minimum on the 3,4-benzopyrene absorption curve at 390, 370 and 380 nm, respectively.

For GLC analysis, the amounts of standard and sample injected were such that they would not give more than 5 ng of 3,4-benzopyrene, that is about half the maximum

TABLE II

GAS CHROMATOGRAPHIC ANALYSIS OF B*a*P; CHANNEL B

Amounts of B*a*P in the TLC fractions and deviations from mean values found by analysis by GC, ECD channel B.

TLC fraction	*Standard addition*							
	400 ng		*200 ng*		*100 ng*		*50 ng*	
	x_i *(BaP found)*	$x_i - \bar{x}$	x_i *(BaP found)*	$x_i - \bar{x}$	x_i *(BaP found)*	$x_i - \bar{x}$	x_i *(BaP found)*	$x_i - \bar{x}$
1	406	24	217	16	121	1	59	−2
	422	40	189	−12	120	0	60	−1
2	390	8	199	−2	122	2	63	2
	343	−39	209	8	117	−3	64	3
3	400	18	212	11	118	−2	64	3
	386	4	202	1	106	−14	63	2
4	379	−3	218	17	119	−1	62	1
	378	−4	219	18	122	2	63	2
5	384	2	208	7	118	−2	63	2
	325	−57	203	2	117	−3	63	2
6	398	16	196	−5	125	5	63	2
	376	−6	197	−4	126	6	62	1
7	389	7	190	−11	117	−3	60	−1
	368	−14	178	−23	107	−13	56	−5
8	422	40	206	5	116	−4	58	−3
	401	19	200	−1	119	−1	59	−2
9	386	4	204	3	126	6	62	1
	346	−36	186	−15	130	10	60	−1
10	383	1	204	3	129	9	61	0
	359	−23	194	−7	127	7	60	−1
11	386	4	197	−4	—	—	59	−2
	386	4	200	−1	—	—	59	−2
12	374	−8	204	3	—	—	64	3
	389	7	188	−13	—	—	62	1
01	34	—	31	—	34	—	19	—
	36	—	31	—	32	—	18	—
02	33	—	29	—	32	—	16	—
	33	—	26	—	32	—	18	—
03	30	—	34	—	31	—	19	—
	31	—	31	—	28	—	17	—

TABLE III

SPECTROPHOTOMETRIC DETERMINATION OF BaP

Values for $A = A\lambda_1 + 1.09\ A\lambda_2 - 2.21\ A\lambda_3$ for the TLC fractions and deviations from the mean values found by absorption spectroscopy.

TLC fraction	*Standard addition*							
	400 ng		*200 ng*		*100 ng*		*50 ng*	
	$A_i \cdot 10^4$	$(A_i - \bar{A}) \cdot 10^4$	$A_i \cdot 10^4$	$(A_i - \bar{A}) \cdot 10^4$	$A_i \cdot 10^4$	$(A_i - \bar{A}) \cdot 10^4$	$A_i \cdot 10^4$	$(A_1 - \bar{A}) \cdot 10^4$
1	2073	21	1136	35	643	21	324	0
	2073	21	1136	35	643	21	302	−22
2	2068	16	1115	14	615	−7	328	4
	2015	−37	1119	18	626	4	327	3
3	2060	8	1098	−3	615	−7	334	10
	2093	41	1119	18	609	−13	333	9
4	2045	−7	1101	0	611	−11	324	0
	2045	−7	1134	33	626	4	317	−7
5	2039	−13	1125	24	615	−7	324	0
	2039	−13	1127	26	615	−7	322	−2
6	2039	−13	1097	−4	600	−22	328	4
	2069	17	1133	32	611	−11	328	4
7	2016	−36	1046	−55	626	4	322	−2
	2069	17	1068	−33	640	18	337	13
8	2023	−29	1112	11	600	−22	314	−10
	2051	−1	1109	8	601	−21	309	−15
9	2078	26	1075	−26	632	10	329	5
	2044	−8	1075	−26	644	22	308	−16
10	2068	16	1064	−37	629	7	323	−1
	2068	16	1109	8	622	0	344	20
11	2010	−42	1080	−21	—	—	317	−7
	2062	10	1080	−21	—	—	328	4
12	2069	17	1093	−8	—	—	335	11
	2038	−14	1068	−33	—	—	323	−1
01	167	—	150	—	162	—	92	—
	172	—	136	—	170	—	81	—
02	157	—	148	—	158	—	86	—
	160	—	154	—	158	—	83	—
03	158	—	176	—	154	—	91	—
	154	—	173	—	163	—	91	—

amount for the linear response on the ECD. When two equal standard injections showed different peak heights, the response for the sample injections inbetween were calculated by interpolation according to the number of injections after the first standard.

The amount of 3,4-benzopyrene in the TLC fractions analyzed determined with the two GLC channels and deviations from mean values are listed in Tables I and II. Table III shows the values found and deviations from mean values for $A = A_{\lambda_1} + 1.09\ A_{\lambda_2} - 2.21\ A_{\lambda_3}$. By subtracting the mean values obtained by the analysis of fractions 01, 02 and 03 from the corresponding total values, the mean recovery values were calculated. They are listed in Table IV together with their standard deviations. Thus Table IV illustrates the results of the present investigation.

It is obvious that, in determinations of 3,4-benzopyrene with the combination of TLC–GLC described, the GLC system is the major source of statistical errors. On the thin layer, some of the 3,4-benzopyrene is only eluted by the mobile phase of the

TABLE IV

COMPARISON OF GAS CHROMATOGRAPHIC AND SPECTROMETRIC DETERMINATION OF B*a*P

Mean total amounts and mean absorbances of B*a*P (and standard deviations) in the TLC fractions found by gas chromatography and absorption spectroscopy. Standard additions of B*a*P and mean recoveries are also listed.

Standard addition	*ECD A*			*ECD B*			*Absorption spectroscopy*		
ng	$\bar{x}$ *(BaP found) (ng)*	$\left(\frac{(x_i-\bar{x})^2}{n(n-1)}\right)^{\frac{1}{2}}$	*Recovery (%)*	$\bar{x}$ *(BaP found) (ng)*	$\left(\frac{(x_i-\bar{x})^2}{n(n-1)}\right)^{\frac{1}{2}}$	*Recovery (%)*	$\bar{A}$ *(absorbance found)*	$\left(\frac{(A_i-\bar{A})^2}{n(n-1)}\right)^{\frac{1}{2}}$	*Recovery (%)*
400	404	3	94 ± 1	382	5	87 ± 2	0.2052	0.0005	92 ± 1
200	209	2	91 ± 1	201	3	85 ± 2	0.1101	0.0006	92 ± 1
100	114	2	85 ± 2	120	2	89 ± 2	0.0621	0.0004	90 ± 1
50	59	1	88 ± 2	61	0.5	87 ± 1	0.0324	0.0003	92 ± 1

second direction. This effect may explain, to some degree, the systematic error of the TLC system.

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Detection of carboxylic acids on thin-layer chromatograms by their reaction with iodide–iodate and amylose

Selective detection reagents provide a useful adjunct to R_F data in identification of compounds separated by thin-layer chromatography. Although a number of reagents for visualizing carboxylic acids have been reported[1–7], interference by residual solvents often limits their sensitivity. This report describes a spray reagent composed of iodide, iodate and amylose which can be used to detect carboxylic acids on silica gel or cellulose after chromatographic separation in acid or basic systems.

Iodide is oxidized rapidly and quantitatively by iodate in the presence of acid by the reaction,

$$IO_3^- + 5I^- + 6H^+ \rightarrow 3I_2 + 3H_2O$$

NOVAK AND DLASK[8] exploited this reaction for detecting acids on paper chromatograms after development in chloroform–acetone–water–formic acid or ethanol–ammonia–water systems. Chromatograms from the acid system were dried for 16 h at room temperature, and the acids were immediately visible as brown spots after spraying with a mixture of potassium iodide, potassium iodate and starch. The papers developed in the basic system were dried for 1 h at room temperature; brown spots developed 1–2 h after spraying. Their reagent contained about 0.01% starch, equivalent to about 0.002% amylose. It has been pointed out[9] that it is the amylose content of starch which affords the characteristic intense blue color of the starch–iodide complex. The reagent described here contains 0.33% amylose. This may account for differences in the results observed. The higher concentration of amylose affords visualization of the acids as blue spots which appear almost immediately after development in either acid or basic systems.

Procedure

Test solutions of each acid were spotted in 10-μl volumes of 5 mg/ml acetone solutions 3 cm from the bottom of 20 × 20 cm Analtech Uniplates®, which consisted

of 0.25 mm layers of microcrystalline cellulose or Silica Gel G on glass. Chromatography chambers were lined on three sides with filter paper and equilibrated with the chosen solvent system 30 min before use. The most satisfactory systems were:

(I) *n*-butanol–anhydrous ethanol–conc. ammonia–water (60:60:60:15) with microcrystalline cellulose plates,

(II) ethanol–conc. ammonia (112:16) with cellulose plates, and

(III) chloroform–acetone–water–90% formic acid (90:90:5:5) with silica gel plates. The chromatograms were developed until the solvent front had ascended about 12 cm from the starting line. Those developed in systems I or II were dried at 105° for 1 h, plates developed in system III were air-dried overnight at room temperature. The separated acids were revealed as dark blue spots on a white or light blue background by spraying the plates with a freshly prepared mixture of equal volumes of 8% potassium iodide, 2% potassium iodate and 1% amylose (Mallinckrodt IndicatAR®). The presence of acid vapors in the laboratory air vitiates the detection by coloring the background.

Results and discussion

Detection of acids was satisfactory with either adsorbent developed in any of the three systems; however, chromatograms on cellulose developed in the formic acid system were streaked. A glacial acetic acid system *viz.*, benzene–methanol–acetic acid (75:18:2) gave good separations with Silica Gel G thin-layers, but a blue background was obtained after spraying even after the plates had been dried at 100° for 2 h in a vacuum oven.

The spots appeared almost immediately after spraying the plates. The background gradually turned brown on standing; however, the acid spots remained easily discernible. NOVAK AND DLASK[8] reported a minimum detection limit of 10 μg for benzoic acid after paper chromatography in the basic system, and they were unable to detect 24 μg after acid system development, ascribing this result to sublimation from the paper during drying. In contrast, we found benzoic acid easily detectable on thin

TABLE I

CHROMATOGRAPHIC RESULTS WITH ACIDS

Acid	pK_a	R_F *values*		
		System I	*System II*	*System III*
p-Aminobenzoic	4.92	0.75	0.48	0.78
Anthranilic	5.00	0.87	0.69	0.88
Benzoic	4.20	0.90	0.90	0.90
Citric	3.08	0.33	0.00	0.17
Fumaric	3.00	0.59	0.20	0.68
Mandelic	3.37	0.88	0.69	0.65
Oxalic	1.19	0.40	0.00	0.09
Phthalic	2.90	0.63	0.19	0.61
Picric[a]	0.82	0.95	0.95	0.45
Salicylic	3.00	0.89	0.78	0.89
Tartaric	2.96	0.45	0.08	0.12

[a] Not detected with the spray reagent.

layers in all three systems. The detection limit using system I was determined to be about 2 μg for a 50-μl spot application. Table I shows that benzoic acid exhibits an R_F value of about 0.9 in all three systems, thus one would expect that less diffuse spots of acids with lower mobility and comparable pK_a values should be detected at least as sensitively. Picric acid, the only noncarboxylic acid of the eleven in Table I and the strongest acid in the group, was the only one which was not detected at the 50-μg load. Some other noncarboxylic acids were tested by spot-test technique after application of 50 μg to Silica Gel G thin layers. Of these, saccharin (pK_a 1.62) was not detected at all, and the bisphenol, bithionol (pK_a 4.82), was barely visualized. Cyclamic and bis (2-ethylhexyl) phosphoric acid were easily detectable. Although not specific, the reagent appears to be highly selective for carboxylic acids.

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Dragendorff reactions for visualization of amino acids and amino acid derivatives separated by paper or thin-layer chromatography

Modifications of the Dragendorff reagent are being widely used for detection of alkaloids separated by paper[1] and thin-layer[2] chromatography. One of these containing ethyl acetate is of particular importance, since it has been shown that the specificity, and especially the sensitivity of the reactions of this reagent may be considerably increased in both PC and TLC[3,4] by spraying the chromatogram with dilute sulfuric acid. The advantages of this reagent and the method of sensitization have been exploited for the specific detection of certain other basic compounds which are not alkaloids, such as adenine[5], choline and betaine[6]. With betaine, similar results could also be obtained if the chromatogram was sensitized by spraying with dilute perchloric acid instead of dilute sulfuric acid.

Furthermore, in an earlier communication[7] we described a characteristic reaction of ε-N-trimethyllysine [Me_3-Lys] on thin layers with Dragendorff reagent. The results obtained with this lysine compound with its dual characteristics of an α-amino acid and a quaternary amine, have led us to study the Dragendorff reactions of amino acids and their derivatives under PC and TLC conditions in general.

Materials and methods

The samples of amino acids used and their derivatives, were commercially available, the ε-N-methylated lysines were synthesized[8,9] and several samples were donated. The chromatographic paper used was Schleicher & Schüll 2043b; the sorbents were cellulose powder MN-300 (Macherey, Nagel & Co., Düren, G.F.R.) and Silica Gel G (Merck AG, Darmstadt, G.F.R.). Solvent 1 consisted of chloroform–methanol–25% ammonia (4:4:1) and solvent 2 of *n*-butanol–glacial acetic acid–water (4:1:5).

The polychromatic ninhydrin reagent was made by dissolving 0.5 g of ninhydrin in acetone and then adding 0.05% cadmium chloride. The Dragendorff reagent containing ethyl acetate was prepared by the method described in earlier communications[3–5]. For PC, the reagent was diluted to twice its initial volume, and for TLC it was diluted 4–6 times.

After spraying with the polychromatic ninhydrin reagent, the thin-layer plates were heated at 90° for 10 min and allowed to stand at room temperature for 12 h. After spraying with the Dragendorff reagent, the papers or the cellulose layers were dried and then sprayed with 0.1 N H_2SO_4 or $HClO_4$. When using silica gel 1.0 N H_2SO_4 or $HClO_4$ was used.

Results and discussion

The R_F values of the amino acids and their derivatives studied under various TLC conditions are given in Table I together with their reaction to the ninhydrin and modified Dragendorff reagent. It appears from this table that only a few of the amino acids or their derivatives give a positive Dragendorff reaction and that many of these are uncertain and short-lived. Those amino acids and their derivatives which give lasting and even specific reactions with the ethyl acetate-containing Dragendorff

TABLE I

COLOR REACTIONS AND $R_F \times 100$ VALUES OF AMINO ACIDS AND THEIR DERIVATIVES

Sorbents: KG = Silica Gel G (Merck); MN-300 = MN-cellulose powder 300 (Macherey, Nagel & Co). Solvent: chloroform–methanol–25% ammonia (4:4:1).

Amino acids and derivatives	*Color reaction*		$R_F \times 100$ *value*[a]	
	Ninhydrin	*Dragendorff + dil. acid*	*KG*	*MN-300*
Aliphatic monoamino acids				
Glycine[c]	+	—	47	33
Sarcosine[l]	+	—	40	38
L(+)-Alanine[c]	+	—	51	51
β-Alanine[c]	+	—	41	31
L-Serine[i]	+	—	42	32
DL-Phosphoserine[c]	+	—		0
DL-α-Amino-*n*-butyric acid[c]	+	—	82	51
α-Amino-isobutyric acid[c]	+	—		57
L-Threonine[i]	+	—	57	47
DL-β-Amino-isobutyric acid[c]	+	—	44	39
γ-Aminobutyric acid[f]	+	—	42	36
L(+)-Norvaline[i]	+	—		
L(+)-Valine[i]	+	—	68	76
L(+)-Norleucine[i]	+	—		85
L(−)-Leucine[i]	+	—	72	84
L(+)-Isoleucine[i]	+	—	70	85
L-Aspartic acid[b]	+	—	20	6
L-Asparagine hydrate[c]	+	—	43	13
L-Glutamic acid[b]	+	—	35	8
L-N-Methyl glutamic acid[k]	+	—		10
L-Glutamine[i]	+	—	50	38
Aliphatic diamino acids				
β,γ-Diaminopropionic acid[d]	+	—	16	11
L-α,γ-Diaminobutyric acid[d]	+	—	5	9
L(+)-Ornithine[i]	+	—	15	45
Citrulline[i]	+	—	48	37
L(+)-Lysine[k]	+	(+)	16	48
DL(+)-*allo*-δ-Hydroxylysine[c]	+	—	5	12
L-Nα-Methyllysine[l]	+	(+)	13	45
DL-Nε-Methyllysine	+	(+)	12	69
DL-Nε-Dimethyllysine	+	+	32	89
DL-Nε-Trimethyllysine	+	+	7	49
DL-α,α-Diaminopimelic acid[d]	+	—		4
S-Containing mono- and diamino acids				
L(+)-Cysteine[i]	+	—		13
L-Cysteic acid[c]	+	—	24	7
DL-*meso*-Homocysteine[c]	+	—	17	5
L-Methionine[i]	+	—	69	73
DL-Ethionine[g]	+	—	77	50
L(+)-*meso*-Lanthionine[c]	+	—	82	
L(−)-Cystine[i]	+	—	2	10
DL-*allo*-Cystathionine[c]	+	—	4	2
L-Djenkolic acid[c]	+	—	25	5
Aromatic monoamino acids				
DL-Phenylglycine[d]	+	—	70	38
DL-Phenylalanine[i]	+	—	72	83
L-N-Methylphenylalanine[l]	+	—	96	83
DL-*o*-Tyrosine[i]	+	—	76	34

TABLE I *(continued)*

Amino acids and derivatives	*Color reaction*		$R_F \times 100$ *value*[a]	
	Ninhydrin	*Dragendorff + dil. acid*	*KG*	*MN-300*
m-Tyrosine[k]	+	—	64	20
L-Tyrosine[i]	+	—	63	53
L-N-Methyltyrosine	+	—		50
DL-3,4-Dihydroxyphenylalanine[c]	+	—	4	10
β-Phenylserine[h]	+	—	63	27
N-Hetero amino and imino acids				
L-2-Azetidine-carboxylic acid[c]	+	—	30	30
L(—)-Proline[i]	+	—	43	64
L-4-Hydroxyproline[c]	+	—	49	43
DL-Pipecolic acid[c]	+	—	33	49
L-Tryptophan[i]	+	—	69	64
DL-5-Methyltryptophan[d]	+	—	90	54
DL-6-Methyltryptophan[d]	+	—	90	52
L(—)-Histidine[i]	+	(+)	56	54
L-1-Methylhistidine[c]	+	+	80	66
L-3-Methylhistidine[c]	+	+	66	70
L-2-Thiohistidine[c]	+	+	73	13
Ergothioneine[j]	—	+		46
L-Carnosine[c]	+	+	26	15
Guanidine derivatives of α-amino acids				
Glycocyanine[d]	—	—		
Creatine[k]	—	—		
L-Arginine[i]	+	(+)	2	22
L-Nα-Methylarginine[l]	+	(+)	16	34
L-Homoarginine[e]	+	(+)	14	19
L-Canavanine sulphate[c]	+	—	36	9
Creatinine[k]	—	+	92	72

[a] R_F values are estimated.
[b] Ajinomoto Co., Inc., Tokyo.
[c] Calbiochem, Los Angeles, U.S.A.
[d] Fluka AG, Buchs SG, Switzerland.
[e] Koch–Light Labs. Ltd., Colnbrook, Bucks., Great Britain.
[f] Kyowa Hakko Kogyo Co., Ltd., Tokyo, Japan.
[g] Mann Research Labs., New York, U.S.A.
[h] Nutritional Biochemicals Co., Cleveland, Ohio, U.S.A.
[i] Reanal Finomvegyszergyár, Budapest, Hungary.
[j] Carl Roth OHG, Karlsruhe, G.F.R.
[k] Sigma Chemical Co., St. Louis, Mo., U.S.A.
[l] Gift of Dr. MITSUO EBATA, Shionogi and Co., Ltd., Osaka, Japan.

reagent when treated with dilute sulfuric acid will be discussed in detail below. It is worth noting that, for most of the compounds, the sensitivity of the Dragendorff reagent, like that of the ninhydrin reagent, is greater on Silica Gel G than on the cellulose layer. In turn, the sensitivity on the cellulose layer is greater than that on the chromatographic paper (see Table II). The compactness of the spot is also greatest on the silica gel layer.

Lysine and its α-N and ε-N-methylated derivatives. Lysine, the three ε-N-methylated lysines and Me[α]-Lys all give a ninhydrin-positive reaction. While the intensity

TABLE II

THIN-LAYER CHROMATOGRAPHY OF AMINO ACIDS

Amino acid	*Sensitivity (μg)*			
	Ninhydrin		*Dragendorff + dil. acid*	
	MN-300	*Silica Gel G*	*MN-300*	*Silica Gel G*
L(+)-Lysine	0.4	0.03	(1.5)	(1.0)
L-$N\alpha$-Methyllysine	0.5	0.04	(1.0)	(0.7)
DL-$N\varepsilon$-Methyllysine	0.4	0.05	(0.8)	(0.7)
DL-$N\varepsilon$-Dimethyllysine	0.8	0.09	0.5	0.4
DL-$N\varepsilon$-Trimethyllysine	1.0	0.5	0.2	0.2
L(−)-Histidine	0.5	0.5	(1.5)	(0.8)
L-1-Methylhistidine	0.7	0.6	0.7	0.6
L-3-Methylhistidine	0.4	0.6	0.3	0.4
L-2-Thiohistidine	0.5	0.1	0.3	0.3
Ergothioneine	—	—	0.5	0.1
L-Carnosine	4.0	2.0	1.0	0.6
L-Arginine	0.4	0.08	(1.5)	(1.0)
L-$N\alpha$-Methylarginine	1.0	0.6	(1.2)	(0.7)
L-Homoarginine	0.4	0.2	(0.8)	(0.1)
Creatinine	—	—	0.5	0.1

of the reaction of Me^{ε}-Lys is approximately the same as that of Lys, the reaction of Me^{ε}_2-Lys, and especially of Me^{ε}_3-Lys, is significantly lower. On the other hand, with the Dragendorff reagent Me^{ε}_3-Lys gave the most characteristic and most intensive reaction, and the order of sensitivity is as follows:

$$Me^{\varepsilon}_3\text{-Lys} > Me^{\varepsilon}_2\text{-Lys} > Me^{\varepsilon}\text{-Lys} > Me^{\alpha}\text{-Lys} > \text{Lys}.$$

While Me^{ε}_3-Lys gives a characteristic betaine reaction (orange-red) with the Dragendorff reagent in visible light, the reaction of the Dragendorff reagent with Me^{ε}_2-Lys and Me^{ε}-Lys is less marked and only gives a pale yellow color. The same may be said about Me^{α}-Lys. The reaction of Lys in visible light is quite weak and hardly perceptible. After spraying with dilute sulfuric acid or perchloric acid, all three ε-N-methylated lysine derivatives give a characteristic purple color, provided that, before spraying with the Dragendorff reagent, the solvents and any traces of salts have been thoroughly removed from the layers. Sensitivities are of the order: Me^{ε}_3-Lys > Me^{ε}_2-Lys > Me^{ε}-Lys. After spraying with the dilute acid, the reaction of Me^{ε}-Lys, and especially of Lys, fades within a few minutes.

The positions of Lys and its ε-N-methylated derivatives together with other proteinogenic amino acids on a two-dimensional chromatogram are shown in Fig. 1. The modified Dragendorff reagent (together with dilute acid) may be advantageously used here for the identification of ε-N-methylated lysines on thin layers, since the other proteinogenic amino acids, with the exception of the methylhistidines, give no permanent reactions.

Methyl histidines. Both 1-Me-His and 3-Me-His give marked Dragendorff reactions which are more pronounced and lasting after spraying with acid. The color of these reactions is easily distinguishable from the colors of the ε-N-methylated Lys reactions. The R_F data in Table III show that the methylated histidines can be separated quite clearly from the methylated lysines on thin layers.

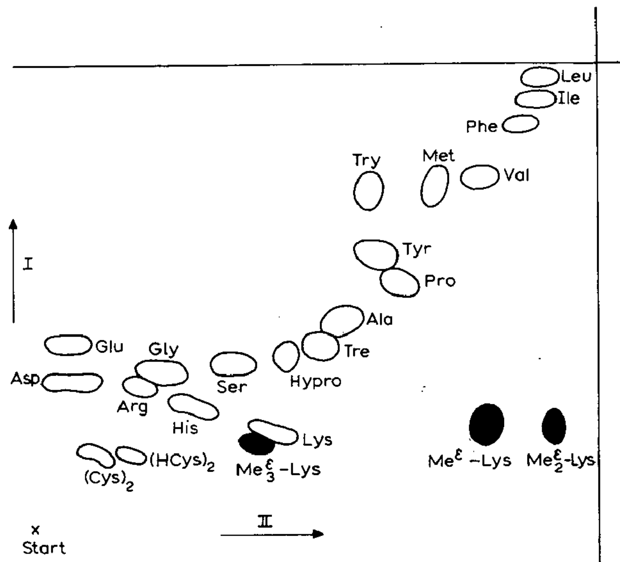

Fig. 1. Two-dimensional thin-layer chromatogram of proteinogenic amino acids. Sorbent: cellulose powder MN-300. Solvent systems: 1 = *n*-butanol–glacial acetic acid–water (4:1:5) (double development); 2 = chloroform–methanol–25% ammonia (4:4:1). White spots are ninhydrin positive; black spots are ninhydrin- and Dragendorff (plus dil. H_2SO_4)-positive.

Ergothioneine, as an α-N fully methylated histidine-like compound, cannot participate in protein formation and thus does not interfere with the examination of the components of protein hydrolysates. It is also shown in Table I that under TLC conditions this compound is easily separated from both methylated histidines and methylated lysines.

Creatine and creatinine and other α-amino acid guanidine derivatives. Me-Arg, and to a lesser degree Homo-Arg, are considered to give positive Dragendorff and Sakaguchi reactions. The Dragendorff reactions, however, are fairly weak and fade within a short time after treatment with sulfuric acid.

TABLE III

R_F × 100 VALUES OF AMINO ACIDS

Amino acid	*MN-300*		*Silica Gel G*	
	Solvent 1[a]	*Solvent 2*[b]	*Solvent 1*	*Solvent 2*
L-Lysine	57	10	14	6
DL-Nε-Methyllysine	69	15	12	5
DL-Nε-Dimethyllysine	89	13	32	4
DL-Nε-Trimethyllysine	49	6	7	3
L-Histidine	54	15	56	8
L-1-Methylhistidine	66	16	80	5
L-3-Methylhistidine	70	18	66	3

[a] Chloroform–methanol–25% ammonia (4:4:1).
[b] *n*-Butanol–glacial acetic acid–water (4:1:5).

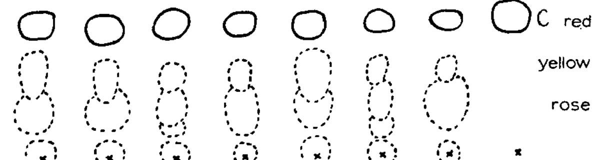

Fig. 2. Thin-layer chromatogram of several urine samples. Sorbent: Silica Gel G. Solvent: *n*-butanol–glacial acetic acid–water (4:1:5). Reagent: ethyl acetate-containing Dragendorff reagent (plus dil. H_2SO_4). C = creatinine (5 μg); 1–7 = urine samples (10 μl).

The behavior of the two guanidine derivatives, namely of creatine and creatinine, deserves special attention. Creatinine gives a characteristic reaction with the Dragendorff reagent which remains and even deepens after treatment with dilute sulfuric acid. Creatine gives no such reaction. Fig. 2 shows the thin-layer chromatograms of human urine when treated with the ethyl acetate-containing Dragendorff reagent followed by spraying with dilute sulfuric acid. 10–20 μl samples were applied. The test can be carried out without preliminary desalting, since none of the other components give this reaction. The Joffe reagent, on the other hand, reacts with the other components of urine as well[10].

Summing up, it seems that the positive Dragendorff reaction with certain amino acids and their derivatives in PC and TLC could be developed into an identification method for these substances.

The authors are grateful to Dr. MITSUO EBATA (Shionogi and Co. Ltd., Japan), Ajinomoto Co. (Tokyo, Japan), Calbiochem (Los Angeles, Calif., U.S.A.), Fluka AG (Buchs, Switzerland), Kyowa Hakko Kogyo (Tokyo, Japan), and Sigma Chemical Co. (St. Louis, Mo., U.S.A.) for several amino acid samples. We also thank Mrs. MARIA MÜLLER and Mrs. EDIT EPER for their technical assistance.

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CHROM. 4672

Solvent extraction and paper chromatography of urinary phenolic amines

Ion-exchange methods for extracting urinary phenolic amines are well known[1-4]; but the possibility of a good solvent extraction method for this category of compounds was excluded by KAKIMOTO AND ARMSTRONG[1].

WEISSBACH[5] has used *n*-butanol for the extraction of 5-hydroxytryptamine. In an attempt to adapt this method for the extraction of urinary phenolic amines, the author came across the following difficulties:

The final residue from the urine (corresponding to 20 μg creatinine) was too bulky to be applied on paper, but by using the first step of the procedure by RODNIGHT[6] it was possible to reduce the bulk without loss of phenolic amines. However, even this extract was too heavy for a good separation and it also contained a number of unwanted phenolic compounds.

The procedure to be described overcomes most of these difficulties and it is possible to obtain a good chromatogram from a urine extract corresponding to 20–25 μg creatinine. Recoveries of five phenolic amines compare well with those reported for previous ion-exchange procedures.

Experimental

Urine is acidified and hydrolysed in an autoclave[2], and then neutralised to pH 5.0 (indicator paper) using NaOH pellets and solid Na_2CO_3 and filtered. Borate buffer*, pH 10.0, is added (3 ml buffer for 7 ml filtrate)[5] to the filtrate corresponding to 20–25 μg creatinine, and the resulting solution (x ml) is then saturated with NaCl and final pH adjusted to 10.0. An equal volume (x ml) of purified *n*-butanol[5] is added to this in a 100 ml (or larger) volumetric flask and the contents are shaken for 10 min. The upper layer is transferred to a 16 × 3 cm tube (A). Another x ml of *n*-butanol are added to the volumetric flask and contents are shaken again for 5 min. The contents of the flask are poured into another 16 × 3 cm tube (B). The upper layer from tube B and the contents of tube A are transferred to the flask originally used for the extraction and 2/3 x ml of borate buffer (pH 10.0, saturated with NaCl and *n*-butanol) are added, and the flask is shaken 4 or 5 times. Part of the contents of the flask are placed in tube A and centrifuged. The upper layer is transferred to a 100-ml beaker. The flask is, then, completely emptied into tube A and after centrifugation the upper layer is again transferred to the 100-ml beaker. 5 ml *n*-butanol used to rinse the extraction flask is also transferred, similarly, to the beaker.

The extract is evaporated to dryness at 40°. The dry residue is taken up in 1 ml (or more) of 60% ethanol which should effect complete solution; the pH of this solution should be between 6 and 7 (indicator paper). To prevent damage to the amines, if pH of this solution is 8.00 or more (the pH of the extract is determined by the temperature at which the extraction with *n*-butanol is carried out), 4 to 5 ml of an 0.02% solution of KH_2PO_4 in 90% aqueous methanol should have been added to the butanol extract before it is evaporated. Acetone (11 ml for each ml of 60% ethanol added[6]) is added, in 2 to 3 ml volumes to the ethanolic solution of the residue, mixing after

* The buffer was prepared according to WEISSBACH[5], but the amount of 10 N NaOH used was only just sufficient.

each addition. The acetone extract is filtered into a 18 ml weighing bottle using a small funnel. It is advisable to carry out this step as far as possible in an enclosed tank, *e.g.* a chromatography tank, saturated with vapour from an acetone–ethanol–water (12:2:1) mixture. The residue in the beaker is once again washed with 5 ml of acetone containing 1% ethanol. The filtrate in the weighing bottle was evaporated to dryness in a small vessel, *e.g.*, a test tube 3 cm in diameter is cut 2 cm from its base and the lower segment is used for the evaporation.

Chromatography of the sample

The residue is taken up in three successive 100-μl volumes of 95% ethanol and spotted in one corner of a 35 × 35 cm sheet of Whatman No. 1 filter paper, shown as ABCD in Fig. 1. The figure illustrates the method of chromatographing the extract, which is spotted as a 2.0 × 1.5 cm spot, centre at point O. Chromatography is carried out in the direction BA (ascending) for 15 h in isopropyl alcohol–ammonia–water (8:1:1) (ref. 1) (solvent A), keeping BC as the lower edge. During this run, a visible streak STRU, starting from the spot and ending at the solvent front, is produced on the paper. The lower one fifth of this streak does not contain any phenolic amines and can safely be removed to reduce the size of the paper for subsequent runs, by cutting along GH.

Next, the paper is subjected to a short run (designated as the wash run) to remove a number of aromatic compounds from the streak STRU, without disturbing the phenolic amines. For this, a strip of filter paper (length AG and breadth 15 cm) is tacked along AG, with closely set stitches. In order to reduce the distance between the

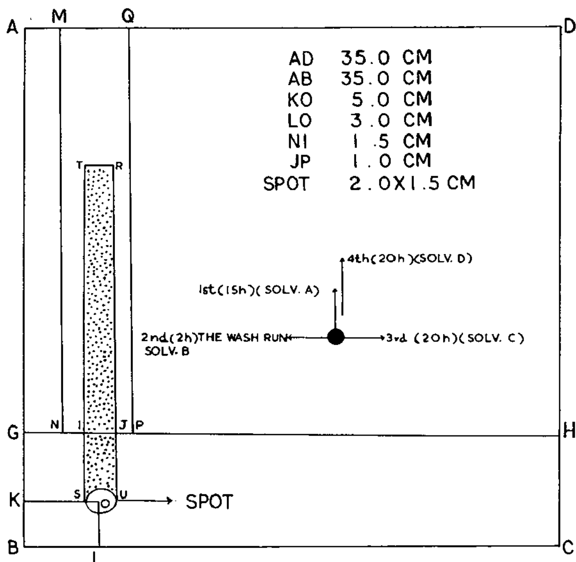

Fig. 1. Diagram illustrating the method of chromatographing the crude extract of phenolic amines. Solvent A = isopropyl alcohol–ammonia–water (8:1:1); solvent B = ether–benzene–formic acid (85%)–water (70:10:8:0.7); solvent C = isopropyl alcohol–*n*-butyl alcohol–isoamyl alcohol–formic acid–water (50:30:20:15:20); solvent D = isopropyl alcohol–*n*-butyl alcohol–*tert.*-butyl alcohol–diethylamine–water (4:2:2:1:2).

streak STRU and level of the solvent in the petri dish, for the ascending wash run, the paper is folded bringing HD on to PQ, and the double flap thus formed is again folded in the middle and the four layers of the filter paper are then stitched along PQ by six well-spaced stitches. This multi-layered side of the paper is placed in the solvent ether–benzene–formic acid (85%)–water (70:10:8:0.7) (solvent B), for the ascending wash run (2 h). The unwanted substances are washed away as streaks on the paper attached along AG, but may be obtained as discrete spots if a slightly different procedure and solvent[7] are used.

After the wash run, the paper is unfolded and cut along MN, 1.5 cm from the edge of the streak STRU, and the paper attached along AG is discarded. MN is now the lower edge of the paper for the next overnight run (20 h, in the direction MD), in the solvent mixture isopropyl alcohol–*n*-butyl alcohol–isoamyl alcohol–formic acid–water (5:3:2:1.5:2) (solvent C). The system *n*-butanol–acetic acid–water (12:3:5)[1], gives

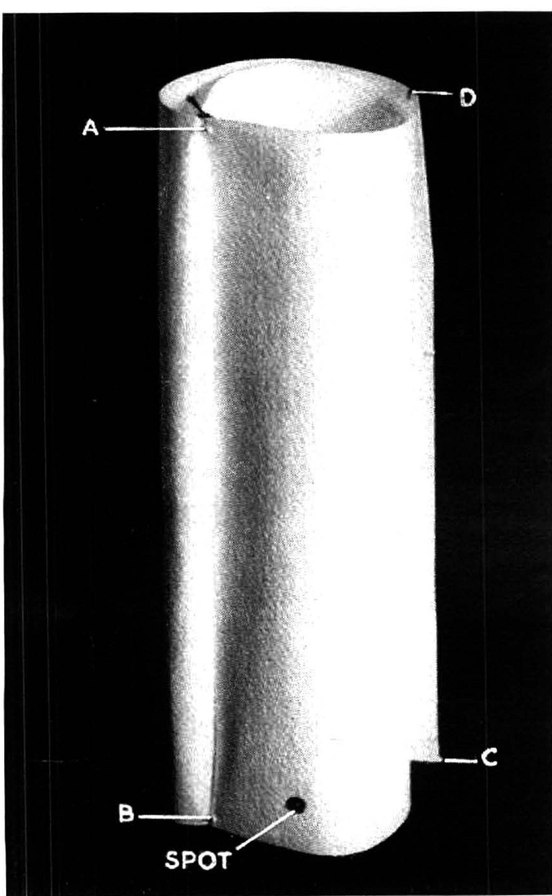

Fig. 2. The method of folding the paper for the first run. Starting with edge AB the paper is folded like a letter S, first in one direction and then in the other. The first fold is held both at its upper and lower ends, by stitches (seen in the illustration), while the second fold is held only by one stitch at the upper end (near end D of the paper). Substances migrate in a perfectly straight line when such a paper is used.

almost similar results if used instead of solvent C. After this, the paper is cut 2 in. below the solvent front line and along PQ (or 1 in. beyond it). HP is the lower edge of the paper for the final overnight run (20 h) in solvent A or in another similar solvent such as isopropyl alcohol–*n*-butyl alcohol–*tert.*-butyl alcohol–water–diethylamine (4:2:2:2:1) (solvent D). The paper is stained with *p*-nitraniline reagent[1] for studying the phenolic amine spots.

For the first ascending run, if the paper is folded as shown in Fig. 2, a 9 cm diameter petri dish will easily accommodate the 35 × 35 cm sheet of filter paper.

Results and discussion

Recoveries of *p*-tyramine, methoxytyramine, metanephrine, normetanephrine and octopamine, range between 50 to 80%, in the extraction procedure described. In the ion-exchange methods[1,2], the recoveries for the different aromatic amines lie between 60 to 90%. In our hands, recoveries with the isoamyl alcohol extraction procedure of SANDLER AND RUTHVEN[4] are lower by more than 20% as compared to those obtained by the present method.

In these recovery experiments 10 to 15 μg of each of the five phenolic amines are added to a borate buffer–water mixture (3:7) saturated with NaCl, which was then adjusted to pH 10.0. The phenolic amines are added to 20 ml and 2 ml of buffer solution, respectively, for the present procedure and for the method of SANDLER AND RUTHVEN[4]. The phenolic amines are extracted according to the respective procedures from these solutions, and the final extracts chromatographed. A standard paper is put up at the same time. After staining the papers with *p*-nitraniline reagent, the spots are quantitatively evaluated by eluting the spots (ref. 4, p. 741) with alkaline methanol and reading the coloured solutions of the metanephrine, normetanephrine and methoxytyramine spots at 540 mμ and those from *p*-tyramine and octopamine spots at 490 mμ.

Some difficulty may be encountered in spotting the extract, prepared as described in this paper, as it may not dry up easily when applied to the chromatographic paper. However, a good separation is obtained even for a spot which may have failed to dry up completely. The explanation for the good separation obtained in the procedure could be as follows. The first run (solvent A) separates phenolic amines as elongated spots because of the effect of contaminants. It, however, helps to remove phenolic amines away from the influence of the contaminants for the subsequent runs. The second wash run (solvent B) does not contribute much to compacting the spots. In the third run (solvent C) the spots are separated; their shape is elongated in the direction of the first run but compact in the direction of solvent C (see Fig. 3B). The final run, which is in the direction of the first run and in solvent A, or some other similar solvent (solvent D), helps to compact the spots in that direction (see Fig. 3A). A compacting effect as a result of multiple runs is well known but, in the present situation, the effect appears to be more pronounced. It must, however, be remembered that in the present case, the first run takes place in the presence of contaminants and the next one in their absence, while in a simple case of multiple runs no such difference is present.

Such a procedure has a scope of general applicability to the chromatography of crude samples. For a particular category of compounds, one has to know a solvent which, in the presence of contaminants, will cause comparatively less distortion of the spots and will separate contaminants from the actual compounds. A similar procedure

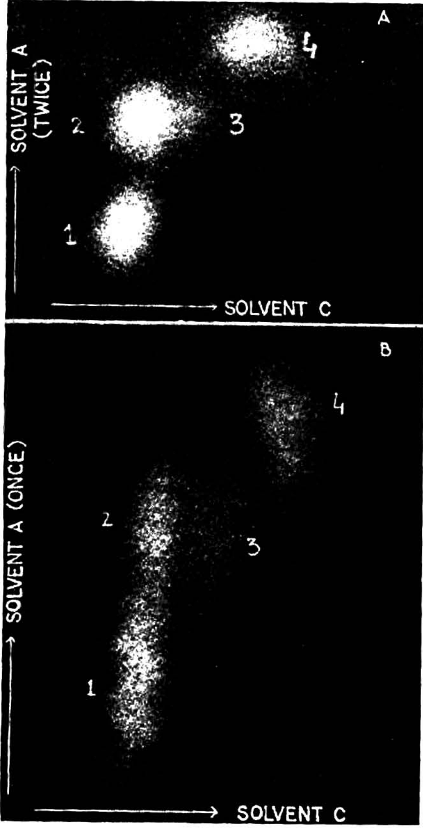

Fig. 3. Chromatograms showing the effect of the final run of the present procedure in compacting the spots. Butanol was shaken with borate buffer pH 10.0, and the residue obtained after evaporating the butanol was spotted on two papers along with a solution of four phenolic amines. 1 = octopamine; 2 = metanephrine; 3 = methoxytyramine; 4 = *P*-tyramine. First run in isopropyl alcohol–ammonia–water (8:1:1) (solvent A) was followed by one (at right angles) in isopropyl alcohol–*n*-butyl alcohol–isoamyl alcohol–water–formic acid (50:30:20:20:15) (solvent C) omitting the wash run of the actual procedure. In paper B, only the above two runs were given while in paper A (as in case of the actual procedure described in this paper) an additional final run was given in the direction of the 1st run using solvent A again.

has already been used for the separation of a particular group of amino acids from untreated urine[8].

The wash run is based on the fact that phenolic and indolic amines have no movement in solvent B or another similar solvent[7]. This is true for *p*-tyramine, methoxytyramine, octopamine, normetanephrine, metanephrine, tryptamine, methoxytryptamine and 5-hydroxytryptamine. Thus, in the wash run, the phenolic amines are not disturbed, whereas a large number of other aromatic compounds which stain with *p*-nitraniline are washed away. In the absence of this run, the known phenolic amine spots in the final chromatograms are overlapped by a number of other *p*-nitroaniline staining spots. However, it should be mentioned that the wash run does not remove some aromatic compounds which are not phenolic amines, since the

final chromatograms contain some *p*-nitraniline staining spots which do not stain with ninhydrin staining reagents[1,2].

Using the extraction and the chromatographic procedure described, the author finds that metanephrine, normetanephrine and *p*-tyramine are always demonstrable in urine samples corresponding to 15–20 μg creatinine. SANDLER AND RUTHVEN[4] needed at least 30 ml of urine for such studies.

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Book Reviews

Methods in Enzymology; Vol. XV, Steroids and Terpenoids, edited by R. B. CLAYTON, Academic Press, New York and London, 1969, xvii + 903 pp., price $ 32.50.

Methods in Enzymology is the major sourcebook and instruction manual for anyone working with enzymes and although each volume is concerned with a particular topic the editors give a thorough and up-to-date coverage of important facets rather than attempt to cover the whole field in less detail. The XVth volume in the series, entitled *Steroids and Terpenoids*, concentrates more on the former but it is of particular interest to readers of this journal because the first and largest of its six sections deals with newer analytical techniques.

Section I is primarily concerned with thin-layer and gas–liquid chromatography but is not, and does not try to be, a definitive manual on the subject. It deals with those aspects which are likely to be required by anyone analysing steroids and the abundant references permit easy access to the original literature. The first and major sub-section, *Thin-layer Chromatography*, is written by B. P. LISBOA, the second, *Gas–liquid Chromatography*, by H. H. WOTIZ AND S. J. CLARK. Then follow shorter discussions of more specific topics such as the gas–liquid chromatography of polycyclic diterpenes, the separation of steroids by means of ion-exchange resins and analytical methods for bile acids.

Sub-section 1 on thin-layer chromatography contains a large compilation of R_F data in tabular form for steroids and also for terpenoids. Detection methods on thin-layer plates and reactions for specific groupings are also fully described. Precautions to prevent destruction of compounds on thin-layer plates (deactivation of the origin before the plate is loaded, for example) are omitted and it is regrettable that no mention is made of procedures for the preparation of small samples for mass spectrometry. The mass spectrometer is widely used for the identification of small quantities of steroids and almost all silica gel and alumina layers are contaminated with hydrocarbons and other organic materials. The thin layers should be washed several times by allowing the solvent to be used to extract the material to run to the top of the plate which can then be reactivated if necessary. If this is not done the mass spectra of compounds which have been chromatographed in small quantities are obscured by the sinusoidal cracking pattern of higher paraffins and confused by other contaminant peaks.

Gas–liquid chromatographic techniques are described in sub-section 2, whose main emphasis is on procedures for the isolation and measurement of different classes of steroid. Surprisingly no mention is made here of combined GLC–mass spectrometry (its use for bile acids is described briefly in a later chapter) because both of these techniques have proved immensely useful in steroid research and in combination promise to extend analysis and identification to sub-microgram quantities.

The second section is on special synthetic methods and gathers together the details of the many elegant chemical and enzymic syntheses that have been developed for the production of the substrates, some stereospecifically labelled, which have become the cornerstone of our understanding of the stereospecificity of enzyme reactions. The procedures are carefully detailed at all stages.

Sections III, IV and V are devoted to the preparation of enzymes of terpenoid and steroid biosynthesis, bile acid formation and steroid hormone metabolism, respectively. The VIth section describes an assay and properties of the corticosteroid- and other steroid-binding proteins. These are the enzymologists' recipes and are as precise as we have come to expect from this invaluable series.

Rapidly expanding subjects, which have relevance to many other disciplines, cannot be completely covered in the detail usually found in this series; wisely the editor concentrated attention on a number of important topics and has succeeded in integrating the contributions of 62 authors into an excellent reference work for those engaged in steroid research.

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CHROM. 4688

Modern Separation Methods of Macromolecules and Particles (Vol. 2 of *Progress in Separations and Purification*), edited by THEO GERRITSON, Wiley/Interscience, New York, 1969, 250 pp., price 140/-.

The biochemist and chemist have been well provided for some years now with refined techniques for the separation and isolation of single molecular species of compounds of molecular weights up to about 1000–2000. Molecules of greater size and also discrete particles are still difficult to handle, however. The volume under review is an assemblage of articles from different authors covering recent work in this area and showing how much progress has been made in the last ten years.

The articles cover: (1) large-pore "disc" electrophoresis, (2) differential elution of trapped molecules, (3) the application of free-flow electrophoresis to the separation of macromolecules and particles, (4) the use of gradients of colloidal silica for the separation of cells and subcellular particles, (5) the separation of lymphocyte populations on glass bead columns, (6) partition in two-phase polymer systems, (7) factors in the partition of blood cells in aqueous dextran–polyethyleneglycol systems, (8) partition of nucleic acids in polymer two-phase systems, (9) equilibrium density gradient separation, (10) gel filtration on agarose gels, (11) theoretical aspects of gel chromatography, and (12) separations based on size and conformation (thin-film dialysis).

A volume built up from contributions from many individuals is bound to be patchy in quality. These papers range from authorative reviews to specific applications but are none the worse for that. The major fields of large molecule and particle separations are covered adequately and this volume provides a good introduction to this difficult but rewarding area. As such it can be recommended. Both biochemists and polymer chemists will find in it useful information.

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A. T. JAMES

News

ITALIAN ASSOCIATION FOR SCIENTIFIC INSTRUMENTS AND EQUIPMENT

Italian technologists working in the field of scientific instruments have founded in Milan the Italian Association for Scientific Instruments and Equipment 'ASTRU' with the aim to divulge the knowledge on new research instruments and to promote the development of the same in all technical–scientific fields and in the field of education, social medecine and economy.

For further information apply to the publisher under reference No. Chrom. N-274.

Apparatus

The Illitron *Protein Sequenator* is now available to provide an automated procedure for protein chemistry research. This protein sequenator is based on the Edman and Begg method for the determination of amino acid sequences in proteins and peptides, and incorporates the latest advances in solid state program control, making possible error-free, fully reliable sequencing. The original method embraces the formation of the phenylthiocarbamyl derivative of the protein, and the splitting off of the N-terminal amino acid as thiazolinone.

The thiazolinones are converted to the corresponding phenyl hydantoins in a separate operation, the latter can then be identified by thin film chromatographic procedures.

For further information apply to the publisher under reference No. Chrom. N-274.

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CHROM. 4728

THE CONTRIBUTION OF EDDY DIFFUSION AND OF THE MACROSCOPIC MOBILE PHASE VELOCITY PROFILE TO PLATE HEIGHT IN CHROMATOGRAPHY

A LITERATURE INVESTIGATION

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SUMMARY

From literature data, the coefficient for radial convective dispersion λ_R and the composite coefficient $(\kappa' + \kappa\varrho^2)$ for longitudinal dispersion by eddy diffusion and the macroscopic velocity profile are estimated as a function of the ratio ϱ of the diameters of the column and of the granules of the packing.

It does not appear to be possible to separate the effects of eddy diffusion and of the macroscopic velocity profile, on the basis of the experimental data available.

INTRODUCTION

The phenomenon of axial and radial dispersion of a solute when it is injected into a packed bed and transported by a moving fluid is both theoretically interesting and practically important, *e.g.*, in the fields of chromatography, chemical engineering and hydrology.

The main lines of the phenomenon appear to be understood now and a fair amount of experimental data has been obtained in the last few years by chromatographers as well as by chemical engineers.

Thus, the moment looks appropriate to present a critical discussion of the results obtained up till now, and to estimate the magnitude of the coefficients occurring in the dispersion equation.

THEORY

A big step forward in the field of interpretation of peak dispersion in chromatography was made in 1956 by VAN DEEMTER *et al.*[1] and by KLINKENBERG AND SJENITZER[2]. Their work led to the well-known VAN DEEMTER equation:

$$H = A + B/u + C_S u + C_M u \tag{1}$$

where:

H = plate height
A = contribution of eddy diffusion
B/u = contribution of diffusion
$C_S u$ = contribution of resistance to mass transfer in the stationary phase
$C_M u$ = contribution of resistance to mass transfer in the mobile phase
u = velocity of the mobile phase

After a couple of years it became apparent that eqn. 1 did not adequately represent the experimental data mainly forthcoming from the field of gas chromatography. Its main shortcomings are:

(1) The experimental values of the contribution of eddy diffusion are much smaller than expected from the physical model of this phenomenon;

(2) The experimental values of the $C_M u$ term are much larger than expected.

BERAN[3] showed, as early as 1957, that dispersion caused by eddy diffusion is independent of the mobile phase velocity only when the latter is large. He derived the equations for the limiting cases of very low and very large velocities and concluded that in the intermediate case "no simple procedure appears to be available and for want of anything better to do (the two limiting expressions for dispersion caused by eddy diffusion) may be added harmonically".

In doing so, he got, essentially, the same result as was obtained two years later by GIDDINGS[4] from a random-walk treatment.

The classical expression for the contribution of eddy diffusion to plate height and the result from GIDDINGS' "coupling theory" can be cast in the following forms:

$$\text{classical theory: } H_{\text{e.d.}} = A = 2\lambda d_p = \frac{2\kappa' d_p^2 u}{\lambda_R d_p u} \tag{2}$$

$$\text{coupling theory: } H_{\text{e.d.}} = \frac{2\lambda d_p}{1 + CD/d_p u} = \frac{2\kappa' d_p^2 u}{\lambda_R d_p u + \gamma D} \tag{3}$$

where:

$\lambda, \lambda_R, \kappa', C, \gamma$ = dimensionless coefficients, depending on the geometry of the column packing and the dynamics of flow
d_p = particle diameter
D = diffusion coefficient of the solute in the mobile phase.

The denominators in eqns. 2 and 3 represent effective diffusion coefficients for radial dispersion. This phenomenon reduces the longitudinal dispersion caused by the non-equivalence of the flow paths around the particles. It can be seen from eqns. 2 and 3 that in the classical theory radial dispersion by convection only is accounted for, whereas in the coupling theory both convection and diffusion are taken into account.

Under normal experimental conditions in gas chromatography it can be expected that $\lambda_R d_p u \ll \gamma D$ so that:

$$H_{\text{e.d.}} \approx \frac{2\kappa' d_p^2 u}{\gamma D} \tag{4}$$

So, both the above mentioned discrepancies between theory and experiment can be reconciled, at least partially, along these lines.

Peak dispersion by eddy diffusion can be regarded as being caused by a mobile phase velocity profile on the scale of the order of a particle diameter. HUYTEN *et al.*[5] in 1960 introduced an analogous term, due to a macroscopic velocity profile:

$$H_{\text{v.p.}} = \frac{2\kappa d_c^2 u}{D_R} \tag{5}$$

where:

κ = dimensionless coefficient, depending on the velocity profile
d_c = column diameter
D_R = effective radial diffusion coefficient.

The denominator was specified later[6] as follows:

$$D_R = \lambda_R d_p u + \gamma D \tag{6}$$

From the work of LITTLEWOOD[7] and HIGGINS AND SMITH[8] it follows that, if du/dr* changes sign at $r = r_0$ ($r_0 < d_c/2$) in wide columns, d_c should be replaced by $2\,r_0$. If this occurs, $H_{\text{v.p.}}$ will be proportional to d_c^2 up to a certain value of the column diameter and then become independent of d_c. Further, LITTLEWOOD showed that eqn. 5 also holds in the case of a one-dimensional velocity profile, as occurs in paper and thin-layer chromatography or electrophoresis. d_c is then equal to the breadth of the paper or thin-layer strip seen by the densitometer, *or* equal to the mean distance between maxima or minima in the velocity profile, whichever is the smaller.

Eqns. 3, 5 and 6 can be combined to:

$$H_{\text{e d.+v.p.}} = 2u\,\frac{\kappa' d_p^2 + \kappa d_c^2}{\lambda_R d_p u + \gamma D} \tag{7}$$

To compare experimental data, obtained for different values of d_p, d_c and D it is advantageous to introduce the following dimensionless quantities:

the reduced plate height $h = H/d_p$
the reduced velocity $v = ud_p/D$
the reduced column diameter $\varrho = d_c/d_p$.

In terms of these variables, eqn. 7 reads as follows:

$$h_{\text{e.d.+v.p.}} = 2v\,\frac{\kappa' + \kappa\varrho^2}{\lambda_R v + \gamma} \tag{8}$$

It follows from eqn. 8 that h should be a universal function of ϱ and v, or nearly so as the values of the coefficients κ, κ', λ_R and γ may depend slightly on the geometry of the column packing and the dynamics of flow.

COMPARISON WITH EXPERIMENTAL DATA

Evidence for the coupling theory of longitudinal dispersion

There is now ample evidence for coupling in longitudinal dispersion but we shall show only one piece of evidence, from the literature on chemical engineering. In the absence of mass transfer between a mobile and stationary phase, and for simplicity neglecting the macroscopic velocity profile, the effective diffusion coefficient for

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* r = distance from the axis of the column.

longitudinal dispersion, accounting for both convective and diffusive dispersion, is (see eqns. 1, 2 and 3):

$$\text{classical theory: } D_L = (A + B/u)u/2 = \lambda d_p u + \gamma D \tag{9}$$

$$\text{coupling theory: } D_L = (A + B/u)u/2 = \frac{\lambda d_p u}{1 + CD/d_p u} + \gamma D \tag{10}$$

or:

$$\text{classical theory: } \frac{D_L}{d_p u} = \lambda + \frac{\gamma D}{d_p u} \tag{11}$$

$$\text{coupling theory: } \frac{D_L}{d_p u} = \frac{\lambda}{1 + CD/d_p u} + \frac{\gamma D}{d_p u} \tag{12}$$

It is customary in chemical engineering literature to present dispersion data as graphs of the logarithm of the longitudinal Peclet number, $Pe_L = d_p u/D_L$, *vs.* the logarithm of the Reynolds number, $Re = d_p u/\nu$*. If eqn. 11 holds, such a graph should rise steadily to a value $-\log \lambda$, whereas the graph should have a maximum if eqn. 12 holds. Fig. 1, from the work of EDWARDS AND RICHARDSON[9], is clearly in favour of the coupling theory.

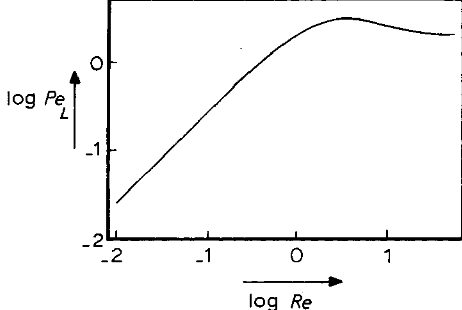

Fig. 1. Longitudinal Peclet number Pe_L as a function of Reynolds number Re. Evidence for coupling in longitudinal dispersion.

The magnitude of λ_R and its dependence on ϱ

FAHIEN AND SMITH[10] have convincingly shown that λ_R depends on ϱ, due to a corresponding dependence of the void fraction on ϱ and to the influence of the column wall on the dynamics of flow. They propose, for gases, the equation:

$$\lambda_R \text{ (gas)} = \frac{\varrho^2}{8\varrho^2 + 155} \tag{13}$$

They investigated the range of ϱ values from $\varrho = 5.6$ to $\varrho = 26$. It follows from Fig. 2 that this equation also holds for the λ_R values at still smaller ϱ values, which can be deduced from an analysis of longitudinal dispersion data by means of eqn. 8. Further, packings of spheres and of irregularly shaped particles appear to yield comparable λ_R values.

* ν = kinematic viscosity.

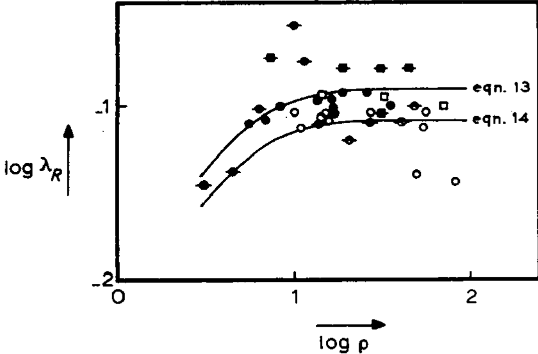

Fig. 2. λ_R as a function of ϱ. ● gas, deduced from radial dispersion, spherical packing[10,11,13]; —●— gas, deduced from longitudinal dispersion, spherical packing[14,15]; —■— gas, deduced from longitudinal dispersion, irregular packing[16]; ○ liquid, deduced from radial dispersion, spherical packing[11,17,18,26]; □ liquid, deduced from radial dispersion, irregular packing[11,19]; —○— liquid, deduced from longitudinal dispersion, spherical packing[12].

Fig. 2 shows also that λ_R values for liquids are substantially smaller than for gases. The values determined from radial dispersion are in good agreement with the values of GORDON *et al.*[12], calculated from longitudinal dispersion data. Again, the shape of the particles appears to have no influence on λ_R.

For the dependence of λ_R on ϱ we propose an equation of the same type as eqn. 13, *viz.*:

$$\frac{3}{2}\lambda_R \text{ (liquid)} = \frac{\varrho^2}{8\varrho^2 + 155} \tag{14}$$

The magnitude of γ

According to STERNBERG AND POULSON[20] γ is equal to 0.73 for massive spherical particles* and to 0.63 for irregularly shaped porous ones, and does not depend on ϱ or d_p. For irregularly shaped massive particles BLACKWELL[19] found a value of 0.65.

The magnitudes of $(\kappa' + \kappa\varrho^2)/\lambda_R$ and of $\kappa' + \kappa\varrho^2$ and their dependence on ϱ

The first mentioned quantity is equal to one half of the right hand side of eqn. 8 when v is so large that diffusion can be neglected, compared to convective dispersion**. In chemical engineering language, $(\kappa' + \kappa\varrho^2)/\lambda_R$ is equal to $1/Pe_L$ for the same condition. This quantity can easily be measured for *liquids*, due to the small value of D in liquids. Fig. 3 shows that the values of $1/Pe_L$ are constant for ϱ values larger than about 20 but decrease somewhat for smaller ϱ values. We propose the equation:

$$1/Pe_L \text{ (liquid)} = (\kappa' + \kappa\varrho^2)/\lambda_R = \frac{5\varrho}{2\varrho + 5} \tag{15}$$

for both spherical and irregularly shaped particles.

Combination of eqns. 14 and 15 yields:

$$\kappa' + \kappa\varrho^2 \text{ (liquid)} = \frac{10\varrho^3}{(6\varrho + 15)(8\varrho^2 + 155)} \tag{16}$$

* Exactly the same value was found by EDWARDS AND RICHARDSON[9].

** However, v should not be so large that turbulence sets in (at R_e 10–100).

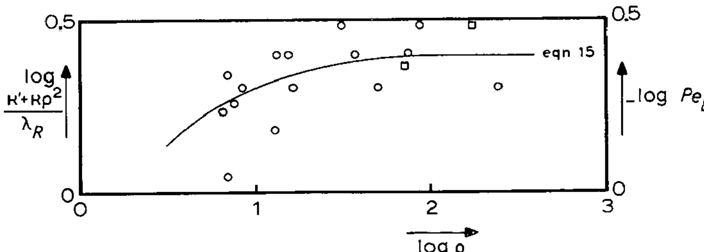

Fig. 3. $(\kappa' + \kappa\varrho^2)/\lambda_R$ as a function of ϱ for liquids. ○ spherical packing[21–25]; □ irregular packing[2,19].

Now we can investigate if h values in liquid chromatography can be described by the deduced values of λ_R, γ and $\kappa' + \kappa\varrho^2$. It must be admitted that for liquid eluents no general agreement exists upon the validity of eqn. 8. It describes perfectly the data of GORDON *et al.*[12] but predicts more curvature in plots of log h *vs.* log v than was found by KELLEY AND BILLMEYER[36] and by KNOX and his collaborators[15,27,28]. These data can be described better if v in eqn. 8 is replaced by $^1/_3v$. However, the resulting equation "does not seem very satisfactory from the theoretical point of view"[28]. Therefore, these data were also analysed by means of eqn. 8. The deduced values of λ_R and γ were substituted in this equation which was then applied to h data at large values of v. The resulting values of $\kappa' + \kappa\varrho^2$ are compared with values calculated from eqn. 16 in Fig. 4. The agreement is reasonable, even perfect for the data from ref. 28.

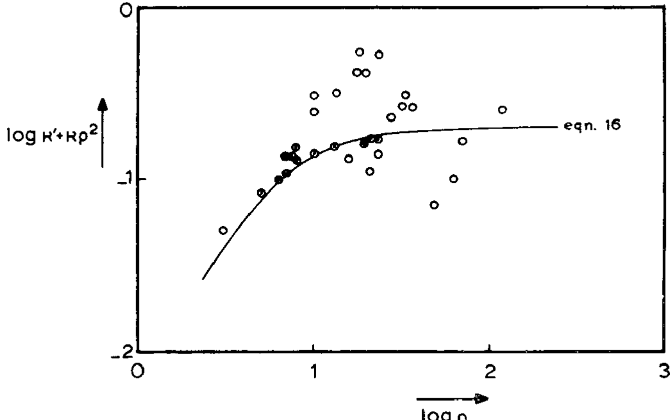

Fig. 4. $\kappa' + \kappa\varrho^2$ as a function of ϱ for liquids. ○ data from refs. 12, 15, 27, 36; ⊘ data from ref. 28.

For *gases* it is difficult to realise v values that are large enough to determine $(\kappa' + \kappa\varrho^2)/\lambda_R$. On the other hand it is easy to realise v values that are so small that the right hand side of eqn. 8 approaches $2v(\kappa' + \kappa\varrho^2)/\gamma$. For gases therefore, $\kappa' + \kappa\varrho^2$ is the more easily accessible quantity. Fig. 5 shows this quantity as a function of ϱ. It appears that the values of $\kappa' + \kappa\varrho^2$ for spherical particles are smaller than those for irregularly shaped ones. We propose the equations:

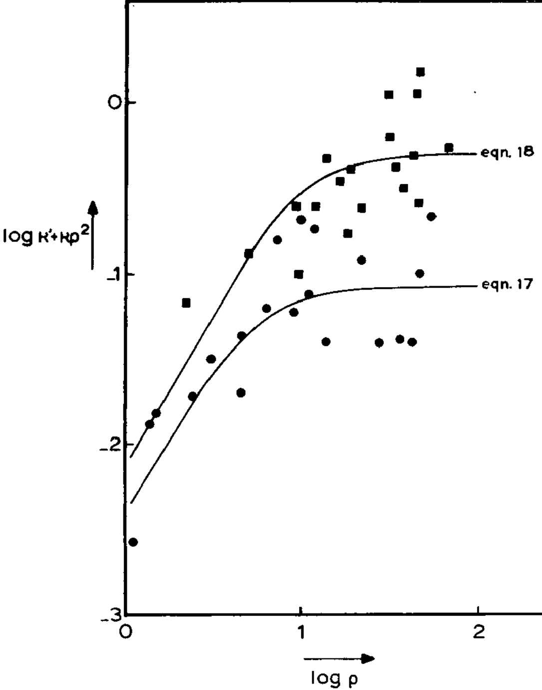

Fig. 5. $\kappa' + \kappa\varrho^2$ as a function of ϱ for gases. ● spherical packing[13–15,20,31,32,35]; ■ irregular packing[7,15,16,20,29–34].

$$\text{spherical particles: } \kappa' + \kappa\varrho^2 \text{ (gas)} = \frac{\varrho^2}{12\varrho^2 + 230} \tag{17}$$

$$\text{irregular particles: } \kappa' + \kappa\varrho^2 \text{ (gas)} = \frac{\varrho^2}{2\varrho^2 + 125} \tag{18}$$

combination of eqns. 13, 17 and 18 yields:

$$\text{spherical particles: } 1/Pe_L \text{ (gas)} = (\kappa' + \kappa\varrho^2)/\lambda_R \simeq \frac{2}{3} \tag{19}$$

$$\text{irregular particles: } 1/Pe_L \text{ (gas)} = (\kappa' + \kappa\varrho^2)/\lambda_R = \frac{8\varrho^2 + 155}{2\varrho^2 + 125} \tag{20}$$

Fig. 6 shows that the experimentally found values of $1/Pe_L$ are in fair accord with eqns. 19 and 20.

The classical eddy diffusion coefficient λ

According to eqn. 2, λ is equal to κ'/λ_R. It follows from eqns. 15, 19 and 20 that it is impossible to make a reliable estimate of λ on the basis of the experimental data that have been obtained up to now: from the values of the right hand sides of these equations at $\varrho = 0$, λ values of 0, 0.7 and 1.2 would follow. Moreover, the validity of these equations at $\varrho \approx 0$ is questionable, as they are derived from data at $\varrho > 6$ for liquids and > 3 for gases.

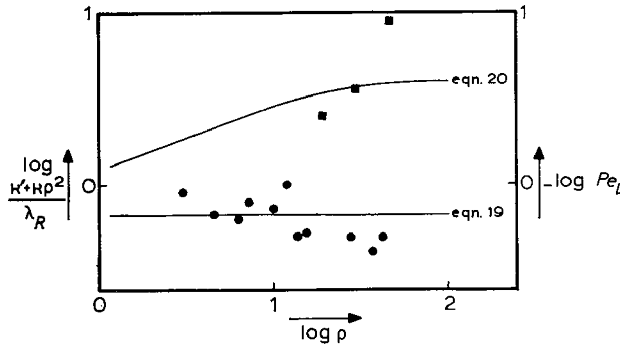

Fig. 6. $(\kappa' + \kappa\varrho^2)/\lambda_R$ as a function of ϱ for gases. ● spherical particles[13–15,35]; ■ irregular particles[16].

Influence of the packing method

KNOX and his collaborators[15] have shown that a more efficient column packing is obtained when dry glass beads are packed into an empty column than when wet beads are packed into a column filled with a liquid. The ratio of h values, obtained for these two packing methods at identical values of v and ϱ, resp., is about 0.7 for liquid eluents.

However, this rather small effect is swamped by the variance between the data of different authors.

The influence of different methods of dry packing has been demonstrated by HIGGINS AND SMITH[8].

However, in view of the relatively small effect of the above mentioned extreme variation in packing method (wet *vs.* dry packing) we may assume that various methods of dry packing, chosen by different investigators as the most efficient one, do not yield significantly different results*.

CONCLUSION

The coefficients of eqn. 8 can be defined as follows in Table I.

TABLE I

Coefficients of eqn. 8	*Liquid eluents*		*Gaseous eluents*	
	Spherical packing	*Irregular packing*	*Spherical packing*	*Irregular packing*
λ_R	$2\varrho^2/3(8\varrho^2 + 155)$	$2\varrho^2/3(8\varrho^2 + 155)$	$\varrho^2/(8\varrho^2 + 155)$	$\varrho^2/(8\varrho^2 + 155)$
γ	0.73	0.64	0.73	0.64
$\kappa' + \kappa\varrho^2$	$10\varrho^3/(6\varrho + 15)(8\varrho^2 + 155)$	$10\varrho^3/(6\varrho + 15)(8\varrho^2 + 155)$	$\varrho^2/(12\varrho^2 + 230)$	$\varrho^2/(2\varrho^2 + 125)$
$(\kappa' + \kappa\varrho^2)/\lambda_R$	$5\varrho/(2\varrho + 5)$	$5\varrho/(2\varrho + 5)$	$2/3$	$(8\varrho^2 + 155)/(2\varrho^2 + 125)$

* *i.e.*, the bias introduced by this factor merges into the variance between the data of different authors.

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RAPID IDENTIFICATION OF LOW MOLECULAR WEIGHT COMPOUNDS IN EXTRACTS OF BIOLOGICAL MATERIAL

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SUMMARY

The problem of obtaining rapid information about the elementary composition of low molecular weight compounds in submicrogram quantities in complex mixtures without prior isolation has been simplified by the development of a new technique, involving the use of a combination of gas chromatography and mass spectrometry (GC–MS). A peak matching accessory allows the subsequent matching of the characteristics of the molecular ion of an unknown compound with that of a reference substance having a known mass adjacent to that of the unknown. The precise molecular weight of the unknown molecular ion can then be calculated with an error of 5–10 p.p.m. This calculated value is then compared with those recorded in appropriate reference manuals in order to obtain the empirical formula of the unknown compound. The mass spectrum of the reference compound corresponding to the most appropriate empirical formula is compared to the spectrum of the unknown to verify its identity. This technique led to the detection and identification of dimethylaminoethanol and piperidine in rat brain.

INTRODUCTION

The identification of unknown compounds in the effluent of a gas chromatograph (GC) has been partially simplified by the use of a system whereby the column effluent passes directly into a mass spectrometer (MS)[1]. However, the analysis of low resolution mass spectra of unknown compounds generally requires a high index of suspicion as to their identity, so that comparisons can be made with the fragmentation patterns of appropriate reference substances. Computer analysis does facilitate the process of identification but is not yet generally available. This paper reports the use of a new GC–MS technique which has been developed as an aid to the rapid identification of unknown volatile substances in rat brain extracts. During long term biological studies of acetylcholine using various techniques we became interested in other cerebral amines. In general, the methods of HOLMSTEDT AND LUNDGREN[2] and HANIN AND

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JENDEN[3] have been used for the brain preparation, extraction and gas chromatographic studies.

MATERIALS AND METHODS

Brain preparation

The brains of albino, male, 200–225-g Sprague–Dawley rats were prepared and extracted according to the method described for acetylcholine by HANIN AND JENDEN[3]. In the latter technique, acetylcholine is demethylated and extracted into chloroform by raising the pH of the water phase to about 9. The more polar amines remaining in the water phase were *not* studied. In our current experiments the highly polar amines were extracted by the addition of 0.1 ml of 13.5 *M* KOH and 0.05 ml chloroform to a 0.1 ml aliquot of the water phase. The combined solution was mixed vigorously for 2 min and centrifuged at $2000 \times g$ for 2 min. A 1-μl aliquot of the chloroform phase was used for the gas chromatographic studies.

Gas chromatography

A Varian model 1200 gas chromatograph equipped with a hydrogen flame ionisation detector was used. The silanised glass column measured 1.5 mm (I.D.) by 1.6 m. Unsilanised, acid- and ammonia-washed Chromosorb W (80–100 mesh) was coated with 4% Amine 220 (1-hydroxy-2-heptodecenyl-imidazoline) and 6% THEED (tetrahydroxyethylenediamine). Injector and detector temperatures were maintained at 150° and 200°, respectively. Flow rates were about 20 ml/min for nitrogen (carrier gas), 200 ml/min for oxygen and 25 ml/min for hydrogen. The detector response was recorded on a Servogor model RE 512 recorder.

Gas chromatography–mass spectrometry

The combined gas chromatograph–mass spectrometer, LKB 9000 (LKB-Produkter AB, Fack, 161 51 Bromma 1, Sweden) was used to scan the mass spectra of the components as they were eluted from the GC column (similar to that described above).

An accessory to this GC–MS system, the LKB 9020 oscilloscopic peak matching device, was used to measure the ratio of the mass of an appropriate ion of an unknown compound to that of a reference agent or *vice versa*.

RESULTS

Gas chromatography

In the initial chromatographic determination 1 μl of the chloroform phase was injected; the column temperature was maintained at 30° until the solvent front and first compound had been completely eluted; the temperature was then increased 8°/min to 70° where it was held until the remaining compounds had been eluted (Fig. 1). The new technique involving the combined gas chromatograph–mass spectrometer–peak matcher system was then used to identify rapidly the compounds eluting as individual peaks (see below). After identification the compounds of interest were studied isothermally at a column temperature of 60° (Fig. 4).

Gas chromatography–mass spectrometry

The identity of one compound only was suspected in the gas chromatogram of the rat brain extract (Fig. 1). Synthetic dimethylaminoethanol (DMAE) has a retention time equal to that of the last peak. In order to establish its identity, a mass spectrum of that peak in the rat brain extract was recorded by means of the combined gas chromatograph–mass spectrometer, LKB 9000. This spectrum was compared to that of synthetic dimethylaminoethanol (Fig. 2). The resemblance was such that the identity could be confirmed.

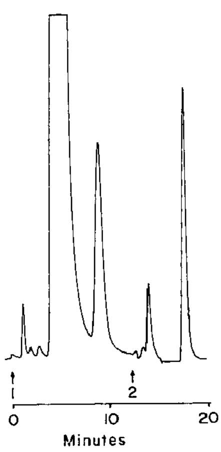

Fig. 1. GC separation of volatile compounds in a 1-μl chloroform aliquot of rat brain extract. Column temperature was kept at 30° between 1 and 2 and at 70° from 2 to the end of the trace. Temperature was raised from 30° to 70° at 2 and the baseline adjusted. First peak after the chloroform solvent front corresponds to the elution of ethanol followed by piperidine and dimethylaminoethanol.

The mass spectrum of the compound eluted just before DMAE was also recorded. The interpretation of the spectrum of this unknown compound was difficult due to interference by unresolved components. Serial scans were recorded, as the compound was eluted, at approximately the same interval to make it possible to distinguish between ions belonging to the interfering compound(s). Instead of scanning successively we could have taken advantage of the selectivity of the mass fragmentographic technique[4] and thus achieve the same picture of the variations of the intensities of the m/e values as shown in Fig. 3.

Twelve successive scans in the mass range 65 to 130 were recorded during the eluting time of the compound. The intensities (in mm) of the different mass numbers were plotted against the successive numbers of scans which are analogous to the time (Fig. 3). The fragments characteristic of the compound vary in intensity while the intensities of the interfering ions are relatively constant. The highest m/e value of the ions of varying intensities, $m/e = 85$, was selected as the most probable parent ion of the unknown compound.

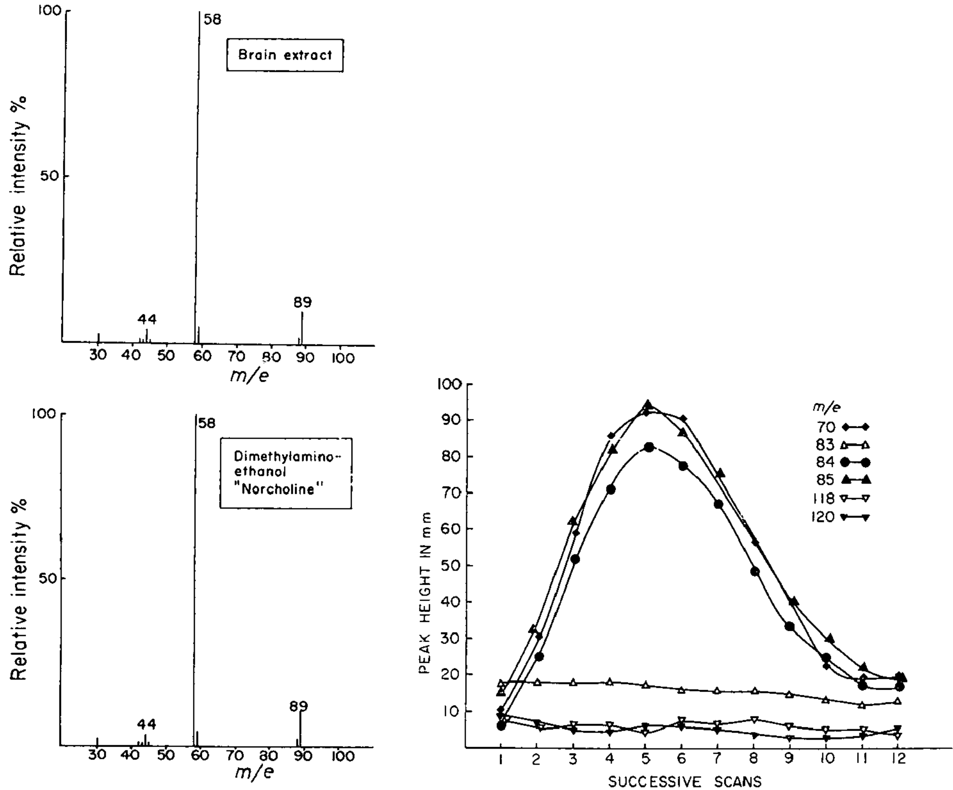

Fig. 2. Upper panel: mass spectrum of a compound extracted from rat brain and having the same retention time as synthetic dimethylaminoethanol. Lower panel: mass spectrum of synthetic dimethylaminoethanol. The mass spectra were recorded at an ionisation energy of 70 eV. GC conditions as in the legend to Fig. 1, except that the temperature was maintained isothermally at 60°.

Fig. 3. Changes in the intensities of six selected masses during twelve successive scans of a compound eluting from the GC column (see text). GC and MS conditions as in the legend to Fig. 2.

New peak matching technique of non-isolated compounds

The usual principles when operating with isolated compounds may be summarised as follows:

The intensity of ions of a certain mass is visualised as a Gaussian curve on the oscilloscope screen of the peak matcher. Ions of lower mass, *e.g.*, the parent ions of the unknown compound, are brought into focus by a manual change of the magnetic field. Ions of higher mass, *e.g.*, those of the reference compound, are then brought into focus by keeping the magnetic field unchanged while the accelerating voltage is decreased by manual adjustment of a high precision decade resistor, calibrated in mass ratio 1.0+++++. The second to the sixth decimals are adjustable. A relay is then set to switch the accelerating voltage between full and reduced, low and high mass (unknown and reference) which makes it possible to watch both masses on the oscilloscopic screen and match them. A six-decimal value for the weight (a.m.u.) of

the unknown compound can then be calculated and an empirical formula deduced. (The last two or three decimals are of little significance.)

This standard peak matching technique was modified to allow peak matching during the elution of an unknown compound from the column. As the time to match the peaks is short and equals the elution time, the precision decade must be set to a pre-selected value of the expected ratio. A list of organic compounds[5] having molecular weights of 85 was inspected and 2-pyrrolidone, C_4H_7NO, with a molecular weight of 85.052761 selected as possible. This value was divided into 86.109545, the molecular weight of the selected reference, *n*-hexane, C_6H_{14}. M^+ hexane/M^+ pyrrolidone = 86.109545/85.052761 = 1.012425. This ratio was pre-set on the precision decade of the peak matcher.

If the unknown compound is pyrrolidone, then, as the compound eluted, the position of the peak appearing on the oscilloscopic screen should be superimposed on that of the reference, *i.e.* match perfectly. This was not the case—the compound was therefore not pyrrolidone. However, the unknown peak did resemble that of $(M_{\text{hexane}}{-1})^+$ (C_6H_{13} = 85.101720) which also appeared on the oscilloscope screen. Reference tables[5] indicate that the organic compound with a mass closest to C_6H_{13} is piperidine, $C_5H_{11}N$ (M = 85.089145).

A new reference, dichloromethane, was selected because it does not interfere with the molecular ion of the unknown. The mass ratio was calculated M^+ piperidine/M^+ dichloromethane = 85.089145/83.953355 = 1.013529 and the value pre-set on the decade. When the peak of the unknown compound appeared on the screen it could be superimposed on that of dichloromethane—strongly suggesting that the unknown compound is piperidine. The identity was then confirmed by gas chromatography (Fig. 4) and gas chromatography–mass spectrometry.

The gas chromatographic peak appearing just after the chloroform at a column temperature of 30° was also examined as described above (Fig. 1). The mass spectrum

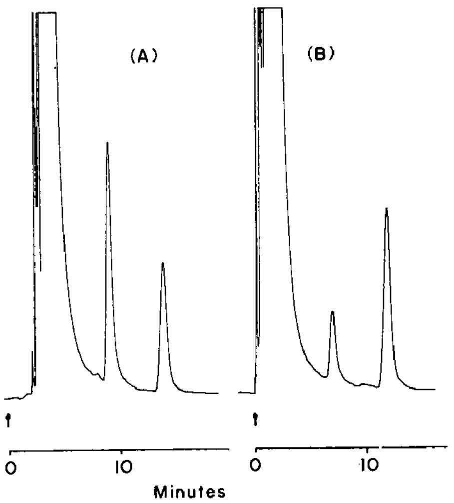

Fig. 4. (A) Gas chromatographic separation of 2 μl of a 5 mM solution of piperidine (first peak) and dimethylaminoethanol (second peak). Conditions are the same as in the legend to Fig. 2. (B) Gas chromatogram of 1 μl rat brain extract (see text). GC conditions as in A.

of the compound eluting at this peak indicated that the highest mass numbers were 46 and 45. We selected from the reference tables an empirical formula for the compound of C_2H_7N, with a mass value of 45.057846 (m/e = 46 could then be $(M+1)^+$). This formula was selected because extraction characteristics of the unknown indicated a basic compound.

CO_2 present in the carrier gas was used as the reference. The pre-set mass ratio was $M^+ C_2H_7N/M^+ CO_2$ = 45.057846/43.989828 = 1.0242787. The choice was incorrect as the Gaussian curve of the eluting compound was not superimposed on that of the reference. The ratio was rapidly altered until the peaks approximately matched. The value of the new ratio was 1.0237. This ratio multiplied by the accurate weight of carbon dioxide gave the mass of the unknown as 45.032386. The closest value in the reference table[5] is 45.034037 with an empirical formula of C_2H_5O. Dividing 45.034037, (C_2H_5O) by 43.989828 (CO_2) gives the ratio 1.023737. This calculated ratio is in very good agreement with the measured value. We concluded that the matched ion had the composition C_2H_5O and instead of being the molecular ion it was the parent ion minus one hydrogen. The molecular ion should then be C_2H_6O, *e.g.* ethanol. This suspicion was confirmed by gas chromatography and mass spectrometry. 1% ethanol had been added to the stock solution of chloroform for stabilisation purposes.

DISCUSSION

Usually peak matching of an unknown compound against a known reference requires that the unknown be isolated in μg-quantities[6]. This isolation may be difficult and time-consuming, especially when working with biological material where the amounts available are usually small. The technique we describe makes it possible to match the peak of an unknown component in a mixture without prior isolation; the matching is done as the component is eluted from the gas chromatographic column into the mass spectrometer. If another ion is present and has almost the same mass as that of the unknown ion the single focussing instrument may lack sufficient resolving power to perform this peak matching technique.

The determination of the mass number of the molecular ion is done by scanning a mass spectrum. This information, the fragmentation pattern and the extraction characteristics of the compound are the basis for the first guess as to the identity of the unknown. The mass ratio between the unknown compound and the reference or *vice versa* is pre-set on the peak matching device. If the guess is right the two peaks representing the two compounds will be superimposed on the oscilloscopic screen. If the guess is incorrect a rapid estimation of the ratio can be made. Then a new assessment, based upon this estimation, is tested. Two or three injections of the unknown solution may be required before the accurate mass ratio is determined. The principal advantage of the technique is that it gives rapid information about the elementary composition of compounds in complex mixtures in submicrogram quantities without prior isolation. In this paper the identity of the unknown compounds was confirmed by comparing their mass spectra to synthetic dimethylaminoethanol and piperidine.

The technique of JENDEN, HANIN AND LAMB[7,8] was modified to permit the recovery of choline, dimethylaminoethanol and piperidine. Simultaneous estimation of dimethylaminoethanol and dimethylaminoethyl acetate (the demethylation product of acetylcholine) is difficult with the 'Polypak 1'–phenyldiethanolamine succinate

support–stationary phase combination originally described[7] because of temperature instability and adsorption effects. Therefore, a stable and less active support, Chromosorb W, and a stationary phase mixture of 4% Amine 220 and 6% THEED was adopted which provides a very satisfactory separation of the volatile amines at low temperature (Figs. 1 and 4). The reduced bleeding greatly facilitated the mass spectrometric studies.

The choline content of rat brain has been estimated by a variety of methods[9,10]. The technique of HANIN AND JENDEN results in the hydrolysis of the dimethylaminoethyl acetate and the subsequent determination of the total dimethylaminoethanol[3]. In the present study both compounds may be determined separately so that the concentration of both may be measured.

Piperidine was first reported by HONEGGER AND HONEGGER[11] as a normal constituent of brain and has previously been found in human CSF[12] and urine[14] as well. Although its origin is not known it is thought to be a product of lysine metabolism through decarboxylation of the intermediate metabolite, pipecolic acid and/or deamination and cyclisation of cadaverine[15]. Certain investigators have suggested that piperidine may function as an endogenous synaptic regulatory substance modulating behaviour[16]. It has been shown to effect markedly the behaviour and activities of human patients and test animals[17]. Other quantitative data and the effect of psychoactive drug treatment and environmental conditions on the levels of these biogenic amines in rat brain will be discussed in future communications.

ACKNOWLEDGEMENTS

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GAS CHROMATOGRAPHY OF HOMOLOGOUS ESTERS

PART III. INFLUENCE OF STATIONARY PHASE POLARITY ON RETENTION OF ALIPHATIC ESTERS

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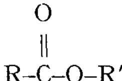
SUMMARY

The retention behaviour of a number of series of saturated homologous esters on polysiloxane stationary phases of increasing polar character is reported.

INTRODUCTION

The retention behaviour of homologous saturated esters on the essentially non-polar Methyl Polysiloxane SE-30 stationary phase has been reported[1]. With little hydrogen bonding occurring, retention was primarily related to the boiling point of the esters. The familiar deviation of the retention of the methyl esters from a linear relationship with carbon number was observed while a similar effect was apparent with isopropyl esters. These variations were largely attributed to the generally higher and lower boiling points, respectively, of the methyl and isopropyl esters with respect to the other members of the homologous series rather than to any column effect.

The esters were conveniently represented as



where the carbon numbers of the acid and alcohol chains are R and R', respectively.

The data of homologous esters on an SE-30 stationary phase when considered with regard to boiling point showed the following features common to retention behaviour:

(1) The slopes of plots representing esters with the same number of carbon atoms in the acid chain (R) decreases as the value of R increases.

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(2) The methyl esters have slightly higher boiling points than expected from the slopes of the corresponding ester plots, while that of methyl formate shows a linear relationship with the alkyl formates.

(3) The isobutyl and isopentyl esters have boiling points relatively constantly lower than the corresponding *n*-alkyl esters, while the slopes of the boiling point plots of these series are parallel to those of the *n*-alkyl esters.

(4) The isopropyl esters have boiling points lower than expected from the slopes of the plots of the higher isoesters.

(5) The boiling points of esters where either R or R′ or both of them have branched chains similarly tend to follow the retention behaviour.

In this work the retention behaviour of aliphatic esters with R and R′ varying between 0 and 6 and between 1 and 8, respectively, on silicone stationary phases of increasing polar character is reported and the results are compared with the earlier observations[1].

The gas chromatography of aliphatic esters has been extensively documented[2] and systematic studies relevant to this work have been previously reviewed[1].

EXPERIMENTAL

Preparation of esters

The esters where available were of commercial quality and of substantial purity. The remainder of the esters were prepared in the laboratory using conventional esterification or transesterification procedures and were purified before use.

Stationary phases

The stationary phases were all polysiloxanes. The Methyl Silicone Polymer SE-30 without effective polar groups on the polymer chain can be considered as essentially non-polar.

TABLE I

ROHRSCHNEIDER CONSTANTS OF STATIONARY PHASES

Phase	*X*	*Y*	*Z*	*U*	*S*
SE-30	0.16	0.20	0.50	0.85	0.48
OV-17	1.30	1.66	1.79	2.83	2.47
OV-25	1.76	2.00	2.15	3.34	2.81
XE-60	2.08	3.85	3.62	5.33	3.45

Replacement of methyl groups by polar or polarisable substituents allows an increase in the polarity factor of the stationary phase. The polysiloxanes OV-17 and OV-25 containing 50 and 75% phenyl groups based on substitution of methyl groups in a dimethyl-polysiloxane polymer have been used together with XE-60 with a 50% replacement of methyl groups by cyanoethyl groups.

Classification of stationary phase characteristics in terms of Rohrschneider constants for the polysiloxanes used[3] is shown in Table I.

Gas chromatography

The retention data were obtained on a modified F & M 810/29 Research Chromatograph with simultaneous flame ionisation and thermal conductivity detection and fitted with an improved flow control system. Two 12 ft. × ¼ in. O.D. aluminium columns were packed with 10% of the stationary phase on 60–80 mesh acid-washed and silanised Celite 560 and operated isothermally at 150°.

With thermal conductivity detection the following conditions were used: injection temperature, 190°; detector temperature, 220°; bridge current, 150 mA; carrier gas, helium; flow rate, 65 ml/min with an inlet pressure of 40 p.s.i.

The retention data of the esters examined are shown in Table II as net retention (V_g), relative retention (V_R) using nonane as standard, and as retention indices (I_R).

DISCUSSION AND RESULTS

Plots of the logarithm of the relative retention volume against the number of carbon atoms in the alcohol chain (R′) for the normal and isoesters produced a series of linear relationships for each of the stationary phases. Plots of the esters examined on OV-25 are shown in Fig. 1.

Positive and negative deviations from linearity existed with the methyl and isopropyl esters on all four stationary phases. With SE-30 and OV-17 the slopes of the plots decreased as the number of carbon atoms in the acid chain increased, *i.e.* R equals 0 to 3, after which the plots are parallel. With OV-25 the greatest slope was observed with the acetate esters while the formate and propionate esters produced

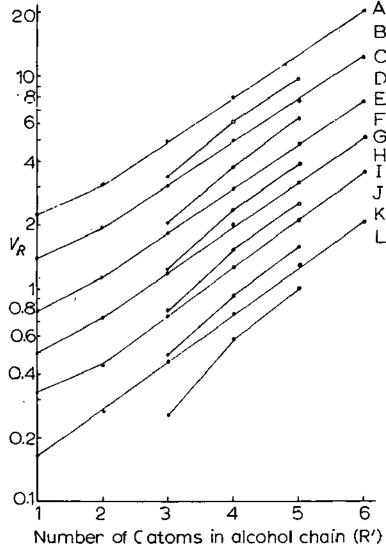


Fig. 1. Plots of the logarithm of the relative retention volume *versus* the number of carbon atoms in the alcohol chain of saturated esters on OV-25 stationary phase with varying alcohol chain (R′) length with R′ forming both normal and isoesters while the acid chain (R) is linear.
A = $C_5H_{11}COOR'$; B = $C_5H_{11}COOisoR'$; C = C_4H_9COOR';
D = $C_4H_9COOisoR'$; E = C_3H_7COOR'; F = $C_3H_7COOisoR'$;
G = C_2H_5COOR'; H = $C_2H_5COOisoR'$; I = CH_3COOR';
J = $CH_3COOisoR'$; K = $HCOOR'$; L = $HCOOisoR'$.

TABLE II

RETENTION DATA FOR ALIPHATIC ESTERS

(a) corrected for dead volume; (b) relative to nonane.

Ester	Stationary phase											
	SE-30			OV-17			OV-25			XE-60		
	$V_{g(a)}$	$V_{R(b)}$	I_R	$V_{g(a)}$	$V_{R(b)}$	I_R	$V_{g(a)}$	$V_{R(b)}$	I_R	$V_{g(a)}$	$V_{R(b)}$	I_R
Methyl formate	0.050	0.075	380	0.037	0.123	493	0.036	0.170	542	0.057	0.350	653
Ethyl formate	0.085	0.129	487	0.066	0.220	650	0.055	0.260	631	0.090	0.560	766
Propyl formate	0.145	0.216	592	0.150	0.380	711	0.090	0.450	740	0.130	0.810	851
Isopropyl formate	0.105	0.162	528	0.375	0.125	494	0.054	0.250	624	0.090	0.560	766
Butyl formate	0.250	0.375	703	0.250	0.630	811	0.160	0.774	840	0.210	1.310	966
2-Methylpropyl formate	0.200	0.301	660	0.110	0.370	705	0.120	0.566	788	0.168	1.040	912
Pentyl formate	0.425	0.645	812	0.410	1.03	905	0.275	1.28	949	0.330	2.060	1070
3-Methylbutyl formate	0.350	0.512	774	0.195	0.650	816	0.215	1.00	894	0.265	1.660	1026
Hexyl formate				0.710	1.79	1013	0.450	2.04	1042	0.490	3.060	1164
Octyl formate				1.480	4.93	1216	1.210	5.50	1244	1.17	7.310	1365
Methyl acetate	0.093	0.140	506	0.080	0.260	632	0.071	0.330	678	0.080	0.500	741
Ethyl acetate	0.130	0.200	571	0.100	0.360	697	0.090	0.440	731	0.110	0.69	816
Propyl acetate	0.225	0.338	683	0.180	0.580	795	0.160	0.780	849	0.170	1.07	918
Isopropyl acetate	0.170	0.259	625	0.115	0.390	716	0.105	0.490	745	0.125	0.78	844
Butyl acetate	0.375	0.585	787	0.300	0.970	895	0.260	1.21	938	0.260	1.63	1024
2-Methylpropyl acetate	0.310	0.468	750	0.240	0.800	857	0.105	0.490	745	0.213	1.32	966
Pentyl acetate	0.645	0.980	898	0.480	1.60	993	0.435	2.00	1040	0.400	2.50	1118
3-Methylbutyl acetate	0.525	0.798	854	0.390	1.30	952	0.335	1.56	990	0.335	2.10	1085
Hexyl acetate				0.810	2.70	1100	0.775	3.60	1158	0.620	3.88	1219
Octyl acetate				2.200	7.96	1313				1.49	9.31	1438
Methyl propionate	0.155	0.235	607	0.120	0.390	716	0.105	0.512	764	0.114	0.76	836
Ethyl propionate	0.220	0.335	679	0.165	0.550	784	0.140	0.700	829	0.150	1.00	900
Propyl propionate	0.375	0.570	787	0.290	0.950	890	0.255	1.18	933	0.230	1.55	1006
Isopropyl propionate	0.265	0.405	717	0.210	0.700	832	0.165	0.770	847	0.190	1.19	941
Butyl propionate	0.630	0.955	891	0.460	1.53	981	0.410	1.95	1035	0.390	2.44	1110
2-Methylpropyl propionate	0.520	0.790	853	0.380	1.26	948	0.310	1.50	983	0.305	2.00	1060
Pentyl propionate	1.060	1.602	998	0.770	2.56	1092	0.685	3.14	1132	0.600	3.75	1210
3-Methylbutyl propionate	0.860	1.304	955	0.620	2.07	1043	0.550	2.50	1086	0.472	3.15	1170
Hexyl propionate				1.250	4.16	1184	1.085	5.05	1228	0.930	5.82	1314
Octyl propionate							2.785	12.95	1420	2.32	14.50	1524

Methyl butyrate	0.260	0.397	714	0.190	0.630	811	0.170	0.78	850	0.170	1.13	930
Ethyl butyrate	0.375	0.565	788	0.260	0.930	887	0.225	1.07	914	0.230	1.44	988
Propyl butyrate	0.605	0.920	884	0.440	1.57	987	0.395	1.80	1018	0.360	2.25	1090
Isopropyl butyrate	0.450	0.680	823	0.320	1.07	915	0.255	1.19	933	0.250	1.66	1026
Butyl butyrate	0.975	1.48	980	0.690	2.47	1081	0.645	2.93	1117	0.535	3.35	1188
2-Methylpropyl butyrate	0.780	1.18	937	0.590	1.97	1036	0.470	2.35	1073	0.435	2.72	1131
Pentyl butyrate	1.550	2.35	1075	1.150	4.11	1182	1.030	4.78	1215	0.820	5.12	1283
3-Methylbutyl butyrate	1.300	1.97	1039	0.995	3.32	1140	0.835	3.80	1171	0.685	4.28	1236
Hexyl butyrate				2.07	6.704	1281	1.630	7.58	1311	1.255	7.80	1381
Octyl butyrate				5.05	18.10	1475	4.135	19.20	1494	2.94	18.37	1576
Methyl isobutyrate	0.210	0.319	669	—	—	—	—	—	—	—	—	—
Ethyl isobutyrate	0.295	0.445	738	0.213	0.710	838	0.175	0.83	865	0.160	1.19	941
Propyl isobutyrate	0.475	0.722	836	0.340	1.130	926	0.280	1.40	970	0.270	1.69	1028
Isopropyl isobutyrate	0.370	0.565	785	0.240	0.770	850	0.185	0.86	868	0.185	1.23	952
Butyl isobutyrate	0.770	1.17	933	0.550	1.83	1024	0.475	2.21	1060	0.615	2.80	1141
2-Methylpropyl isobutyrate	0.660	0.997	900	0.460	1.490	980	0.380	1.73	1010	0.360	2.25	1090
Pentyl isobutyrate	1.265	1.87	1032	0.930	3.10	1125	0.780	3.54	1152	0.660	4.10	1244
3-Methylbutyl isobutyrate	1.050	1.59	998	0.760	2.46	1080	0.615	2.86	1108	0.550	3.44	1192
Hexyl isobutyrate				1.482	4.94	1220	1.225	5.70	1250	1.050	6.57	1341
Octyl isobutyrate				4.110	13.28	1416	3.125	14.55	1443	2.325	15.50	1541
Methyl pentanoate	0.430	0.650	814	0.290	1.07	915	0.300	1.40	970	0.300	2.00	1060
Ethyl pentanoate	0.595	0.900	879	0.450	1.50	978	0.410	1.90	1029	0.370	2.31	1100
Propyl pentanoate	0.975	1.480	980	0.760	2.46	1080	0.645	3.00	1126	0.580	3.63	1205
Isopropyl pentanoate	0.725	1.110	921	0.505	1.68	1003	0.430	2.00	1040	0.400	2.50	1118
Butyl pentanoate	1.545	2.34	1074	1.240	4.01	1178	1.070	4.86	1218	0.850	5.67	1304
2-Methylpropyl pentanoate	1.300	1.97	1027	0.990	3.30	1138	0.785	3.74	1166	0.710	4.44	1251
Pentyl pentanoate	2.455	3.72	1170	1.850	6.40	1270	1.615	7.50	1312	1.260	8.40	1395
3-Methylbutyl pentanoate	2.060	3.12	1134	1.585	5.28	1232	1.335	6.21	1268	1.00	6.65	1344
Hexyl pentanoate				3.06	10.20	1360	2.555	12.0	1404	1.870	12.46	1491
Octyl pentanoate				—	—	—	6.315	29.8	1589	4.230	28.15	1679

(continued on p. 414)

TABLE II (*continued*)

Ester	*Stationary phase*											
	SE-30			*OV-17*			*OV-25*			*XE-60*		
	$V_{g(a)}$	$V_{R(b)}$	I_R	$V_{g(a)}$	$V_{R(b)}$	I_R	$V_{g(a)}$	$V_{R(b)}$	I_R	$V_{g(a)}$	$V_{R(b)}$	I_R
Methyl 3-methylbutyrate	0.325	0.508	764	0.270	0.900	880	0.235	1.10	915	0.215	1.41	982
Ethyl 3-methylbutyrate	0.490	0.740	840	0.339	1.130	926	0.315	1.44	972	0.275	1.72	1029
Propyl 3-methylbutyrate	0.785	1.19	937	0.570	1.900	1028	0.490	2.25	1063	0.420	2.62	1126
Isopropyl 3-methylbutyrate	0.605	0.92	883	0.370	1.230	943	0.320	1.49	976	0.305	1.91	1053
Butyl 3-methylbutyrate	1.250	1.89	1030	0.920	3.06	1128	0.780	3.58	1156	0.630	4.05	1225
2-Methylpropyl 3-methylbutyrate	1.040	1.58	994	0.730	2.43	1073	0.585	2.86	1108	0.520	3.25	1179
Pentyl 3-methylbutyrate	1.975	2.99	1125	1.650	5.30	1230	1.145	5.45	1243	0.980	6.12	1320
3-Methylbutyl 3-methylbutyrate	1.665	2.52	1088	—	3.95	1175	1.040	4.70	1200	0.795	4.96	1276
Hexyl 3-methylbutyrate	—	—	—	2.590	8.35	1320	1.920	9.14	1345	1.465	9.16	1418
Octyl 3-methylbutyrate	—	—	—				—			3.425	21.42	1616
Methyl hexanoate	0.700	1.06	913	0.550	1.83	1024	0.490	2.25	1063	0.434	2.70	1130
Ethyl hexanoate	0.970	1.47	980	0.750	2.68	1100	0.645	3.08	1128	0.520	3.26	1179
Propyl hexanoate	1.560	2.36	1077	1.390	4.35	1196	1.065	4.90	1219	0.830	5.19	1286
Isopropyl hexanoate	1.175	1.78	1018	0.920	3.07	1123	0.725	3.30	1143	0.580	3.63	1205
Butyl hexanoate	2.475	3.75	1170	2.340	7.30	1296	1.665	7.64	1310	1.260	7.88	1382
2-Methylpropyl hexanoate	2.035	3.08	1131	1.695	5.65	1245	1.315	6.02	1258	1.040	6.51	1339
Pentyl hexanoate	3.940	5.96	1268	3.630	11.72	1392	2.645	12.12	1406	1.960	12.20	1482
3-Methylbutyl hexanoate	3.250	4.93	1227	2.755	9.18	1342	2.020	9.52	1357	1.565	9.79	1433
Hexyl hexanoate	—						4.225	19.68	1508	2.720	18.09	1577
Octyl hexanoate	—									6.220	41.40	1766
Methyl 4-methylpentanoate	0.580	0.88	876	0.500	1.65	1000	0.395	1.84	1020	0.355	2.25	1090
Ethyl 4-methylpentanoate	0.825	1.25	947	0.648	2.16	1051	0.550	2.56	1090	0.455	2.84	1142
Propyl 4-methylpentanoate	1.295	1.96	1038	1.050	3.50	1152	0.850	3.96	1180	0.700	4.38	1244
Isopropyl 4-methylpentanoate	0.995	1.51	985	0.770	2.49	1082	0.585	2.66	1095	0.517	3.20	1173
Butyl 4-methylpentanoate	2.105	3.20	1138	1.655	5.52	1246	1.400	6.37	1270	1.060	6.63	1344
2-Methylpropyl 4-methylpentanoate	1.750	2.65	1100	1.500	4.70	1210	1.055	4.80	1220	0.855	5.35	1294
Pentyl 4-methylpentanoate	3.315	5.02	1232	3.080	9.45	1350	2.185	10.00	1360	1.615	10.10	1441
3-Methylbutyl 4-methylpentanoate	2.805	4.25	1198	2.470	7.70	1308	1.635	7.79	1316	1.380	8.03	1391
Hexyl 4-methylpentanoate				3.975	13.25	1415	3.435	15.80	1466	2.450	15.30	1540
Octyl 4-methylpentanoate	—			—			8.735	40.60	1650	5.625	35.20	1734

parallel plots as did the butyrate, pentanoate and hexanoate esters, but of slightly lower slope. A different behaviour is observed with XE-60, where the slopes are all very similar but with the propionate esters having a slightly greater slope than the other ester series.

When the number of carbon atoms in the acid chain was three or more, an incremental increase in the carbon number in either the acid or alcohol chain resulted in an increase of 95, 97, 96 and 100 retention index units per methylene group using the stationary phases SE-30, OV-17, OV-25 and XE-60, respectively. With the alkyl formates on SE-30 an increase of about 108 retention index units per methylene group in the alcohol chain was observed while on the other phases the differences in slope of the formate and other ester lines were lower and the differences in retention indices were accordingly less.

The increased and decreased retention of the methyl and isopropyl esters on the four phases are shown in Table III.

TABLE III

RETENTION INDEX VARIATION FOR METHYL AND ISOPROPYL ESTERS

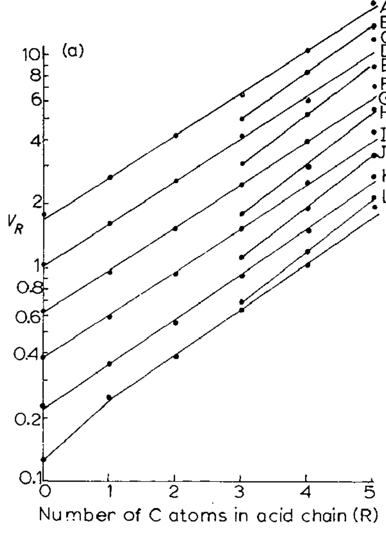
Esters	*Stationary phase*			
	SE-30	*OV-17*	*OV-25*	*XE-60*
Methyl	30	35	30–40	25–50
Isopropyl	20	25	25–35[a]	15–30

[a] Isopropyl formate exhibited a decreased retention of nearly 50 index units.

The relative retention of the various esters when plotted against the number of carbon atoms in the acid chain (R) produced a series of essentially linear relationships on the various stationary phases. The plots for esters on the OV-25 and XE-60 phases are shown in Fig. 2.

The normal esters where R = 4, 5 and 6 produced parallel plots while esters where R = 0 to 3 showed a gradual decrease in slope from R = 0 to R = 3.

Plots of this type have been shown[1] to accentuate the difference in retention behaviour of methyl formate and the other methyl esters on SE-30. The same effect is apparent with the OV-17 and OV-25 phases but with XE-60 the reverse behaviour occurs, *i.e.* methyl formate and the other methyl esters produce a linear relationship while the formates in the other plots show an increased retention in Figs. 2a and b. The difference is shown in Figs. 2a and b, with results on OV-25 and XE-60 stationary phases. With chromatography on the XE-60 stationary phase and to a lesser extent on OV-25 it was apparent that the fit of the points to the various plots was not as satisfactory as with SE-30 and OV-17 or with the α-alkylacrylic esters on the SE-30 stationary phase where linear plots with the acid carbon chain (R) as an ordinate were first reported[4]. Further studies are being undertaken using polysiloxane stationary phase with Rohrschneider constants intermediate between OV-25 and XE-60 and greater than XE-60 using both aliphatic and olefinic esters such that the applicability of the relationship shown in Fig. 2 can be more widely examined.



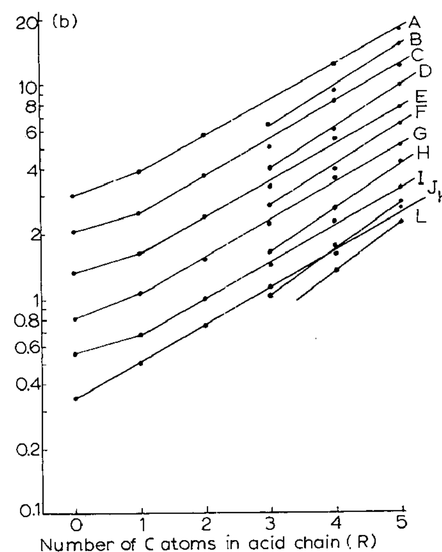


Fig. 2. Plots of the logarithm of the relative retention volume *versus* the number of carbon atoms in the acid chain of saturated esters on (a) OV-25 and (b) XE-60 stationary phases with R of both normal and isostructure while the alcohol chain is linear.
A = $RCOOC_6H_{13}$; B = iso$RCOOC_6H_{13}$; C = $RCOOC_5H_{11}$;
D = iso$RCOOC_5H_{11}$; E = $RCOOC_4H_9$; F = iso$RCOOC_4H_9$;
G = $RCOOC_3H_7$; H = iso$RCOOC_3H_7$; I = $RCOOC_2H_5$;
J = iso$RCOOC_2H_5$; K = $RCOOCH_3$; L = iso$RCOOCH_3$.

It has been shown with SE-30 that normal and isoesters having the same carbon number in the acid chain (R) exhibit a constant decrease in retention index, but the decrease varies for esters of different acid chain length[1]. A similar situation exists with chromatography on the other phases and the incremental decreases for the various ester series on each phase is shown in Table IV.

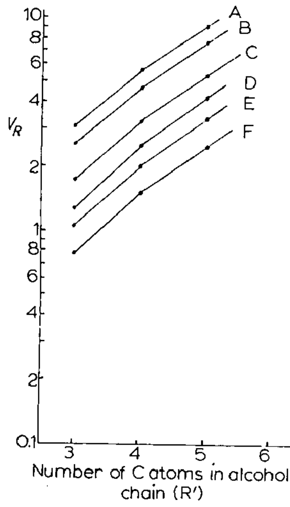
No worthwhile conclusion can be drawn from this comparison. It is evident, however, that the earlier observation[1] that as the number of carbon atoms in the acid chain increases, the decrease in retention due to branching in the acid chain is reduced, is not generally apparent.

The effect of branching in the alkyl groups on both sides of the carbonyl group are shown in Figs. 3 and 4.

TABLE IV

RETENTION INDEX DECREASES ON BRANCHING IN THE ACID CHAIN

Carbon number in acid chain R	*Stationary phase*			
	SE-30	*OV-17*	*OV-25*	*XE-60*
3	47	59	55	45
4	43	47	60	75
5	35	49	41	39



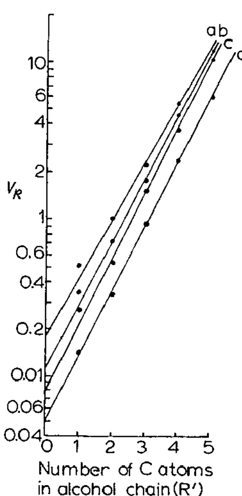


Fig. 3. Plots of logarithm of relative retention *versus* number of carbon atom in the alcohol chain of saturated esters on OV-17 stationary phase with isoalcohol chains (R') and with normal and isoacid chains.

A = $CH_3CH_2CH_2CH_2CH_2COOisoR'$; B = $CH_3—CH(CH_3)—CH_2—CH_2COOisoR'$

C = $CH_3CH_2CH_2CH_2COOisoR'$; D = $CH_3—CH(CH_3)—CH_2COOisoR'$;

E = $CH_3CH_2CH_2COOisoR'$; F = $CH_3—CH(CH_3)—COOisoR'$

Fig. 4. Plots of logarithm of relative retention *versus* number of carbon atoms in the alcohol chain of normal saturated esters where R = R' on (a) SE-30, (b) OV-17, (c) OV-25 and (d) XE-60 stationary phases.

Plots of relative retention for the isoalkyl esters with respect to the isoalkyl branched acid chain esters are shown in Fig. 3 for the OV-17 phase and it is evident that branching of the acid chain reduces the retention of the isoalkyl esters by a constant factor for esters with a constant number of carbon atoms in the acid chain. The retention index differences for the four phases are shown in Table V.

The effect of the carboxyl group on the retention of the *n*-alkyl esters is apparent from Fig. 4. An essentially linear relationship for esters with the same number

TABLE V

EFFECT OF BRANCHING OF ISOALKYL ESTERS

Acid chain length	*Stationary phase*			
	SE-30	*OV-17*	*OV-25*	*XE-60*
3	40	60	63	57
4	46	59	64	52
5	30	35	48	46

of carbon atoms in the R and R′ chains is achieved and a constant effect of the carboxyl group by both R and R′ is indicated.

By extrapolation of the plot to zero, *i.e.* R = R′ = 0, the net retention volume of the carboxyl group was obtained. The net retention of the group increased with increasing polar character of the column and is approximately 304, 410, 450 and 520 retention index units for the SE-30, OV-17, OV-25 and XE-60 stationary phases, respectively.

Examination of the actual retention characteristics on the four stationary phases shows that relative retention volumes on less polar phases are smaller. The retention volume of the hydrocarbon reference compounds decreases with increasing polar character of the column such that esters on the XE-60 phase had the smallest denominator and thereby the highest V_R and I_R, the net retention being highest on the least polar phase.

ACKNOWLEDGEMENT

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CHROM. 4707

GAS CHROMATOGRAPHY OF PARTIALLY METHYLATED ALDITOLS AS TRIFLUOROACETYL DERIVATIVES

I. SEPARATION OF MONO-O-METHYL-PER-O-TFA-D-GLUCITOLS

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SUMMARY

The reduction of mono-O-methyl-D-glucoses to their corresponding mono-O-methyl-D-glucitols and gas chromatography of the trifluoroacetyl derivatives of the latter resulted in an excellent separation of the compounds under investigation. Trimethylsilyl derivatives of the same compounds were not satisfactorily separated. The retention times of the mono-O-methyl-per-O-trifluoroacetyl-D-glucitols were comparable with those of trimethylsilyl derivatives. Trifluoroacetylation of mono-O-methyl-D-glucitols was found to be sufficiently fast for the procedure to be used for routine analyses.

INTRODUCTION

Gas chromatography is a useful tool for both the qualitative and quantitative evaluation of the analysis of polysaccharides by methylation[1,2]. Methanolysis of polysaccharides, and even of monosaccharides, usually results in the formation of complicated mixtures of glycosides, amongst which the methylglycosides of mono-O-methylhexoses are poorly volatile, and when gas chromatographed are almost completely retained in the column.

The problem of poor volatility of the methyl-mono-O-methyl-hexosides was solved by the development of the silylation technique and its application to carbohydrate chemistry[3]. However, the problem of overlapping peaks corresponding to individual anomers and stereoisomers remained. Reduction of monosaccharides to their corresponding sugar alcohols and subsequent gas chromatography as their trimethylsilyl ethers has met with little success[3]. Alditols have been separated by SAWARDEKER *et al.*[4] as their more polar acetates on a copolymerized stationary phase (ECNSS-M). Similar results have also been obtained by SJÖSTRÖM *et al.*[5] using mixed stationary phases. After reduction to the corresponding alditols, BJÖRNDAL *et al.*[6] successfully separated partially methylated aldoses as partially methylated alditol

acetates. Gas chromatography of the acetates of some other D-glucose and D-glucitol methyl ethers was recently reported by JONES AND JONES[7]. The main difficulty encountered in gas chromatography of alditolacetates is the high temperature necessary to obtain reasonable retention times, as usually the temperature is very close to that of the stability of the stationary phase. The possibility of the stationary phase bleeding, which results in an increased noise level when using a flame ionization detector (FID), then becomes an additional hazard.

Only a few workers[8–10] have investigated the use of trifluoroacetates for the gas chromatography of carbohydrates. The trifluoroacetates of some common alditols have been successfully separated by SHAPIRA[11] at a reasonable temperature and with a short analysis time.

The present paper describes the separation of mono-O-methyl-D-glucoses after reduction to their corresponding D-glucitols as per-O-trifluoroacetyl derivatives.

EXPERIMENTAL

Apparatus

A Hewlett-Packard Research Chromatograph, Model 5750 G, with a dual column system and flame ionization detectors was employed. The composition of equilibrium mixtures of monosaccharides was determined quantitatively using Hewlett-Packard Integrator 3370 A.

Operating conditions

The gas chromatography of TMS derivatives was carried out on various columns: (A) 6 ft. × 1/8 in. (O.D.) Al, packed with 10% Apiezon L on Gas-Chrom Z 80–100 mesh; (B) 6 ft. × 1/8 in. (O.D.) Al, packed with 3% ECNSS-M on Chromatone N AW HMDS 70–80 mesh (Lachema, Brno); (C) 10 ft. × 1/8 in. (O.D.) stainless steel, packed with 1% XE 60 on Gas-Chrom Z 80–100 mesh.

Helium was used as a carrier gas. The flow rate was adjusted so that the columns showed the maximum efficiency.

Derivatives

Chromatographically pure mono-O-methyl-D-glucoses, synthesized according to known procedures[12], were used. Before derivatization, the free monosaccharides (3–5 mg) were equilibrated in 1 N H_2SO_4 (3–5 ml) at 100° for 2 h which was found to be sufficient time for the ratio of the α- and β-forms of the sugars to become

TABLE I

COMPOSITION OF EQUILIBRIUM MIXTURES OF MONO-O-METHYL-D-GLUCOSES AS FOUND BY GLC OF TMS ETHERS

Compound	α- (%)	β- (%)
2-O-Me[a]	54.8	45.2
3-O-Me	41.2	58.8
4-O-Me	47.0	53.0
6-O-Me	45.7	54.3

[a] 2-O-Me-D-glucopyranose etc.

constant. Sulfuric acid was removed by an anion-exchange resin (Ionenaustauscher I OH^-, Merck A.G.) and water was removed under reduced pressure below 40°. The composition of the equilibrium mixtures is shown in Table I.

The TMS derivatives were prepared by dissolving the dry residue, after equilibration or reduction to sugar alcohols, in 0.3 ml of pyridine and adding 0.1 ml of Tri-Sil Concentrate (Pierce Chemical Co.). The mixture was shaken vigorously for about 30 sec and then left at room temperature for 1 h. The samples on storage in a desiccator over P_2O_5 at room temperature showed no change in composition over a period of three months.

The reduction of the mono-O-methyl-D-glucoses into the corresponding D-glucitols and the preparation of the TFA derivatives was carried out in the following manner: 5 mg of each component was dissolved in 2 ml of water, 50 mg of sodium borohydride was added and the mixture was left at room temperature for 4 h. Dilution with water was followed by demineralization with a cation-exchange resin (Dowex 50 W H^+) and evaporation of the boric acid with methanol. The residue was left in a desiccator over P_2O_5 overnight and trifluoroacetylated with 0.3 ml of trifluoroacetic anhydride in the presence of 3 μl of pyridine. The reaction mixture was shaken vigorously for about 30 sec and left at room temperature for 1 h which was found to be sufficient time for the complete conversion of mono-O-methyl-D-glucitols into per-O-trifluoroacetates.

Both TMS and TFA derivatives were prepared in 10 ml drop-shaped vials equipped with polyethylene caps.

RESULTS AND DISCUSSION

Since, up to the present, a satisfactory method for the gas chromatographic separation of a mixture of mono-O-methyl-D-glucoses had not been found we first tried to solve the problem by using the known silylation technique. We employed various stationary phases and several combinations of operating conditions. As shown in Table II the separation achieved was not satisfactory for quantitative work. Our next attempt was to try to separate a mixture of mono-O-methyl-D-glucoses as their TMS ethers after reduction to their respective mono-O-methyl-D-glucitols. Although the reduction to sugar alcohols reduces the number of possible components by half,

TABLE II

RELATIVE RETENTION TIMES OF EQUILIBRATED MONO-O-METHYL-D-GLUCOSES AS TMS ETHERS

Compound	*Column A (210°)*	*Column B (120°)*	*Column C (145°)*
α-2-O-Me	0.82	0.80	0.56
β-2-O-Me	1.17	1.17	0.79
α-3-O-Me	0.65	0.57	0.41
β-3-O-Me	1.03	0.88	0.67
α-4-O-Me	0.78	0.66	0.47
β-4-O-Me	0.89	1.17	0.82
α-6-O-Me	1.04	1.00	0.79
β-6-O-Me	—	1.37	1.00
α-Glucose	1.00 (7.25 min)	1.00 (2.97 min)	1.00 (6.97 min)
Inositol	—	2.86 (8.5 min)	1.64 (11.42 min)

TABLE III

RELATIVE RETENTION TIMES OF EQUILIBRATED MONO-O-METHYL-D-GLUCOSES AS PER-O-TRIFLUORO-ACETATES

Compound	*Column C temp. programmed 130–150° (1°/min)*	*Column C temp. programmed 120° (4 min)–150° (2°/min)*
α-2-O-Me	0.44	0.56
β-2-O-Me	0.50	0.63
α-3-O-Me	0.33	0.44
β-3-O-Me	0.55	0.66
α-4-O-Me	0.37	0.48
β-4-O-Me	0.50	0.62
α-6-O-Me	0.18	0.24
β-6-O-Me	0.26	0.34
Inositol	1.00 (13.8 min)	1.00 (17.7 min)

the separation achieved was still not satisfactory on either of the columns used. It was thus obvious that in order to achieve a satisfactory separation the TMS group had to be replaced by a more polar group. Since BJÖRNDAL *et al.*[6] had previously shown that the acetyl group did not give rise to alditol derivatives having retention times comparable with those of TMS derivatives, we selected the trifluoroacetyl group, which was known to give satisfactory results with common alditols[11] and amino acids[13].

In our experience, trifluoroacetic anhydride fulfills all the conditions necessary as a derivatization agent in the gas chromatography of carbohydrates. Trifluoroacetylation was found to be a fast reaction, the derivatives formed were sufficiently stable and gave retention times comparable with those of TMS ethers. Thus, the present paper demonstrates the possibility of separating a mixture of mono-O-methyl-D-glucoses as the TFA derivatives of the corresponding mono-O-methyl-D-glucitols.

Tables III and IV show the retention times of mono-O-methyl-per-O-trifluoroacetyl-D-glucoses and mono-O-methyl-per-O-trifluoroacetyl-D-glucitols relative to that of inositol which we chose as an internal standard because it never occurres in polysaccharide hydrolyzates.

For comparison we show the separation of a mixture of the equilibrated mixture of mono-O-methyl-D-glucoses as per-O-TMS ethers in Fig. 1; Fig. 2 shows the sepa-

TABLE IV

RELATIVE RETENTION TIMES OF MONO-O-METHYL-PER-O-TRIFLUOROACETYL-D-GLUCITOLS

Compound	*Column C temp. programmed 130–150° (1°/min)*
2-O-Me	0.63
3-O-Me	0.79
4-O-Me	0.88
6-O-Me	0.29
Inositol	1.00 (15.7 min)

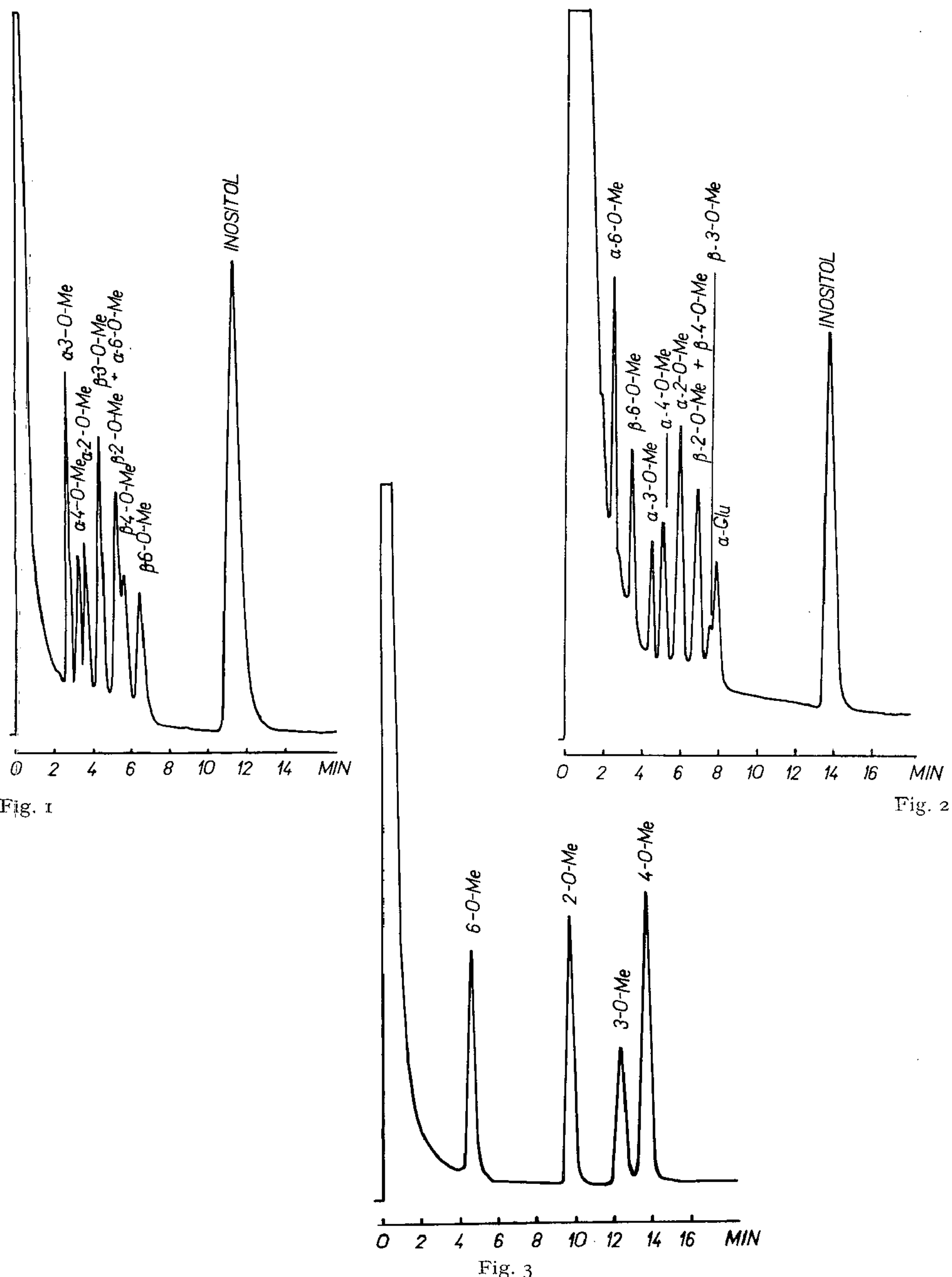

Fig. 1. GLC of an equilibrated mixture of mono-O-methyl-D-glucoses as their per-O-TMS ethers. Column C; 145°. Carrier gas flow rate 20 ml/min.

Fig. 2. GLC of an equilibrated mixture of mono-O-methyl-D-glucoses as their per-O-TFA derivatives. Column C; 130–150° (1°/min). Carrier gas flow rate 30 ml/min.

Fig. 3. GLC of mono-O-methyl-per-O-TFA-D-glucitols. Column C; 130–150° (1°/min). Carrier gas flow rate 25 ml/min.

ration of the same compounds as their per-O-TFA derivatives, and Fig. 3 the separation of a mixture of mono-O-methyl-D-glucoses after reduction to mono-O-methyl-D-glucitols and GC as the per-O-TFA derivatives.

For quantitative work it is necessary that the resolution of consecutive peaks is of the highest possible value (possibly higher than 1). It can be seen from the presented data that the resolution of the TFA mono-O-methyl-D-glucitols was better than 1.5 which corresponds to a separation better than 99.7%.

It is clear that, for both the qualitative and quantitative investigation of mixtures of mono-O-methyl-D-glucoses, the per-O-TFA derivatives are more advantageous than the per-O-TMS-ethers. It is even better to reduce mono-O-methyl-D-glucoses in the mixture to the corresponding D-glucitols and gas chromatograph these as per-O-TFA derivatives. The separation is excellent and the gas chromatography needs less time and a shorter column than in the case of the acetates. The procedure described is suitable for routine analyses. The work on separation of other carbohydrate derivatives as trifluoroacetates is in progress and will be published later.

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CHROM. 4684

DETERMINATION OF O-METHYLATION PRODUCTS OF NORADRENALINE

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SUMMARY

A method for the determination of normetanephrine, norparanephrine and 3,4-dimethoxyphenylethanolamine in aqueous solution is described. Periodate oxidation converts these O-methylated amines to aldehydes which are then extracted with benzene and separated by gas chromatography. Sensitivity is greatly increased if labeled amines are used.

The errors from the oxidation, extraction and chromatographic steps are reported for the unlabeled and labeled compounds. Special attention is given to the estimation of the O-methylated amines when they are derived from [^{14}C]noradrenaline in a mixture containing the competent enzyme.

An enzymatic preparation of [7-^{14}C]normetanephrine and [7-^{14}C]norparanephrine is also described.

INTRODUCTION

Although a mixture of normetanephrine (NM; 3-methoxy-4-hydroxy-β-phenylethanolamine) and norparanephrine (NP; 3-hydroxy-4-methoxy-β-phenylethanolamine) has never been completely resolved in its components, periodate oxidation converts NM and NP into the aldehydes vanillin (V) and isovanillin (IV) respectively, which have been separated by paper chromatography[1,2]. We show that, similarly, periodate oxidation of 3,4-dimethoxy-β-phenylethanolamine (DMPE) yields veratraldehyde (VA).

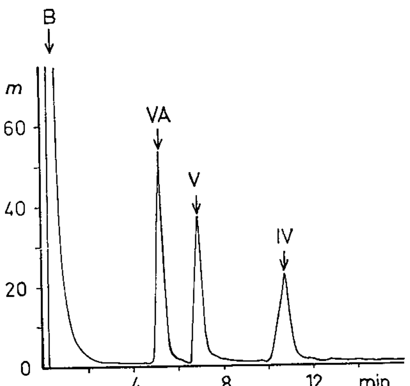
In this report, we describe an analytical method involving periodate oxidation, benzene extraction and gas chromatography, which determines the concentration of NM, NP and DMPE, the three possible O-methylation products of noradrenaline (NA). The accuracy of the method, which uses radioisotopes, is studied not only when the O-methylated amines are dissolved in water, but also when they are in the incubation mixture utilized for the assay of catechol-O-methyltransferase.

EXPERIMENTAL AND RESULTS

Vanillin, isovanillin and veratraldehyde separation by gas chromatography

An Aerograph Autoprep A-700 gas chromatograph equipped with a thermal

* Aspirant du Fonds National Belge de la Recherche Scientifique.



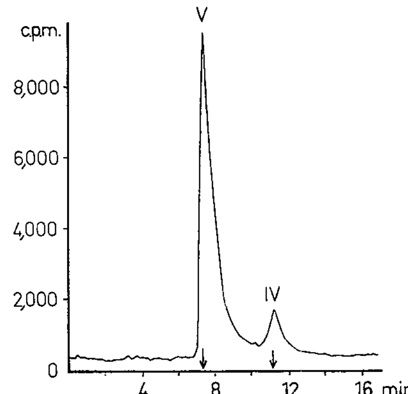


Fig. 1. Gas chromatographic separation of vanillin (V), isovanillin (IV) and veratraldehyde (VA). The injected mixture contains 120 μg of each aldehyde in a total volume of 50 μl of benzene (B). Mass (*m*) *vs.* elution time (min).

Fig. 2. Gas chromatographic separation of ^{14}C-vanillin (V) and ^{14}C-isovanillin (IV). 50 μl of benzene solution containing 15,000 d.p.m. Radioactivity (c.p.m.) *vs.* elution time (min). The arrows indicate the position of the mass peaks. Recording conditions: detector operating voltage: 2100 V; time constant: 10 sec; sensitivity: 10,000 c.p.m. full scale; speed of recording chart: 25 mm/min.

conductivity detector was used. The column was a 180×0.35 cm steel tube; the stationary phase was a mixture of 1% HIEFF (Applied Science Laboratories) and 1% diethylene glycol succinate on Gas-Chrom Q (Applied Science Laboratories). Helium (60 ml/min) was used as the carrier gas; the injection port and the detector were at 220°, while the column temperature was maintained at 168°. Fig. 1 shows a typical separation following the injection of a benzene solution of a mixture of V (Fisher Scientific Co.), IV (K & K Laboratories) and VA (synthesized according to the method of PERKIN AND ROBINSON[3]).

When the aldehydes were radioactive, the helium eluent from the chromatographic column was mixed with methane (60 ml/min) before passing through the 200 ml chrome-plated brass gas-flow proportional detector of a Model 45 Gas Radiochromatograph Nuclear Chicago. The connecting tube was maintained at 225° and the detector at 250°. The gas-flow detector was coupled with a ratemeter and a recorder. The areas under the radioactivity peaks were measured by planimetry. Fig. 2 reproduces the radioactivity trace obtained after injection of a mixture of ^{14}C-V and ^{14}C-IV.

Between 15,000–20,000 d.p.m. in 50 μl benzene were required to obtain good tracings for planimetric measurements. As expected, Table I shows that, as the input d.p.m. decreases, the relative error increases.

Periodate oxidation of the unlabeled O-methylated amines and benzene extraction of the aldehydes

Standard procedure. Between 5–12 ml of an aqueous solution of NM, NP or DMPE was brought above pH 10.5 by the addition of concentrated NH_4OH. Then, a greater than ten-fold excess of $NaIO_4$ was added. After 10 min at room temperature, the solution was cooled to 0° in an ice bath and the pH lowered to 6.5 by the addition

TABLE I

REPRODUCIBILITY OF THE MEASURED ^{14}C-V/^{14}C-IV RATIO WHEN VARIOUS ALIQUOTS OF THE SAME SOLUTION ARE INJECTED SEVERAL TIMES

The solution contains 5 mg of each aldehyde and 825,000 d.p.m. per ml.

Approximate injected volume (µl)	*Approximate radio-activity (d.p.m.)*	*Peak area*[a]		*Number of injections*	*V/IV ratio*
		V	*IV*		
45	37,000	75	6	6	13.1 ± 1.0
30	25,000	50	4	5	13.7 ± 1.0
20	16,000	34	2.5	4	14.3 ± 1.9
10	8,000	15	1.1	6	14.3 ± 4.6
5	4,000	7	<0.5	6	—

[a] Planimeter arbitrary units: 1 cm^2 = 2.68 units.

of 10 *N* H_3PO_4. The cold solution was extracted by 10, 5, 5 and 5 ml of benzene, the organic phases combined and the total volume adjusted to 25 ml with benzene. The concentration of the aldehydes in the benzene solution was determined by gas chromatography by comparison with standard solutions.

Extraction yield. Between 25–30 mg of pure V, IV or VA were dissolved in 12 ml of 2.7 *N* NH_4OH containing 1% $NaIO_4$. Five determinations were performed for each aldehyde and the yield of the benzene extraction was 100 ± 5% for each of them.

Oxidation and extraction yield. Between 30–40 mg of NM·HCl (Calbiochem) or 5–6 mg of DMPE·HCl (synthesized in our laboratory) were dissolved in 2.5 ml of 3 *N* NH_4OH and oxidized by the addition of 5 ml of 5% $NaIO_4$. The yield with NM was 98 ± 5% (five determinations). With DMPE, two extractions with 5 ml of benzene each were sufficient (a third extraction gave no detectable VA, *i.e.* $< 1\%$) and the volume was adjusted to 10 ml. The yield was 94 ± 5% (four determinations).

Oxidation and extraction of labeled O-methylated amines

(A) [7-^{3}H]Normetanephrine

7-^{3}H-NM (0.1 µCi; 23 mCi/mmole, NEC) was dissolved in 5 ml 3 *N* NH_4OH containing 0.4% $NaIO_4$. Benzene extraction was performed according to the standard procedure. The radioactivity of the benzene solution was determined in a Packard Tri-Carb spectrometer after addition of toluene and scintillators (PPO, dimethyl-POPOP); a [^{3}H]hexadecane standard was used to calculate the counting efficiency. The radiochemical yield of the oxidation–extraction procedure was only 79%. Therefore, the experiment was repeated with the addition of 30 to 40 mg of NM·HCl as a carrier, using 5% $NaIO_4$. The chemical yield, determined by gas chromatography, was 98%, while the radiochemical yield still remained around 80%. The missing label was found in the aqueous phase which lacked V.

The percentage loss of tritium was reproducible; seven experiments with 0.1 to 0.2 µCi of 7-^{3}H-NM gave a mean radiochemical yield of 83.6 ± 1.4%.

(B) Preparation of ^{14}C-labeled O-methylated amines

(i) [7-^{14}C]Normetanephrine. 0.2 µmoles of 7-^{14}C-NA (48 mCi/mmole; Schwartz Bioresearch) were incubated for 2 h at 37° with 0.7 ml 0.6 *M* phosphate buffer (pH 7.8),

5.0 ml of a catechol-O-methyltransferase preparation (ammonium sulfate fractionation of the supernatant from rat liver, followed by dialysis of the dissolved precipitate[4]), 100 μmoles $MgCl_2$, 10 μmoles (—)S-adenosyl-L-methionine (P.L. Biochemicals) and 5 μmoles cysteine·HCl (total volume = 7.0 ml). The reaction was terminated by the addition of 7 ml 5% trichloroacetic acid (TCA). The precipitate was centrifuged and the supernatant lyophilized. The lyophilization residue was then dissolved in 2 ml of 0.2 *M* ammonium acetate buffer (pH 6.1) and the solution was applied on a Rexyn 102 (H^+) column (35×2 cm) packed and equilibrated according to KIRSHNER AND McGOODALL[5]. The column was eluted by 0.4 *M* ammonium acetate buffer (pH 5.2); four 4.0 ml fractions were collected every hour and the radioactivity of a 0.1 ml aliquot of each fraction was determined (Fig. 3).

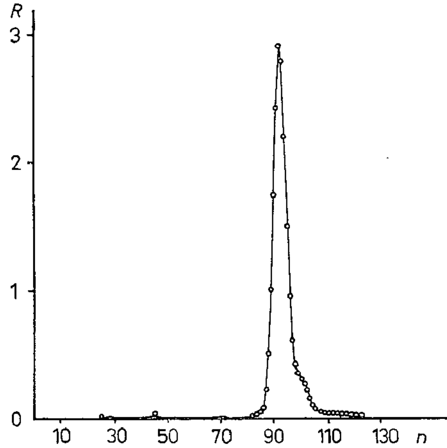


Fig. 3. Chromatography of the enzymatically synthesized ^{14}C-labeled normetanephrine and norparanephrine. Radioactivity (*R* in 10^6 d.p.m./0.1 ml) of fractions (*n*) eluted from the Rexyn 102 column. The main peak (maximum at 93) contains 7-^{14}C-NM, while the shoulder (beginning with fraction 100) also contains 7-^{14}C-NP.

Paper chromatography[6] of an aliquot of the pooled fractions 92–95 yielded only one radioactive spot which migrated at an R_F identical to pure NM. Periodate oxidation, benzene extraction and gas chromatography also yielded one radioactive peak, identified as V. From these results, we concluded that the pooled 92–95 fractions contained 7-^{14}C-NM with a radiochemical purity above 98%.

(ii) [7-^{14}C]Norparanephrine. Paper chromatography of the pooled fractions from the 100–102 Rexyn column yielded two radioactive spots. One spot (61% of the total radioactivity) migrated with an R_F identical to pure NM. This spot was eluted, an aliquot removed for immediate counting and another aliquot subjected to periodate oxidation and benzene extraction. The radiochemical yield of the oxidation-extraction was 92 ± 10%. Gas chromatography of the organic extract gave both radioactive V and IV, but the peaks were too small to be accurately measured.

The second spot (39% of the total radioactivity), eluted from the paper, was likewise treated. The radiochemical yield after oxidation and extraction was 12% and no radioactive V, IV or VA peaks were observed after gas chromatography. The second spot thus contained unidentified radiolabeled contaminants.

To determine the ratio of NM to NP, a sample of the pooled fractions 100–102 was oxidized by periodate, the solution was benzene extracted and the organic phase studied by gas chromatography. The radioactive peaks were measured and the ratio V/IV was found to be 0.23. Since the radioactivity of the mixture NM + NP amounted to 61% of the total, this ratio indicates that the ^{14}C of the pooled fractions 100–102 consisted approximately of 50% 7-^{14}C-NP, 11% 7-^{14}C-NM and 39% of unknown impurities. Further attempts to purify the 7-^{14}C-NM were unsuccessful because the amount involved was too small.

(C) Oxidation-extraction yield with 7-^{14}C-NM and 7-^{14}C-NP

Using 0.026 μCi of the 7-^{14}C-NM prepared in section *B (i)* in four different experiments, the oxidation-extraction yield was found to be 95 ± 1%. The impure preparation of NP described in section *B (ii)* was used to determine the yield of the oxidation-extraction procedure for ^{14}C-NP. In computing the yields, the radioactivity of the starting solution was corrected for the 39% of impurities observed by paper chromatography; the radioactivity of the organic phase was further corrected to account for the 12% of these impurities which were extracted in the organic phase. The combined yields for the labeled NP and NM were calculated to be 89 ± 2% (five experiments with 0.0294 μCi of the mixture, *i.e.* 0.0147 μCi of NP).

Determination of the labeled O-methylated amines in an incubation mixture containing catechol-O-methyltransferase

(A) The standard mixture used in the experiments with catechol-O-methyltransferase contained, in a total volume of 2.5 ml, 2 ml of 0.06 *M* sodium phosphate buffer (pH 7.8), 5 mg of proteins, 2 μmoles $MgCl_2$, 2 μmoles (—)S-adenosyl-L-methionine, 0.1 μmole cysteine·HCl. After addition of the labeled O-methylated amine, 2.5 ml 5% TCA were added. The precipitate was collected by centrifugation and washed twice with 1.3 ml 2.5% TCA. To the three combined supernatants, the following reagents were added: 5 μg NM·HCl, 10 μg NA·bitartrate hydrate, 1.25 ml concentrated NH_4OH and 1 ml 1% $NaIO_4$ (the quantity of periodate must be adjusted to the quantity of substances to be oxidized). After 30 min, the mixture was cooled to 0° in an ice bath, the pH adjusted to 6.5 and the solution extracted according to the standard procedure. Table II shows that, for 7-^{14}C-NM (0.025 μCi), the yield was about 94%.

(B) When determining catachol-O-methyltransferase activity, the incubation mixture also contains unreacted ^{14}C-NA. Five experiments with 0.11 μCi of 7-^{14}C-NA as the only labeled compound in the incubation mixture showed that 0.6 ± 0.06% of the periodate degradation products contaminated the organic phase.

The yields of oxidation-extraction for ^{14}C-NM and ^{14}C-NP were then studied in the presence of ^{14}C-NA; the values, corrected for the contamination by NA degradation products, appear in Table II.

(C) In most cases, when the labeled O-methylated amines were produced by the enzyme from ^{14}C-NA, the benzene solution of the radioactive aldehydes was too dilute for direct chromatographic analysis. Therefore, a concentration step was added.

The solution containing a mixture of ^{14}C-NM and ^{14}C-NP was oxidized with periodate and extracted with benzene. The radioactivity of an aliquot of the organic phase was determined and 100 μg of V, IV and VA were added per 20,000 d.p.m. to

TABLE II

RADIOCHEMICAL YIELD AFTER OXIDATION OF 7-^{14}C-NM AND 7-^{14}C-NP PRESENT IN CATECHOL-O-METHYLTRANSFERASE STANDARD INCUBATION MIXTURE AND BENZENE EXTRACTION

Substance	*Quantity (μCi)*	*Number of experiments*	*7-^{14}C-NA (μCi)*	*Yield (%)*
7-^{14}C-NM	0.025	5	0	93.8 ± 1.7
	0.0068 to 0.0135	13	0.11	93.2 ± 2.1
7-^{14}C-NP	0.0147[a]	5	0.54	85 ± 3.5

[a] 0.0294 μCi of the impure preparation.

the organic phase which was then dried on sodium sulfate and concentrated by vacuum distillation to have 400,000 d.p.m. per ml. 50 μl were injected in the gas chromatograph; mass and radioactivity tracings were simultaneously recorded. The ratio of the areas under the V and IV radioactivity peaks gave the NM/NP ratio in the primary solution.

The aldehydes evaporated during the concentration process, but it was shown by the mass peaks that the ratio V/IV did not change.

DISCUSSION

NM, NP and DMPE concentrations in aqueous solution are determined by periodate oxidation to V, IV and VA, followed by benzene extraction and gas chromatographic separation of the aldehydes. Trace amounts of these O-methylated amines can be determined if they are labeled. Addition of carrier aldehydes to the benzene solution and simultaneous records of mass and radioactivity in the effluent from the chromatographic column allow the identification of the radioactive peaks and the estimation of their isotope content by planimetric measurement of their areas.

Above pH 10.5, the yield of the periodate oxidation of milligram amounts of NM into V or of DMPE into VA is at least 95%. At pH 6.5 at 0°, the aldehydes (V, IV, VA) are quantitatively extracted from aqueous solutions into benzene. At a lower pH or a higher temperature, the yield of the oxidation step decreases due to the increasing oxidizing power of the periodate.

When using 7-^{3}H-NM, the radiochemical yield of the oxidation into 7-^{3}H-V is lower (79%) than the chemical yield (98%). As the ^{3}H-C bond is stable both in NM and V, the exchange of the ^{3}H must take place during the oxidation process itself, probably at the level of the intermediary complex. Although the loss of ^{3}H was reproducible (16.4 ± 1.4%), we nevertheless decided to use the ^{14}C-labeled amines instead.

7-^{14}C-NM and 7-^{14}C-NP were prepared enzymatically from 7-^{14}C-NA. The final 7-^{14}C-NM was radiochemically pure (more than 98%), while we obtained a preparation of 7-^{14}C-NP whose radiochemical purity was only 50%. It was shown with 7-^{14}C-NM that the radiochemical yield of oxidation into V was the same as the chemical yield. The presence of impurities in the 7-^{14}C-NP preparation necessitated corrections in the computation of the yield of oxidation into IV; nevertheless, the computed radiochemical yield closely agrees with the experimental data obtained for 7-^{14}C-NM.

The yield for ^{14}C-DMPE could not be verified because this compound was unavailable. However, as the chemical yield for the oxidation of the unlabeled amine is about 95%, it is very probable that the radiochemical yield for the labeled product will be of the same order of magnitude.

The three aldehydes (V, IV, VA) can be separated by gas–liquid chromatography. The separation is completed in less than 15 min and the amount of radioactivity associated with each aldehyde can be computed from the areas of their respective peaks. This procedure can thus be used to estimate the *para* and *meta*-O-methylated, and the *meta,para*-O-dimethylated products eventually obtained by the action of catechol-O-methyltransferase or other enzyme preparations on ^{14}C-NA.

When the benzene solution is concentrated before the chromatographic analysis, a loss of the aldehydes occurs, but the ratio V/IV remains unchanged; thus, the ^{14}C-NM/^{14}C-NP can still be determined. As the radioactive assay before concentration yields the NM + NP value, it is therefore possible to determine ^{14}C-NM and ^{14}C-NP separately in the incubation mixture.

Since periodate oxidation of the O-methylated derivatives of adrenaline (metanephrine, paranephrine and N-methyl DMPE) also yields the same aldehydes (V, IV, VA respectively), this method may also be applicable if adrenaline serves as substrate for the enzyme; however, the method will not permit to differentiate between the products obtained from the two catecholamines if both are present unless they are labeled with different isotopes and the proportional counter equipped with a two channel analyzer. 3-Methoxy-4-hydroxymandelic (VMA) and 3-hydroxy-4-methoxymandelic (iso-VMA) acids also yield V and IV on periodate oxidation[7] so that the method can also be used to determine these two compounds.

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NOVEL DETERMINATIONS OF PICLORAM BY GAS–LIQUID CHROMATOGRAPHY*

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SUMMARY

The herbicide 4-amino-3,5,6-trichloropicolinic acid (Picloram, Tordon) can be analyzed by gas–liquid chromatography both in trimethylsilylated and decarboxylated forms. The latter compound, 4-amino-3,5,6-trichloropyridine, may be obtained either directly from soil samples or from suitable extracts. It is easily purified by column chromatography on silica gel and can be found in a minimum amount of three picograms by Ni-63 electron capture detection. Using the decarboxylation of soil extracts, linear calibration curves can be established for the range between 10 and 1000 p.p.b. The minimum residue level detectable by this method is less than 5 p.p.b., starting from a 1 g sample.

INTRODUCTION

The herbicide Picloram is widely used in agricultural and military applications. Its main target in the United States is the control of woody plants and broad-leaved weeds. While most grasses are unaffected by high levels of Picloram, certain crops such as beans or tomatoes are extremely susceptible. Consequently, a reliable and highly sensitive method for the determination of Picloram residues in soil is of some importance.

Most present methods use electron capture gas–liquid chromatography (EC–GLC) of the Picloram methyl ester. This approach has obvious advantages, since Picloram responds strongly in the EC detector. Esterification then confers the volatility required for GLC and also allows a more efficient column chromatographic clean-up.

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** Data in this paper are taken from Doctoral Thesis.

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LEAHY AND TAYLOR[1] extracted soil with 0.05 N KOH in 15% aqueous KCl, acidified and partitioned into ethyl acetate. They esterified the extract prior to a Florisil clean-up and analyzed by EC–GLC. Alkaline extraction is quite effective; the free acid Picloram can be recovered from various types of soils by 0.1 N NaOH up to 97.5%, as YOUNGSON *et al.*[2] have shown with labeled material. BJERKE *et al.*[3] used a similar extraction technique, but cleaned up the free acid on alumina before esterification and EC–GLC. MERKLE *et al.*[4] and SAHA AND GADALLAH[5] extracted Picloram from soil with acidic acetone and analyzed the methyl ester by EC–GLC without prior column clean-up.

In our hands, these methods became difficult to operate, and ambiguous to interpret, at Picloram levels between 10 and 100 p.p.b., a concentration range of considerable importance. Not only was good quantitation difficult to achieve, but positive identification of peaks as originating from Picloram demanded additional amounts of work, expertise, and/or faith.

Consequently, we investigated methods for the analysis of Picloram by GLC other than the ones based on the methyl ester. The trimethylsilyl ether (TMS) derivative and the decarboxylated Picloram presented themselves as obvious possibilities. The latter compound had been found and characterized by PLIMMER AND KEARNEY[6] as a by-product in the extraction of Picloram containing soils with acetonitrile. (In two recent notes, HALL *et al.*[7,13] reported the use of a pyrolysis technique for on-column decarboxylation of Picloram.) The following paragraphs describe our attempts to develop these possibilities into methods suitable for the determination of Picloram in standard solutions and soil extracts.

EXPERIMENTAL

Trimethylsilylation

Using a closed-vial technique similar to one described by GEHRKE *et al.*[8], optimum time and temperature conditions were determined for the reaction of Picloram with bis(trimethylsilyl)trifluoroacetamide (BSTFA, ref. 9). The optimum conditions were 100° for 15 min, resulting in a single, symmetrical GLC peak by flame ionization detection. The structure of the compound formed was not further investigated; however, we assumed it to represent Picloram trimethylsilylated at the carboxyl group. This assumption was based on the fact that under similar trimethylsilylation conditions 4-amino-3,5,6-trichloropyridine (decarboxylated Picloram) remained unaffected. This assumption seems further justified when steric hindrance by the chlorine atoms vicinal to the amino group is considered. The reactivity of the amino group is further reduced by the inductive effects of the three chlorine atoms and the carboxyl group.

$$\text{Picloram (}NH_2\text{, Cl, Cl, Cl, COOH pyridine)} + CF_3CON[Si(CH_3)_3]_2 \xrightarrow[15\,\text{min}]{100^\circ} \text{(}NH_2\text{, Cl, Cl, Cl pyridine)}COOSi(CH_3)_3$$

At optimized conditions, varying amounts of Picloram from 5 to 500 μg were derivatized with 0.2 ml of BSTFA, which functioned both as a reagent and as a solvent. Injecting 2 μl onto a 10% OV-17 on Chromosorb W-HP, 100/120 mesh column, the

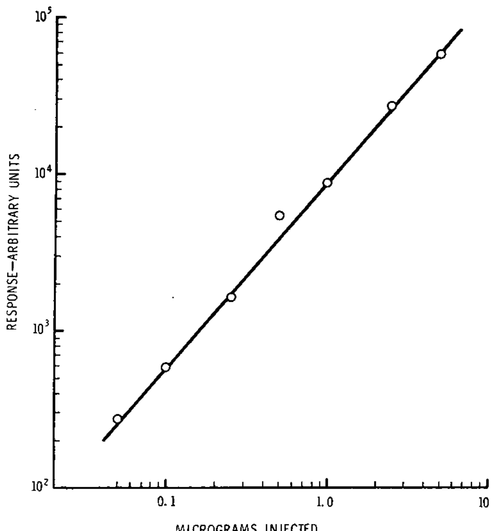

Fig. 1. Standard curve for Picloram-BSTFA. Reaction conditions: 0.2 ml BSTFA, 100°, 15 min. Injection: 2 μl. Column: 10% OV-17 on 100/120 mesh Chromosorb W-HP, 2.0 m × 4 mm I.D. Pyrex. Oven temperature: 220° isothermal. N_2 flow rate: 60 ml/min. MicroTek model MT-220, FID.

standard curve shown in Fig. 1 was obtained. It indicates that the reaction *per se* is suitable for application in a quantitative analysis.

Extracts of natural materials, however, often present serious GLC background problems after trimethylsilylation. BSTFA in particular is an extremely efficient reagent, encompassing a wide spectrum of possible substrates. Unless a rigorous clean-up is possible, low residue levels can often not be determined in complex matrixes (*e.g.* see ref. 10).

Consequently, the detection of the TMS derivative by a selective means, electron capture, was investigated. The great excess of BSTFA, however, seriously disturbed the Ni-63 EC detector used in this study. An attempt to remove the excess BSTFA and dissolve the derivative in an aprotic, non-polar solvent met with failure, since the derivative hydrolyzed very easily and serious errors in quantitation resulted.

At present, the use of the trimethylsilyl derivative of Picloram for analytical GLC is confined to the hydrogen flame detector. Due to the success of the decarboxylation method as described in the following paragraphs, no further studies concerning the trimethylsilylation of Picloram present in biological extracts were attempted.

Decarboxylation

The decarboxylated product, 4-amino-3,5,6-trichloropyridine, possesses unique advantages for low-level residue analysis. It responds strongly in the EC detector and can be synthesized and kept in reasonable purity as a standard. It is not prone to hydrolysis like many other types of derivatives and is formed in a very specific reaction. Easily purified by column chromatography, it chromatographs well on a variety of

GLC columns. At least in our hands, however, difficulties associated with the decarboxylation reaction required careful control of the reaction conditions.

The decarboxylation of Picloram was conducted at different temperatures and reaction times, with or without added catalysts, in culture tubes with teflon-lined screw-caps. About 3 h at 150° were necessary to effect a maximum of decarboxylated product. With 5 to 10 µl of concentrated HCl added per ml acetonitrile, maximum decarboxylation was achieved in 15 min at 150°. Fig. 2 shows a representative yield study in this series of experiments.

Under these conditions, aqueous HCl hydrolyzed the solvent acetonitrile to acetamide and finally acetic acid and ammonium chloride. Small amounts of these products, however, did not seriously interfere with the GLC analysis. In contrast to the trimethylsilylation reaction, the solvent used in decarboxylation could be evaporated under a stream of dry nitrogen without loss of decarboxylated Picloram. The compound was then redissolved in hexane or a suitable non-polar solvent and injected into the gas chromatograph. This change of solvent resulted in much better performance of the EC detector. With pure, recrystallized material, the standard curve shown in Fig. 3 was obtained. It illustrates the range of linear response of the EC detector. The minimum detectable amount was approximately 3 pg injected. For undetermined reasons, the extrapolated calibration curve does not go through the origin.

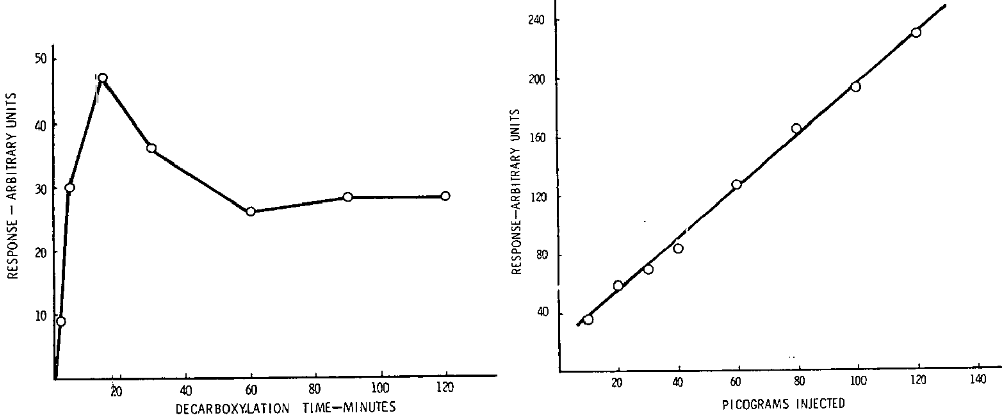

Fig. 2. Decarboxylation of 10 ng of Picloram as a function of time. Reaction conditions: 1 ml of acetonitrile, 10 µl of conc. HCl, 150°. Injection: 5 µl hexane solution. Column: 1.5% OV-17 + 1.95% QF-1 on 100/120 mesh Chromosorb W-HP, 1.8 m × 4 mm I.D. Pyrex. Oven temperature: 155°. N_2 flow rate: 45 ml/min. MicroTek model MT-220, Ni-63 detector, d.c. mode.

Fig. 3. Standard curve for 4-amino-3,5,6-trichloropyridine. Injection: 5 µl hexane solution. Column: 1.5% OV-17 + 1.95% QF-1 on 100/120 mesh, Chromosorb W-HP, 1.8 m × 4 mm I.D. Pyrex. Oven temperature: 155°. N_2 flow rate: 45 ml/min. MicroTek model MT-220, Ni-63 detector, d.c. mode.

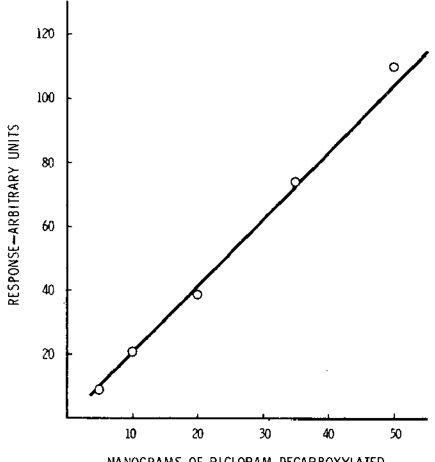

Fig. 4. Standard curve for the decarboxylation of Picloram. Reaction conditions: 1 ml acetonitrile, 10 μl conc. HCl. 150° for 15 min, 5 μl injection. Column: 1.5% OV-17 + 1.95% QF-1 on 100/120 mesh Chromosorb W-HP, 1.8 m × 4 mm I.D. Pyrex. Oven temperature: 155°. N_2 flow rate: 45 ml/min. MicroTek model MT-220, Ni-63 detector, d.c. mode.

Varying amounts of the free acid Picloram were decarboxylated under identical conditions with the results shown in Fig. 4. These, and all other response data in this paper, represent peak heights. The curve indicates that—even though the yield of decarboxylated product is less than theoretical—the reaction can serve as the basis for an analytical method.

Decarboxylation on soil

The decarboxylation of Picloram on soil was tested with Menfro silt loam, a soil of medium organic content. 5 ml of acetonitrile, 50 μl of conc. HCl, and 1 g of soil were put into a 16 × 75 mm culture tube (Corning Glass Company), and the tube capped with a teflon-lined plastic screw-cap and heated at 150° for ½ h in an oil bath behind a safety shield. After cooling, the sample was centrifuged, the supernatant decanted, and the residue resuspended twice in 5 ml portions each of acetonitrile and centrifuged. The combined extracts were evaporated to dryness and the residue taken up in hexane for injection into the gas chromatograph. All organic solvents used were Mallinckrodt, Nanograde. In this method, the right amount of HCl depends, of course, on the type of soil. While the method is fast, it extracts more interfering material than the following one, and as a result, the minimum detectable limit is only 0.1 p.p.m.

Decarboxylation of a soil extract

This more sensitive, albeit somewhat lengthier, method uses a modification of extraction and partition steps described in the literature[2]. The solvent is then changed from ethyl acetate to acetonitrile, and the Picloram decarboxylated and purified on a silica gel column. The minimum detectable limit is 5 p.p.b., and the minimum limit of quantitation about 10 p.p.b. Column clean-up is necessary only at these extremely low levels; from 30 p.p.b. upward this step can be omitted and the analysis consider-

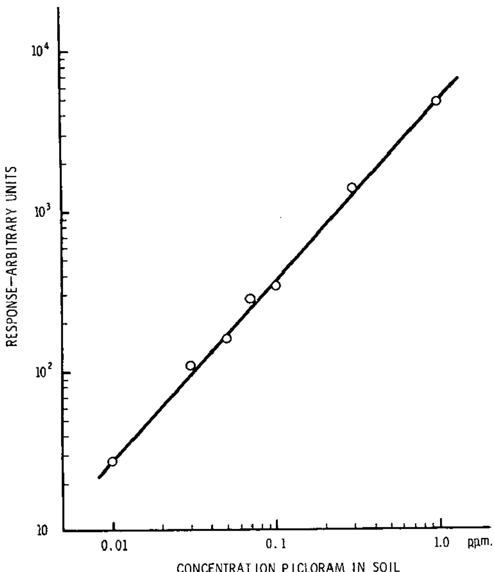

Fig. 5. Standard curve for the extraction of Picloram from soil. Column: 1.5% OV-17 + 1.95% QF-1 on 100/120 mesh Chromosorb W-HP, 1.8 m × 4 mm I.D. Pyrex. Oven temperature: 155°. N_2 flow rate: 45 ml/min. MicroTek model MT-220, Ni-63 detector, d.c. mode.

ably accelerated. In repeated checks, recovery of decarboxylated Picloram from the silica gel column was always higher than 90%.

Fig. 5 shows a standard curve for Picloram recovered from Menfro silt loam spiked with 10 to 1000 p.p.b. of the herbicide. Quadruplicate analyses were done at the 10, 100, and 1000 p.p.b. levels, resulting in relative standard deviations of 23%, 15%, and 5%, respectively. The other points shown are single analyses. A 5 p.p.b. sample gave a clearly discernible peak for Picloram but could not be quantitated with reasonable accuracy. Fig. 6 shows two representative chromatograms from soils with low and high organic content spiked with 10 p.p.b. of Picloram. In the following paragraph a typical procedure of analysis is outlined in detail.

Typical soil analysis

Shake 1 g of soil for 3 min with 3 ml of 1 *N* KOH in a 16 × 75 mm culture tube on a Vortex mixer (Scientific Industries, Springfield, Mass). Centrifuge, decant the supernatant into a 50 ml tube, and repeat twice. Acidify the combined supernatants with 1 ml conc. HCl, and partition twice with 10 ml portions of ethyl acetate. Evaporate the combined ethyl acetate layers to dryness in a 16 × 75 mm screw-cap culture tube, using a 90° sand bath and a gentle stream of nitrogen. Add 1 ml acetonitrile and 5 µl conc. HCl, cap the tube securely and keep in an oil bath at 150° for 15 min. (This part of the procedure should be carried out behind a safety shield with due caution. Samples with leaking caps must be discarded.) Remove the tube from the oil bath, cool, open, and evaporate the acetonitrile under a stream of nitrogen with gentle heating, not exceeding 40°. Prepare a column for liquid chromatography by loosely plugging a 230 mm Pasteur disposable pipet (Fisher Scientific Company) with glass wool and fill

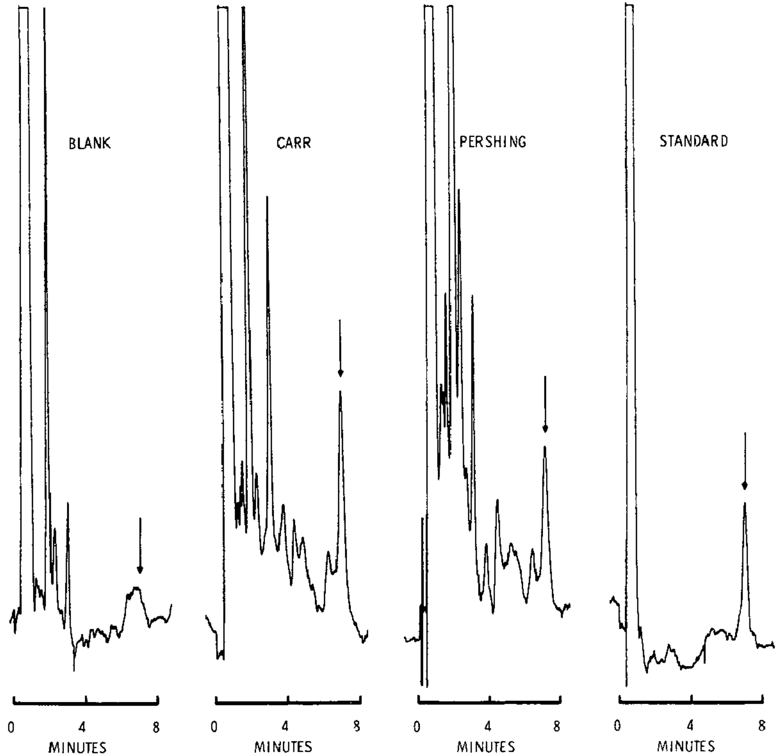

Fig. 6. Chromatograms of Pershing and Carr soils spiked with 0.01 p.p.m. Picloram. Extracts cleaned up on silica gel. Injection: 4 μl out of 1 ml. Column: 3.5% OV-17 + 4.5% QF-1 on 80/100 mesh Chromosorb W-HP, 1.8 m × 4 mm I.D. Pyrex. Oven temperature: 160°. N_2 flow rate: 40 ml/min. MicroTek model MT-220, Ni-63 detector, R.F. mode, 60 V, 270 μsec interval, 10 μsec width.

it about three fourths full with a slurry of No. 923 Silica Gel (Fisher Scientific Company) in hexane. (Pre-extract the silica gel with acetone for 3–4 h in a soxhlet to remove interfering materials.) Remove air bubbles from the column and introduce the residue dissolved in hexane. Elute with 15 ml of 5% acetone in hexane, evaporate the eluate to dryness under a stream of nitrogen, redissolve in hexane and inject a suitable aliquot into the gas chromatograph. The following GLC conditions have been used in our study, but are by no means the only ones possible: MicroTek MT 220 gas chromatograph with 1.8 m × 4 mm I.D. Pyrex U-tube column filled with 3.5% OV-17 + 4.5% QF-1 (1.5% OV-17 + 1.95% QF-1 in some experiments) on Chromosorb W-HP, 80/100 mesh[11,12]. Column temperature 155°, injector 235°, detector 290°; nitrogen flow rate 45 ml/min; d.c. voltage at 90% of standing current, or R.F. potential 60 V, pulse interval 270 μsec, pulse width 10 μsec. Run samples interspersed with standards to account for changes in detector sensitivity. Calculate the amounts of Picloram present from peak heights.

Reliability studies

The described method was tested on four different soils: (1) Carr loamy fine

TABLE I

PERCENT RECOVERY OF PICLORAM FROM FORTIFIED SOILS

Average of three determinations.

Soil type	*Organic content*	*100 p.p.b.*	*10 p.p.b.*
Pershing silt loam	High	94	68
Menfro silt loam	Medium	94	62
Leta silty clay loam	Medium	93	74
Carr loamy fine sand	Low	101	72

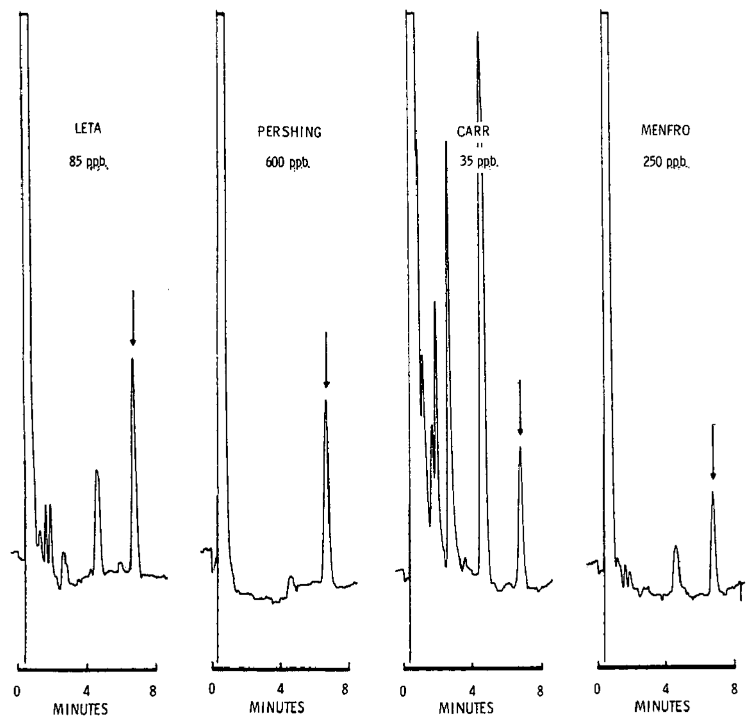

Fig. 7. Chromatograms of Pershing, Menfro, Leta and Carr soils spiked at levels unknown to the analyst. Extracts not cleaned up on silica gel. Column: 3.5% OV-17 + 4.5% QF-1 on 80/100 mesh Chromosorb W-HP, 1.8 m × 4 mm I.D. Pyrex. Oven temperature: 160°. N_2 flow rate: 40 ml/min. MicroTek model MT-220, Ni-63 detector, R.F. mode, 60 V, 270 μsec interval, 10 μsec width. Sample volume and injection size adjusted as required.

TABLE II

ANALYSIS OF SOILS OF UNKNOWN PICLORAM CONTENT

Soil	*p.p.b. present*	*p.p.b. found*	
Pershing silt loam	600	600	[a]
Menfro silt loam	250	190	190
Leta silty clay loam	85	85	67
Carr loamy fine sand	35	44	38

[a] Sample lost during decarboxylation due to leaking cap.

sand (low in organic matter) from the Missouri river bottom; (2) Leta silty clay loam (moderate organic matter) from the Missouri river bottom; (3) Menfro silt loam, courtesy of the Plant Pathology Department, University of Missouri; (4) Pershing silt loam (moderately high organic matter) from an upland area. All four soils were collected in Boone County, Mo.

The results of the analyses at 10 and 100 p.p.b. levels are shown in Table I. Clean-up on the silica gel column was used only for the 10 p.p.b. samples.

As a final test, each of the four soils was fortified with an amount of Picloram unknown to the analyst. Duplicate determinations were run on each soil according to the described procedure. The analyst decided in each case that silica gel clean-up was not required. The results are shown in Table II, and representative chromatograms appear in Fig. 7. The average time spent by the analyst in this study was 1.25 h per sample (= total time spent divided by number of samples).

DISCUSSION

The use of two derivatives of the herbicide Picloram for determination by GLC has been investigated. The trimethylsilylated product can be determined by flame ionization, but causes appreciable difficulties when the EC detector is used. If a good clean-up method for biological extracts could be found and the detection difficulties could be circumvented with another type of selective detector, this approach should develop into a suitable analytical method. A procedure to remove the excess silylation reagent before GLC would permit the use of the EC detector and thus provide an additional, sensitive means of confirming the presence or absence of Picloram.

The decarboxylation of Picloram in its two versions proved far more successful. Of these methods, the decarboxylation on soil is faster and eliminates the need for separate extraction and partition steps. The present minimum level of quantitation, however, is only around 100 p.p.b. The method depends to a great degree on the nature of the matrix (the soil) and suitable modifications must be made for each particular type of sample.

The most careful control of parameters during the analytical procedure is called for in the decarboxylation step. If standards, samples, and blanks are not treated exactly alike, the reliability of the data is bound to suffer.

Using the steps of extraction and partition as described in the literature and adding a silica gel column clean-up prior to decarboxylation allows quantitation at the 10 p.p.b. level. We have obtained much "cleaner" chromatograms by decarboxylation than by formation of the methyl ester according to reported procedures. This may, however, be due to our greater familiarity with decarboxylation. If positive identification of a suspected residue in soil is required, concurrent determinations of Picloram both as the methyl ester and as the decarboxylated product provide, in our opinion, the best test for its presence.

Thus, the described method should offer attractive alternatives to the common analysis of Picloram as the methyl ester. The use of the decarboxylated compound allows a fast and reliable determination of Picloram and unequivocal confirmation of results obtained by other procedures.

ACKNOWLEDGEMENTS

The authors are pleased to acknowledge the competent technical assistance of Mr. ERNEST LEAKE and want to thank Dow Chemical Company, Midland, Mich., U.S.A., for several samples of analytical grade Tordon.

They also gratefully recognize the valuable discussions with the Department of Agronomy, University of Missouri, Columbia, Mo.

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CHROM. 4708

GAS CHROMATOGRAPHIC MEASUREMENT OF LEVELS OF FENFLURAMINE AND NORFENFLURAMINE IN HUMAN PLASMA, RED CELLS AND URINE FOLLOWING THERAPEUTIC DOSES

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SUMMARY

A specific and sensitive procedure for the determination of fenfluramine and norfenfluramine at therapeutic levels in human plasma, red cells and urine has been developed. The method involves extraction of the drugs from alkalinised samples into diethyl ether or dichloroethane, with subsequent separation and detection on a gas–liquid chromatograph fitted with a flame ionisation detector. N,N-Diethylaniline is used as an internal standard for quantitation by the relative peak area technique. Levels encountered after oral ingestion of a single therapeutic dose are reported.

INTRODUCTION

Fenfluramine (N-ethyl-α-methyl-3-trifluoromethyl-phenethylamine) is widely prescribed in the hydrochloride form as "Ponderax" for the treatment of obesity. A gas–liquid chromatographic (GLC) procedure has been described for the determination of the parent drug and its de-ethylated metabolite, norfenfluramine, in human urine after therapeutic dosage[1]. This method lacks the sensitivity required for the accurate estimation of these compounds in plasma at therapeutic levels[2,3] and has the further disadvantage that the use of amphetamine as an internal standard may lead to erroneous results in patients who may have ingested this compound as well. BRUCE AND MAYNARD[4] have reported a GLC method with blood involving the formation of a heptafluorobutyryl derivative of fenfluramine and the use of an electron-capture detector. Although sensitive to picogram quantities, this procedure is time consuming and is limited to a small linear range of detection, so making it unsuitable for the analysis of a large number of samples with a wide range of levels. The method described in the present work is comparatively quick and simple, one sample taking an hour, and has been successfully applied to the measurement of fenfluramine and norfenfluramine in blood and urine after therapeutic administration, as well as to cases of overdosage.

EXPERIMENTAL

Reagents

Diethyl ether was purified by re-distillation over sodium wire. 1 *N* and 2 *N* sodium hydroxide and 1 *N* sulphuric acid were washed with re-distilled diethyl ether before use. The internal standard was N,N-diethylaniline. All reagents were supplied by Hopkin & Williams Co. Ltd., Chadwell Heath, Essex, Great Britain.

Gas chromatography

A Pye 104 chromatograph equipped with dual-flame ionisation detectors was used. The signal was recorded on a Honeywell −0.1 to 1.0 mV recorder. The column support was 80–100 mesh acid-washed, dimethyldichlorosilane-treated Chromosorb G, coated with 10% potassium hydroxide from methanol and 10% Apiezon L from methylene chloride as stationary phase. This was packed into silanised glass columns (1.5 m long × 4 mm I.D.) and conditioned at 200° in a stream of nitrogen for 24 h before use. The instrument settings were as follows: temperature, injection port 205° and column 155°; gas flow rates, hydrogen 30 ml/min, nitrogen 30 ml/min and oxygen 350 ml/min.

The high degree of electrical "background noise" encountered at low attenuations (10^{-11} A) mitigated against accurate determinations. By using oxygen in place of air in the detector, an improvement of up to 80% in the detector sensitivity enabled higher attenuations to be used. A high flow rate of oxygen was found to be essential in order to prevent burning out of the detector[5].

Extraction procedure

Plasma. Plasma (10 ml) was made alkaline by the addition of 1.0 ml of 1 *N* sodium hydroxide and extracted by gentle shaking with 10 ml diethyl ether and 1 ml of internal standard solution (0.05 mg% N,N-diethylaniline in diethyl ether) in a mechanical shaker for 5 min. After centrifugation at 4,000 r.p.m., the ether phase was removed from the aqueous phase and stored in a glass-stoppered centrifuge tube at −5° to freeze out dissolved aqueous phase together with plasma constituents which would interfere with subsequent detection. (Drying with anhydrous sodium sulphate resulted in losses of up to 25% fenfluramine.) The organic phase was decanted into a centrifuge tube containing 0.5 ml of 1 *N* sulphuric acid, and the mixture agitated for 5 min on a Fisons "Whirlimixer". The phases were separated by centrifugation at 4,000 r.p.m. for 5 min and the ether layer discarded. The residual aqueous phase was made alkaline by adding 0.5 ml of 2 *N* sodium hydroxide and extracted with 1 ml of diethyl ether by shaking for 1 min on the "Whirlimixer". After further centrifugation at 4,000 r.p.m. for 5 min, the organic layer was transferred to a 15-ml tapered centrifuge tube and carefully concentrated to approx. 50 μl with a slow stream of nitrogen. Evaporation to dryness was avoided, since this resulted in apparent increase of up to 40% in the amount of fenfluramine due to preferential evaporation of the internal standard. The final extracts were stored at −5° in glass-stoppered tubes; this temperature precipitates any aqueous contaminant and prevents evaporation of the organic phase. For analysis the extract was removed from the cold and 3–5 μl were injected immediately onto the gas chromatograph.

Red cells. For the initial extraction of the red cells diethyl ether was found to

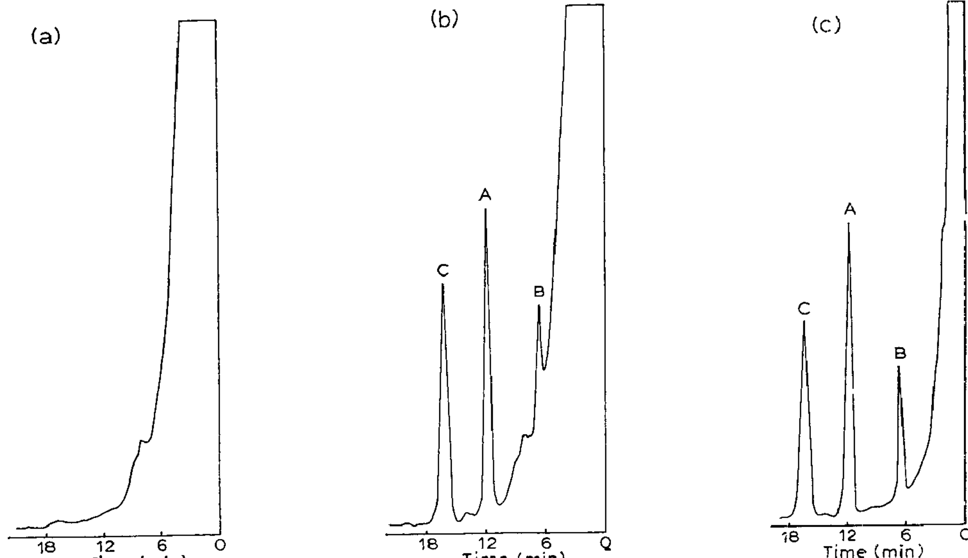

Fig. 1. (a) Gas chromatogram of an ether extract of normal plasma without fenfluramine or norfenfluramine. (b) Gas chromatogram of an ether extract of plasma containing 50 ng/ml of fenfluramine (A), 12 ng/ml of norfenfluramine (B) and N,N-diethylaniline (C) as internal standard. (c) Gas chromatogram of an ether extract of urine containing 4.2 μg/ml fenfluramine (A), 1.3 μg/ml of norfenfluramine (B) and N,N-diethylaniline (C) as internal standard.

remove substances which produced interfering peaks on the chromatograph. By substituting the less polar solvent 1,2-dichloroethane this difficulty was eliminated.

Urine. Urine (5 ml) was made alkaline with 0.5 ml of 1 *N* sodium hydroxide and extracted with 5 ml diethyl ether and 1 ml of the internal standard (1.0 mg% N,N-diethylaniline in diethyl ether) by gentle shaking for 5 min. After centrifugation at 4,000 r.p.m. for 5 min, the organic layer was transferred to a 15-ml tapered cen-

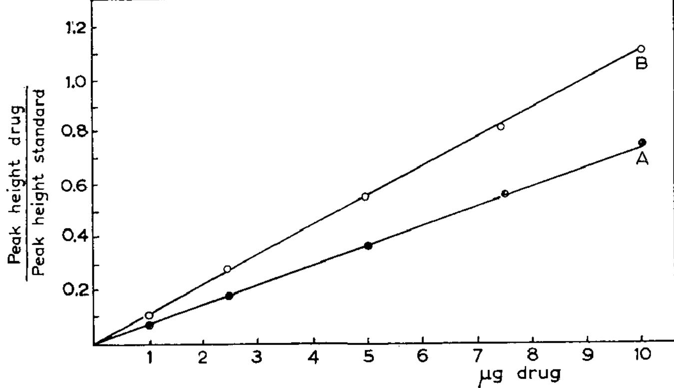

Fig. 2 The standard calibration graph for urine relating the ratio of the peak heights of fenfluramine (A), norfenfluramine (B) and N,N-diethylaniline.

trifuge tube, carefully evaporated to about 50 μl with a stream of nitrogen and 3–5 μl of this extract were injected onto the gas chromatograph.

Measurement

Losses of the compounds due to adsorption on the solid phase of the column were minimised by presaturating the active adsorption sites prior to each set of determinations. This was achieved by repeated injection of an ethereal solution of nicotine (1 μg/ml) until a constant base-line was maintained. The retention times of fenfluramine, norfenfluramine and N,N-diethylaniline were 11.8 min, 6.5 min and 16.2 min, respectively (Fig. 1). Over the range 1–10 μg of fenfluramine and norfenfluramine, the ratios of peak heights of the compounds to that of the internal standard were linear (Fig. 2). At the lower range of 0.02–1.0 μg, a plot of the ratios of peak areas (height × width at half height) gave a more linear calibration curve (Fig. 3). Recoveries of fenfluramine and norfenfluramine from plasma and red cells over the range 0.1–0.5 μg were 85 ± 4% and from urine over the range 2.0–20 μg, 99 ± 0.5%. The limit of detection of both compounds in plasma and red cells was 2 ng/ml.

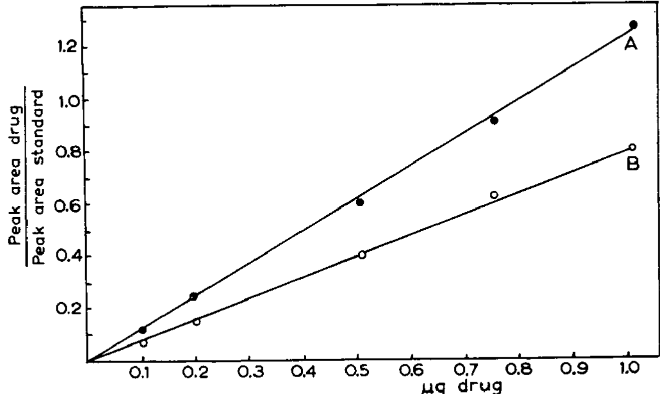

Fig. 3. The standard calibration graph for blood relating the ratio of peak areas of fenfluramine (A), norfenfluramine (B) and N,N-diethylaniline.

Specificity

The chromatographic system gave a separation of fenfluramine and norfenfluramine from the related congeners, amphetamine and methylamphetamine, which had retention times of 9.4 min and 13.0 min, respectively, under the same conditions.

Application

Two male subjects each received oral doses of 60 mg of fenfluramine hydrochloride. Venous blood samples were withdrawn at hourly intervals over a period of 8 h; and three further samples at 24 h, 30 h and 50 h. The red cells and plasma were separated immediately by centrifugation and both were analysed for fenfluramine and norfenfluramine. Urine samples were collected at hourly intervals between blood sampling for the initial 8½ h after dosage, and thereafter at intervals between 2 and 4 h over a period of 48 h.

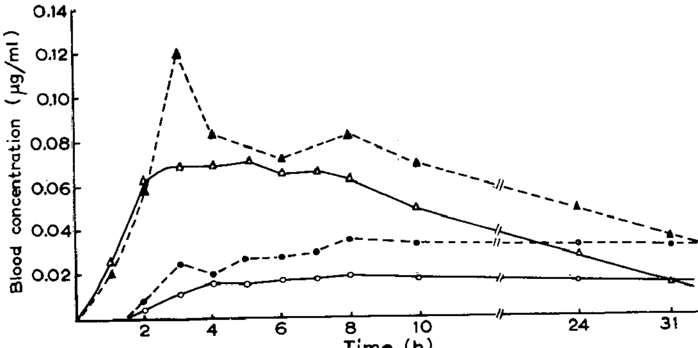

Fig. 4. Plasma and red cell concentration–time curves indicating levels found in subject A following an oral dose of 60 mg of fenfluramine hydrochloride. △—△, plasma fenfluramine; ▲— —▲, red cell fenfluramine; ○—○, plasma norfenfluramine; ●— —●, red cell norfenfluramine.

After immediate measurement of the pH and volume, urine samples were stored at −20° prior to analysis.

RESULTS AND DISCUSSION

Plasma levels of fenfluramine reached a peak of between 0.05–0.07 μg/ml approximately 3 h after oral administration of fenfluramine hydrochloride. For norfenfluramine, peak plasma levels of between 0.015–0.02 μg/ml occurred approximately 4 h after dosage (Fig. 4).

BROOKES[2] reported a peak plasma fenfluramine level of 0.16 μg/ml occurring 3–4 h after oral administration of three times the daily therapeutic dose (3 × 60 mg)

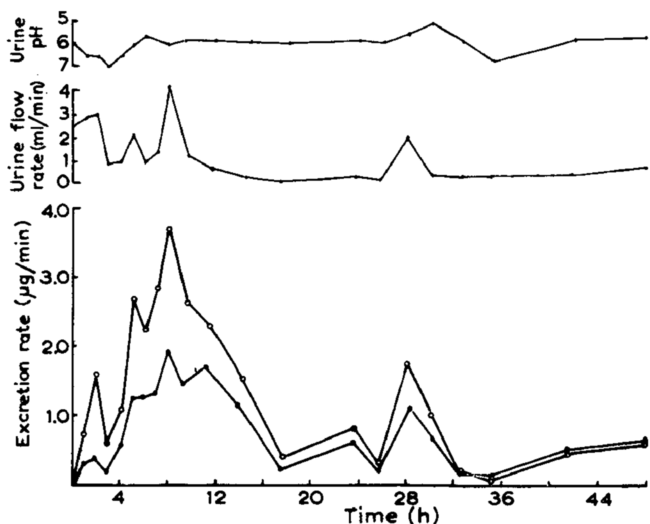

Fig. 5. The corresponding urinary excretion rates of fenfluramine (○—○) and norfenfluramine (●—●) from subject A.

to a male subject. Using the same procedure, BRUCE AND MAYNARD[3] quoted a peak plasma fenfluramine level of only 0.03 μg/ml after oral ingestion of 60 mg of the drug. Their refined technique, however, when applied to a subject who ingested 5 mg of the drug, indicated a peak plasma fenfluramine level of 0.005 μg/ml (ref. 4). It would seem that the initial findings of BROOKES[2] and the latter findings of BRUCE AND MAYNARD[4] have been substantiated by the present work. Plasma levels of fenfluramine and norfenfluramine remained relatively constant for 5 h after peak levels were achieved and then declined at a constant rate. Fenfluramine and norfenfluramine could be detected in plasma and red cells over 50 h after a single therapeutic dose. In human subjects, following the ingestion of 60 mg of fenfluramine hydrochloride, the distribution of fenfluramine and norfenfluramine between plasma and red cells was found to be 40% and 60% for both compounds.

The rate of urinary excretion of these compounds fluctuated considerably over the same period (Fig. 5), this finding being in agreement with previous work of BECKETT AND BROOKES[1], which showed that the rate of excretion of fenfluramine and norfenfluramine was a function of urinary volume and pH.

Work presented elsewhere[6–8] has demonstrated the applicability of this procedure in cases of fenfluramine overdosage.

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Thanks are also due to Selpharm Laboratories Ltd. (London) for their generous gifts of fenfluramine and norfenfluramine hydrochlorides.

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CHROM. 4697

THE BEHAVIOUR OF SOME SEPHADEX GELS IN DIOXANE–WATER MIXTURES

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SUMMARY

The uptake of dioxane and water by Sephadex G-25 and Sephadex LH-20 from mixtures of dioxane and water is determined, and the elution characteristics of sodium chromate and *p*-benzoquinone are reported on columns prepared from the two gels swollen in mixtures of the two solvents. The results are used to formulate a method of design of gel filtration systems using mixed solvents which will be free from adsorption and partition effects. The merits of use of gels for partition chromatography are discussed.

INTRODUCTION

It is well known that separations on synthetic xerogels such as Sephadex do not occur solely by a simple exclusion mechanism; adsorption and partition effects also play a part, especially with the more highly cross-linked gels and additionally when mixed solvents are used. On account of the varying solubility characteristics of different substrates the use of mixed solvents is sometimes desirable; further, using mixed solvents, it is possible to cause only partially swelling of a gel so that its fractionation range is altered. Various attempts have been made to elucidate adsorption and partition effects[1–6] and it has recently been shown fairly conclusively that adsorption is by hydrogen bonding between the solute and ether or hydroxyl groups on the Sephadex gels[7,8]. The question of adsorption of solvent has been examined in order to find true values for the volume of the imbibed solute (V_i) to allow true distribution coefficients (K_D) to be established from a knowledge of solvent regain, since if part of the imbibed solvent is adsorbed to the gel matrix, it is unlikely that it will be available

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for dissolution of solutes within the gel matrix. In none of the previous studies was the actual composition of the imbibed solvent determined and hence variations in its composition could not be used to explain the observed phenomena; this is the purpose of the present work.

Sephadex G-25 and LH-20 were chosen for study because their degree of cross-linking and exclusion characteristics are similar. The latter gel is a propylene oxide derivative of the former, the degree of substitution of the hydroxyl groups being approximately 60% (ref. 9). The solvents examined were dioxane and water and their mixtures. The chromatographic characteristics of the gels were determined using low molecular weight substances of widely disparate polarity, namely sodium chromate and benzoquinone.

EXPERIMENTAL

Determination of composition of imbibed solvent

The gel dried at 70° was allowed to swell in a large volume of the solvent or solvent mixture being examined and the regain was determined by the method employed by PEPPER *et al.*[10] and modified by GRANATH AND FLODIN[11]. The centrifuged gel was allowed to equilibrate with a large volume of "Specially Dried" methanol (Hopkin and Williams Ltd.); the methanolic solution was then analysed for water by the Karl Fischer method. The dioxane content of the imbibed solvent was found by difference from the total solvent regain and the water content.

Chromatographic conditions

Columns were prepared from the swollen gel (column dimensions: 1 × 20 cm)[12] allowed to settle while a flow of the solvent used for swelling was maintained (0.2 ml/min) for 6 h, and the bed volume was then measured. The void volume (V_0) for the column was determined using Blue Dextran (Pharmacia Fine Chemicals Ltd.) and also for water-rich solvents with colloidal carbon (obtained by dilution of Indian ink).

The elution volume (V_e) of aliquots (0.3 ml) of sodium chromate (0.3 M) and benzoquinone (0.3 M) each dissolved in the solvent used for swelling the sample was measured. The volume as the centre of the yellow zone emerged from the column was taken to be V_e. Apparent K_D values for the two solutes in the various systems were calculated by assuming that V_i for the column equalled the weight of dry gel in the column multiplied by the solvent regain for the particular system.

RESULTS AND DISCUSSION

The reproducibility of the method for the determination of solvent regain was tested using a standard centrifugation speed of 6000 r.p.m. with a 45° microangle centrifuge of radius 4 cm.

Behaviour of G-25

Fig. 1 shows the variation of solvent regain with changing solvent composition for G-25 Coarse and the corresponding variation of dioxane and water content whilst Fig. 2 shows the variation in solvent composition inside the gel with composition outside the gel. It is seen that there is always an excess of water inside the gel; this

TABLE I

WATER REGAIN OF BATCHES OF SEPHADEX GELS

Gel	*Regain (g/g dry gel)*	*S.D. (20 results each) (g)*
G-25 Fine	1.98	± 0.12
G-25 Coarse Lot No. To 6249c	2.00	± 0.07
G-25 Coarse Lot No. 8298	2.16	± 0.06
LH-20 Coarse	1.90	± 0.15

excess is plotted against external solvent composition in Fig. 3. Extrapolation of the curve shows that there is a permanently retained quantity of water, of the order of 0.2 g/g of dry gel, at all solvent compositions. This represents a layer of water molecules hydrating the hydroxyl groups in the gel. Extrapolation to 100% water gives a value of 0.24 g/g of dry gel, but at 100% dioxane the value is lower. This may be explained by the fact that the gel shrinks in dioxane-rich solvents so that hydrogen bonding can occur between adjacent hydroxyl groups on the gel thus excluding water. The repeating unit of the polymer[4] has a molecular weight of 1028 and contains 17 hydroxyl groups; if one molecule of water is bonded to each hydroxyl group this would correspond to 0.29 g/g of dry gel, which agrees reasonably with the observed value, 0.24 g/g, and also with the figure suggested by LATHE AND RUTHVEN[1]. The increase in the excess water in mixed solvent compositions can be explained because it is probable that the hydrogen-bonded water will itself bond to more water to form domains around the hydroxyl groups. It has been shown that these domains around the hydroxyl groups would be expected to be broken up by sodium chromate at the concentrations employed in these experiments[3]. The water hydrogen-bonded directly to the

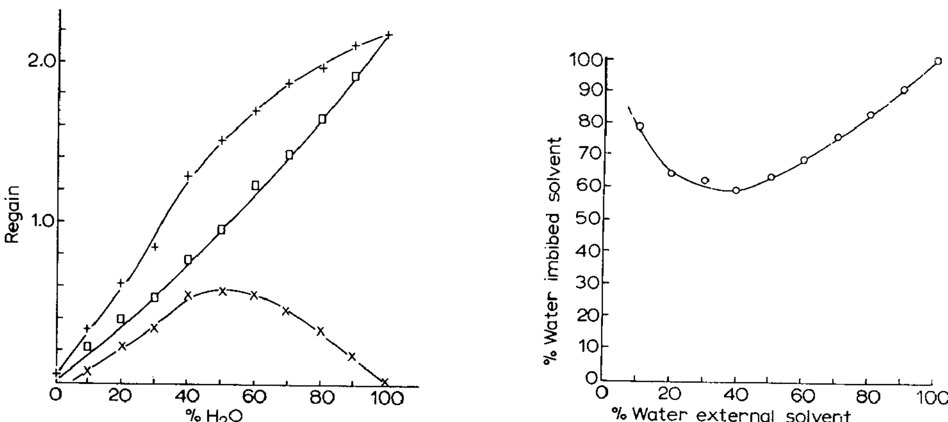

Fig. 1. Total regain (+), water regain (□), and dioxane regain (×) for different swelling solvent compositions.

Fig. 2. Variation of internal solvent composition with external solvent composition for Sephadex G-25.

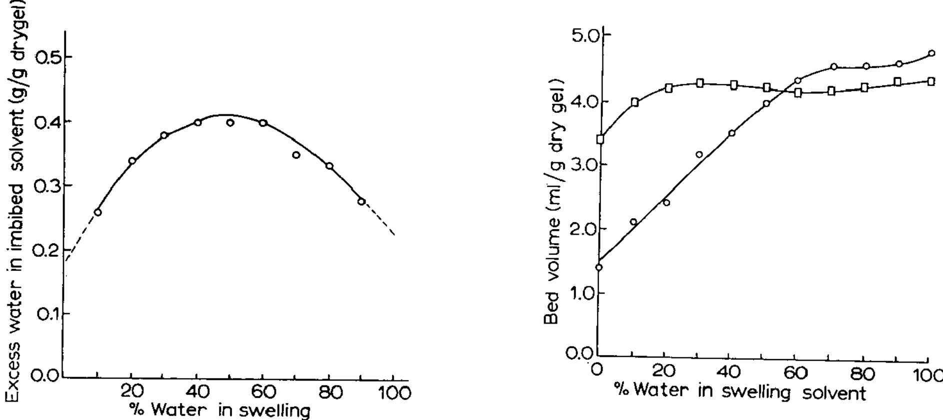

Fig. 3. Variation of excess water of swelling solvent for G-25.

Fig. 4. Variation of bed volume with composition of swelling solvent. ○, G-25; □, H-20.

gel would not be available to this solute for solution, so that a discrepancy may be expected between the V_i of a column determined from solvent regain experiments and that obtained by elution of sodium chromate from a column. K_D for sodium chromate was found to be 0.88 using solvent regain data (Fig. 6). Correction for the unavailable water of 0.24 g/g of dry gel gives a value of 0.98. It is therefore concluded that for determination of true K_D values on G-25 gel in water, the solvent regain should be corrected by 0.24 g/g of dry gel to find the true value of the volume of imbibed solvent available to solutes; if very weak solutions are employed so that the ordered regions are not destroyed by the solute this correction factor should be increased and Fig. 3 shows the order of the correction factors to be applied.

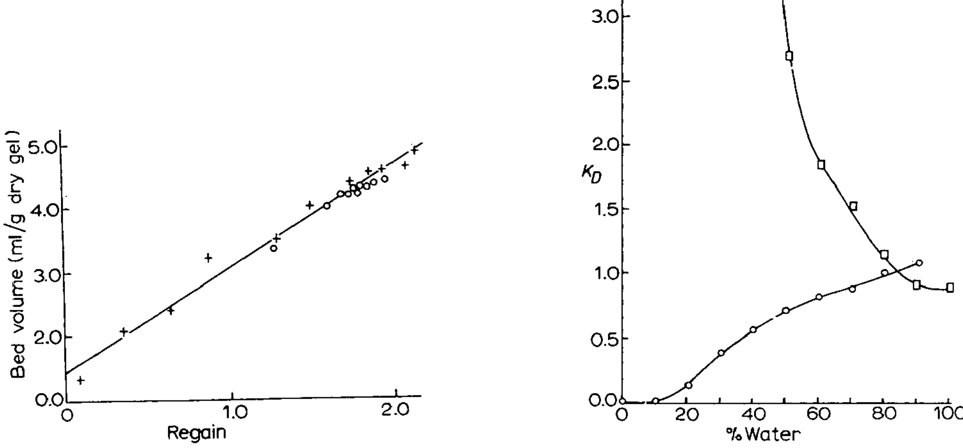

Fig. 5. Variation of bed volume with regain. +, G-25; ○, LH-20.

Fig. 6. Elution characteristics of *p*-benzoquinone (○) and sodium chromate (□) on G-25 in different solvent compositions.

Fig. 4 shows the variation of bed volume with composition of the swelling solvent and Fig. 5 shows the variation of bed volume with solvent regain. It is clear from Fig. 5 that determination of bed volume (a relatively simple procedure) can be used to determine solvent regain to a first approximation (slope of line 1.72 ± 0.2 ml g^{-1}).

Fig. 6 gives the elution characteristics of the two solutes in varying solvent compositions. As expected, the K_D value of the sodium chromate increases as the excess water inside the gel increases indicating straight phase partitioning, until, in compositions containing less than 40% water, the chromate ceases to move down the column. The quinone behaves conversely and below 10% water, no quinone enters the gel at all. The K_D value of quinone at 100% water (extrapolated) indicates that it has some affinity for the gel itself. True exclusion chromatography for both substances appears to occur with solvent compositions in the region of 85% water in dioxane.

Behaviour of LH-20

The variation of bed volume with solvent composition for LH-20 is observed to be quite different from that of G-25 (Fig. 5) as is the change in regain with solvent composition (Fig. 7). It is apparent that the gel has an affinity for both the polar and the non-polar solvent, and consequently domains of each solvent can be expected to form around hydrophilic and hydrophobic sites, respectively, in the gel. There is in addition a third effect which must be taken into account, especially with lipophilic–hydrophilic gels such as LH-20; this is the ordering effect which hydrophobic solutes have upon water[13]. Water could be expected to be forced into domains both by this effect, and by hydrogen-bonding to hydroxyl and ether groups on the gel. Dioxane is thus forced into the region of the hydrophobic sites. When an ionised solute is introduced into such a system, it is difficult to predict its behaviour quantitatively; however, the elution behaviour of the two solutes (Fig. 8) follows the trends which would be expected from an inspection of the internal and external solvent compositions which are shown in Fig. 7.

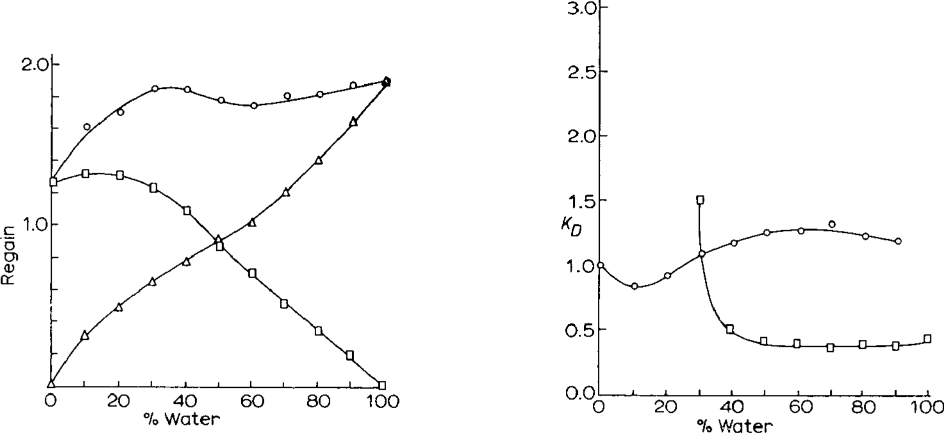

Fig. 7. Total regain (○), water regain (△), and dioxane regain (□) for different swelling solvent compositions.

Fig. 8. Elution characteristics of *p*-benzoquinone (○) and sodium chromate (□) on LH-20.

CONCLUSIONS

The results show that attempts to isolate individual effects in separations on xerogels and to attribute them to exclusion, partition or adsorption, as a comparison of gel filtration with adsorption and partition chromatography would suggest, are likely to fail. The results reported do, however, suggest a practical procedure which may be followed in order to design a system using mixed solvents to separate series of chemically similar compounds according to their molecular weight alone. The simplest model for a gel filtration system would be to regard it as a pure partition system, where the stationary phase is a stationary solution of the gel in the solvent, and the mobile phase is the solvent or solvent mixture. More highly cross-linked gels will give more concentrated stationary solutions than looser gels, so that less polar solutes will be expected to partition from an aqueous mobile phase into tightly cross-linked gels to a greater extent than into looser gels, regardless of the molecular sieve mechanism. In order to counteract this effect the mobile phase may be made less polar by incorporation of a miscible non-polar solvent such as dioxane. However, this non-polar solvent will itself then become distributed between the two phases until equilibrium is reached; chromatography of a solute will then be between a stationary solution of the gel plus the non-polar solvent plus water, and the mobile solution of the non-polar solvent plus water; large molecules will thus be subjected to exclusion effects as well as to partition effects. In order to design a system which will produce separation by the exclusion mechanism alone, it is not necessary to carry out the whole procedure reported earlier in this paper. All that is required is the preparation of a series of columns from gel swollen in a range of solvent mixtures; V_i for each column may then be estimated with sufficient accuracy for this purpose from a measurement of the bed volume for a given weight of dry gel whilst determination of V_0 may be made using Blue Dextran. The variation of the K_D value with solvent composition for a small molecule of similar polarity to the series of molecules to be separated may be observed on the columns, and a plot similar to Figs. 6 and 8 obtained. Where the resultant curve crosses the $K_D = 1$ ordinate the system is balanced, *i.e.* the chemical affinity of the solute for the stationary solution and the chemical affinity for the mobile solution are equal, and separations due to molecular sieving alone can be expected. As gel columns are frequently employed for repeated investigations which require separations of large numbers of samples whose composition varies only slightly, the effort required to design the system properly as outlined above would be well worth while; accurate determination of V_i from solvent regain at each solvent composition near to the balance point, as roughly determined using the V_i to bed volume relationship, would allow the balance point to be found precisely.

The employment of synthetic xerogels such as Sephadex for partition chromatography represents only a minor part of their present total application; partition chromatography has been carried out in the main either on cellulose or on non-adsorptive supports such as celite. When a swollen xerogel is visualised as a stationary solution instead of as a rigid inert support such as celite, its merits compared to inert supports become clear. No loading of the stationary phase is necessary, the gel is simply swollen in the proposed mobile phase and once swollen, its solution properties will differ from those of the swelling solvent, particularly if a mixed swelling solvent was employed with a tightly cross-linked gel. When a column is packed, high chro-

matographic efficiency is achieved due to the uniform particle shapes and sizes of synthetic gels. The whole column will be involved in the chromatographic process so that the capacity of the columns will be higher using a gel than using a conventional system with an inert support. We have also observed that when a gel is swollen in a solvent which gives maximum regain, a column packed with such a gel retains its bed volume when a solvent which swells the gel slightly less is substituted for the original solvent on the column[14]. Hence, if after one chromatographic run separation is unsatisfactory, the mobile solvent composition may be changed slightly to improve separation. Using a conventional partition system such solvent changes are limited by the need to maintain immiscibility between the two phases: with xerogels, miscibility is impossible. Such an application of synthetic xerogels has great potential in organic chemical research. Gel chromatography has as yet not found wide application in this field because separation is required not according to molecular weight, as in much biochemical work, but according to functional group. PORATH has shown that retention chromatography on Sephadex gels compares favourably with gas chromatography as a generally applicable technique[15]. Partition chromatography with mixed solvents on highly cross-linked gels, together with suitable pumps and detectors, compares even more favourably with gas chromatography for organic chemical separations, particularly when working with sensitive or involatile samples. A liquid chromatograph comprising a peristaltic pump, a small xerogel column (20–30 $\times$ 1 cm), a simple light-absorbing or refractive index detector, and a fraction collector may in the future well find as many uses as a gas chromatograph in the organic chemical laboratory.

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CHROM. 4700

SEPARATION OF POLAR LIPIDS BY COLUMN CHROMATOGRAPHY ON HYDROXYLAPATITE*

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SUMMARY

The separation of various lipids by column chromatography on hydroxylapatite was investigated. Selective elution of lipid classes was effected by varying the ratio of acetone to methanol in the eluant. Separations achieved compared favorably to those obtained with silicic acid or DEAE-cellulose.

Column chromatography on hydroxylapatite afforded complete recovery of polar lipids, judged by the 99–100% recovery of total phosphorus. Careful examination of eluted fractions did not indicate degradation of lipids during column development.

INTRODUCTION

Hydroxylapatite, $Ca_{10}(OH)_2(PO_4)_6$, an adsorbent introduced in 1956 by TISELIUS *et al.*[1], has been widely used for separation of proteins[2], lipoproteins[3] and polynucleotides[4] by CC. This adsorbent has been used also for the separation of polar lipids by TLC with 7% calcium sulfate as a binder[5].

In our work on the separation of immunologically-active polar lipids from cattle serum, we found that CC with DEAE-cellulose or silicic acid gives inadequate separation of certain individual polar lipid components. This led us to explore the use of CC on HTP to achieve improved separation of individual polar lipids. We report here details of the method of CC of polar lipids on HTP and the advantages gained from the application of this method.

METHODS

Reagents

All reagents used were analytical grade. Triolein was a gift from Dr. N. PAYZA, New York Medical College; L-α-cephalin dipalmitoyl (synth) and phosphatidyl-L-serine (according to Folch) from Mann Research Laboratories, Inc., New York;

* Abbreviations: HTP = hydroxylapatite; DEAE-cellulose = diethylaminoethyl cellulose; TLC = thin-layer chromatography; CC = column chromatography; Fr = fraction.

cerebrosides (beef brain), phosphatidylinositide (Fr I from beef brain), and sphingomyelin from General Biochemicals, Chagrin Falls, Ohio; L-α-lecithin and cardiolipin (extract bovine heart) from Pierce Chemical Company, Rockford, Ill.; hydroxylapatite Bio-gel HTP was from Bio-Rad Lab., Richmond, Calif. TLC plate Silica Gel HR was purchased from Analtech, Inc., Wilmington, Del.; bovine J^{cs} serum was supplied kindly by Dr. W. STONE, Madison, Wisc.

Extraction of lipids from serum

The powder obtained from lyophilizing 500 ml of dialyzed bovine serum was washed with 500 ml of acetone. The acetone-insoluble residue was extracted with 2 l of chloroform–methanol (2:1) at room temperature under a stream of nitrogen for 48 h using a magnetic stirrer[6]. The extract was clarified by filtration on a sintered glass funnel (grade M) and then was concentrated to near dryness under nitrogen. The concentrate was dissolved in 20 ml of diethyl ether, and the polar lipids were precipitated with an excess of acetone containing 1 ml of acetic acid. The precipitated polar lipids were collected by centrifugation at 2,000 r.p.m. at 5° for 20 min in the International Centrifuge Model PR-2. The supernatant which contained neutral lipids and pigments was discarded.

The precipitated polar lipids were dried to constant weight over P_2O_5 at 8° in a vacuum desiccator previously flushed with nitrogen.

Hydroxylapatite column chromatography

Column preparation. Twenty grams of Bio-gel HTP were washed three times with 150 ml of methanol, two times with 150 ml of acetone and two times with 100 ml of diethyl ether. After each washing, the supernatant was decanted. Residual ether was removed in a stream of nitrogen which was bubbled for 10 min through the adsorbent.

The ether-free adsorbent was activated in an oven at 120° for 18 h. Activated HTP was than cooled in a desiccator previously flushed with nitrogen, and then the adsorbent was suspended in diethyl ether. The slurry was poured into a glass column (1.2 × 35 cm) containing a fritted disc (grade M) which was covered with fine glass wool. The column bed was then washed with 100 ml of chloroform.

Chromatography of lipids. A 250-mg mixture of standard lipids dissolved in 7 ml of chloroform was applied to the column for chromatography of the standard lipids. A 300-mg sample of serum polar lipids dissolved in 10 ml of chloroform was applied to a separate identical column. The lipids were eluted successively from the columns under slight nitrogen pressure by the following solvents: (I) chloroform, 250 ml; (II) acetone–methanol (9:1), 250 ml; (III) acetone–methanol (7:3), 300 ml; (IV) acetone–methanol (5:5), 250 ml; (V) acetone-methanol (3:7), 250 ml; (VI) methanol, 300 ml; (VII) diethyl ether–ethanol–aqueous 0.04 *M* KOH (10:7:5), 650 ml (Solvent mixture VII previously was used to advantage for the chromatography of lipids on alumina[7].) Total phosphorus in eluted fractions was determined by the method of BARTLETT[8]. Recovery from the column was judged by total phosphorus recovery of serum lipids after subtraction of the amount of phosphorus eluted by these solvents from a blank column to which no sample was added.

Analytical methods. After acid-methanolysis, long-chain bases and sugars were analyzed by gas–liquid chromatography on SE-30 (ref. 9).

Serological assay. Hemolysis test and inhibition of hemolysis of cattle J^{cs} red cells were performed according to STONE AND IRWIN[10].

Isolation of lipids from Fr VII. Fractions of 35 ml were collected. After the eleventh fraction, the eluate became alkaline and was immediately brought to pH 8.3 (colorless to phenolphthalein) with glacial acetic acid. After complete elution with the total 650 ml of eluant, fractions were combined and evaporated to a small volume under nitrogen. To the concentrate was added 40 ml of aqueous 0.1 *M* KCl and the mixture was extracted twice with 200 ml of chloroform–methanol (2:1). Then the aqueous phase was further extracted two times with equal volumes of chloroform–methanol (4:1). The lipids were contained in the combined chloroform extracts.

Thin-layer chromatography. Silica Gel HR plates, 250-μ coating thickness, were activated at 130° for 2 h and cooled under nitrogen. Column fractions were evaporated to dryness under nitrogen, dissolved in chloroform–methanol (2:1) and then applied to the plate. Individual lipid standards also were applied to the plate as reference compounds. The chromatography tank was lined with equilibration paper and was equilibrated with developing mixture for 2 h before TLC. The mixture chloroform–methanol–water (65:25:4)[11] was used for TLC of the various lipids. After chromatography, individual lipids were visualized by either iodine vapors, ammonium bisulfate or ninhydrin reagent.

RESULTS

Hydroxylapatite column chromatography

The lipid fractions eluted from this column were monitored and identified by TLC. Solvent was changed when no further elution of lipids occurred. After concentration under reduced nitrogen pressure, column fractions and lipid standards were applied to TLC plates and developed with chloroform–methanol–water (65:25:4).

Standard lipid fractionation

Standard lipids after elution from the column were identified in each fraction by TLC (Fig. 1). On the basis of the information obtained by comparing the migration

TABLE I

ELUTION OF STANDARD LIPID MIXTURE FROM HYDROXYLAPATITE

Volumes of solvents are for a column 35 × 1.2 (I.D.) cm prepared with 20 g of HTP; a 250-mg sample of standard lipids was applied.

Fraction No.	*Eluent*	*Volume (ml)*	*Lipids eluted (monitored by TLC)*
I	Chloroform	250	Triglycerides (neutral lipids)
II	Acetone–methanol (9:1)	250	Cardiolipin
III	Acetone–methanol (7:3)	300	Lecithin
IV	Acetone–methanol (5:5)	250	Sphingomyelin
V	Acetone–methanol (3:7)	250	Upper spot of cerebrosides mostly
VI	Methanol	300	Phosphatidylethanolamine, cerebrosides
VII	Diethyl ether–ethanol–aqueous 0.04 *N* KOH (10:7:5)	650	Phosphatidylserine, phosphatidylinositol

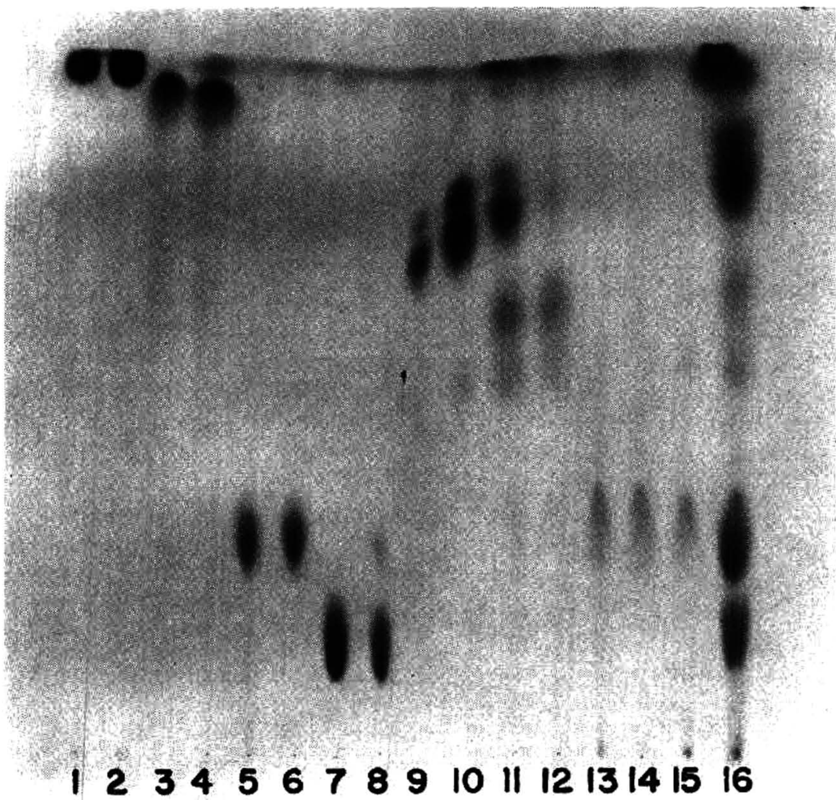

Fig. 1. Thin-layer chromatogram of lipid standards and fractions of standard lipid mixture eluted from a HTP column. Adsorbent: Silica Gel HR. Solvent: chloroform–methanol–water (65:25:4). Visualization: ammonium bisulfate spray. 1 = triolein; 2 = Fr I; 3 = cardiolipin; 4 = Fr II; 5 = lecithin; 6 = Fr III; 7 = sphingomyelin; 8 = Fr IV; 9 = Fr V; 10 = cerebrosides; 11 = Fr VI; 12 = phosphatidylethanolamine; 13 = phosphatidylserine; 14 = Fr VII; 15 = phosphatidylinositol; 16 = mixture of 1, 3, 5, 7, 10, 12, 13, 15.

of the components of each fraction with those of standard lipids, the generalizations in Table I were made.

A 300-mg sample of polar lipids extracted from serum was fractionated on an identical hydroxylapatite column to test the usefulness of the method for resolving complex mixtures of lipids. A recovery of 99–100% of the serum lipids placed on the column was obtained, based on total P determinations. The components in each eluted fraction were identified by TLC using authentic lipid standards as reference compounds. The resulting separation is shown in Fig. 2.

Fr II (spot 5) contained only traces of lipids not visible on this plate. Analysis of Fr II showed the presence of a small amount of hexoside ceramides. The lipids in Fr V (spot 10) contained only traces of sphingosine, but Fr V contained considerable amounts of long-chain bases which differed from sphingosine by showing a greater retention on SE-30. Spots 11 and 12 contained mainly phosphatidylethanolamine, which in this TLC system always gave two spots. Fr VII (spot 14) which was adjusted

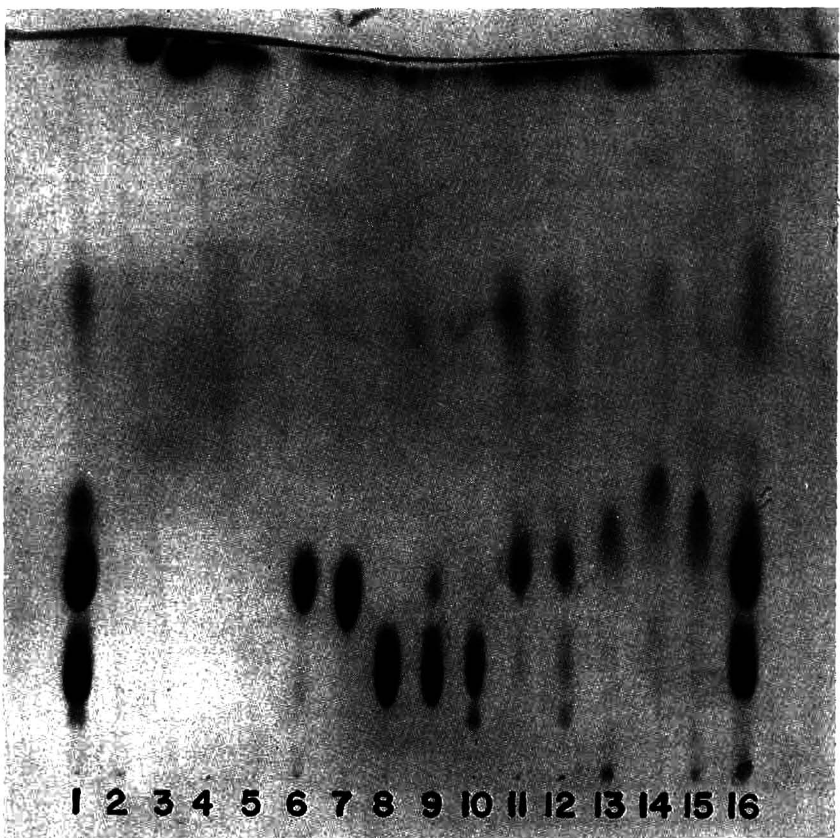

Fig. 2. Thin-layer chromatogram of lipids separated from bovine J^{cs} serum by HTP column chromatography. Adsorbent: Silica Gel HR. Solvent: chloroform–methanol–water (65:25:4). Visualization: ammonium bisulfate spray. 1 = bovine serum lipids; 2 = triolein; 3 = Fr I; 4 = cardiolipin; 5 = Fr II; 6 = lecithin; 7 = Fr III; 8 = sphingomyelin; 9 = Fr IV; 10 = Fr V; 11 = phosphatidylethanolamine; 12 = Fr VI; 13 = phosphatidylinositol; 14 = Fr VII; 15 = phosphatidylserine; 16 = mixture of 2, 4, 6, 8, 11, 13, 15.

with acid till colorless to the phenolphthalein indicator showed the phenolphthalein spot near the solvent front.

The immunologically active bovine J hapten was eluted with Fr VII from HTP.

The sheep brain cerebroside standard gives two spots by TLC. This probably results from the different fatty acids present in these cerebrosides[12]. An attempt was made to resolve further these cerebrosides by chromatography on a HTP column with mixtures of acetone and methanol. Careful selection of the ratio of acetone to methanol afforded separation of the cerebrosides into three groups: (1) cerebrosides corresponding mostly to the upper spot on TLC (Fig. 1, cerebroside spot), eluted from column by acetone–methanol (3:7); (2) cerebrosides corresponding to both spots, eluted from column by acetone–methanol (1:9); (3) cerebrosides corresponding to the lower spot of the thin-layer chromatogram, eluted from column by methanol.

DISCUSSION

Chromatography on columns of silicic acid[13] and DEAE-cellulose[14,15] is commonly used as the initial step in separation of various lipid classes from crude polar lipids. However, the fractionation of phospholipids by silicic acid CC does not resolve phosphatidylserine from phosphatidylethanolamine. Furthermore, overlapping of certain lipids such as lecithin and sphingomyelin in the eluted fractions is difficult to avoid. On the other hand, CC of lipids on DEAE-cellulose does not separate lecithin from sphingomyelin and cerebrosides, nor cardiolipin from phosphatidylinositol.

Column chromatography on HTP may be used advantageously for resolving mixtures containing these compounds. This can be seen readily by referring to Table II, where the resolving ability of HTP is compared with the resolving abilities of DEAE-cellulose and silicic acid, as given in the literature[13,14].

TABLE II

COMPARISON OF THE RESOLVING POWER OF SILICIC ACID, DEAE-CELLULOSE AND HYDROXYLAPATITE COLUMNS[a]

Compound	Silicic acid column (13)				DEAE-cellulose column (14)							Hydroxylapatite column						
	1	2	3	4	1	2	3	4	5	6	7	1	2	3	4	5	6	7
Lecithin			■		■									■				
Phosphatidylethanolamine		■	■			■											■	
Phosphatidylinositol			■								■							■
Phosphatidylserine		■						■										■
Cerebroside					■											■	■	
Cardiolipin											■		■					
Sphingomyelin				■	■										■			

[a] Blackened square denotes elution of component; numbers denote fractions.

The commonly employed eluting mixtures of chloroform and methanol fail to give good resolution of lipids on HTP columns. Mixtures of acetone and methanol, similar to those used by VANCE AND SWEELEY[9] for eluting hexoside ceramides from a silicic acid column, exhibited good resolving power on HTP. Selectivity was obtained by varying the ratio of acetone to methanol.

Occasionally, lecithin is incompletely desorbed from HTP by solvent mixture III, and acetone–methanol (6.5:3.5) may then be substituted to achieve the desired resolution of lecithin from sphingomyelin. Variations in the preparation of the gel and in the activation procedures may be responsible for the occasional aberrant behavior of lecithin on HTP columns.

Several methodological points warrant discussion. Methanol-soluble particles of HTP may appear in the column effluent. We recommend that the gel be washed with methanol and decanted at least three times. This treatment not only greatly reduces the amount of particles present in the effluent but also facilitates a fast flow rate from the column. We have been able to complete the elution of the various lipid

classes from a HTP column, operated under slight nitrogen pressure within 8–9 h.

TLC of the cerebrosides usually gives an elongate-dumbell shaped zone or two spots; fatty acid heterogeneity is believed to contribute to this chromatographic heterogeneity[12]. Hydroxylapatite CC affords partial resolution of these cerebrosides.

No degradation of the lipids occurred during chromatography on HTP as judged by the absence of new spots on TLC examination of the eluted fractions.

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CHROM. 4705

QUANTITATIVE CHROMATOGRAPHY OF THE OLIGOSACCHARIDES IN WORT, BEER AND BREWING SYRUPS

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SUMMARY

A method for the separation and quantitative analysis of the oligosaccharides from four to fifteen glucose units in brewing materials is described. The procedure is to separate the oligosaccharides on a heated cellulose column by elution with a water–*n*-butanol–ethanol gradient mixture. Quantitative analysis is then achieved by an automatic analyser which mixes and reacts the eluent stream with anthrone/sulphuric acid and then measures the absorbance on an integrating recorder.

INTRODUCTION

The "dextrin" fraction in wort and beer has been investigated by many workers using such techniques as acetone precipitation[1], ethanol extraction[2], paper[3–7] and charcoal column[8,9] chromatography. More recently ENEVOLDSEN[10] has used paper chromatography to separate wort oligosaccharides both by molecular weight and into α-1,4-linked and α-1,6-linked fractions.

DELLWEG *et al.*[11–13] have successfully separated up to fifteen homologous glucose oligomers on polyacrylamide gel columns using an Auto-Analyzer. The lower sugars can be readily separated by gas chromatography[14–18] and this technique has been extended for the analysis of starch hydrolysates of up to seven glucose units[19].

THOMA *et al.*[20] have used partition chromatography on cellulose columns for the preparative separation of the oligo (1–10 glucose units) and megalosaccharides (10–20 glucose units) from starch hydrolysates. This technique has been developed into a manual analytical method for the quantitative analysis of oligosaccharides in brewing materials and was described in our previous report[21].

This present paper describes the further development of a faster automated method for the separation and determination of the oligosaccharides derived from malt and other cereals encountered in the brewing industry.

EXPERIMENTAL

The chromatographic and analyser system is illustrated in Fig. 1. All tubing unless otherwise stated is of 1-mm-bore Teflon. Between tube connections were made from 1/16 in. I.D. Viton tubing (Watson-Marlow) made air-tight by twisting Nichrome wire tightly around the tubing.

Gradient elution

The column eluent was pumped from a gradient elution device consisting of a 250-ml conical flask filled to 200 ml with a mixture of water–ethanol–*n*-butanol (24.5:23:52.5) connected to an empty 50-ml flask, which is connected in turn to a 1-l flask containing 800 ml of water–ethanol–*n*-butanol (42:25:33). All solvents were de-aerated and kept air-free by immersing the gradient elution apparatus in a water bath maintained at 60° by circulating water through the bath by means of a Churchill thermo-circulator. The eluent was pumped to the top of the chromatographic column, by means of a micro-pump at a fixed rate of 28 ml/h.

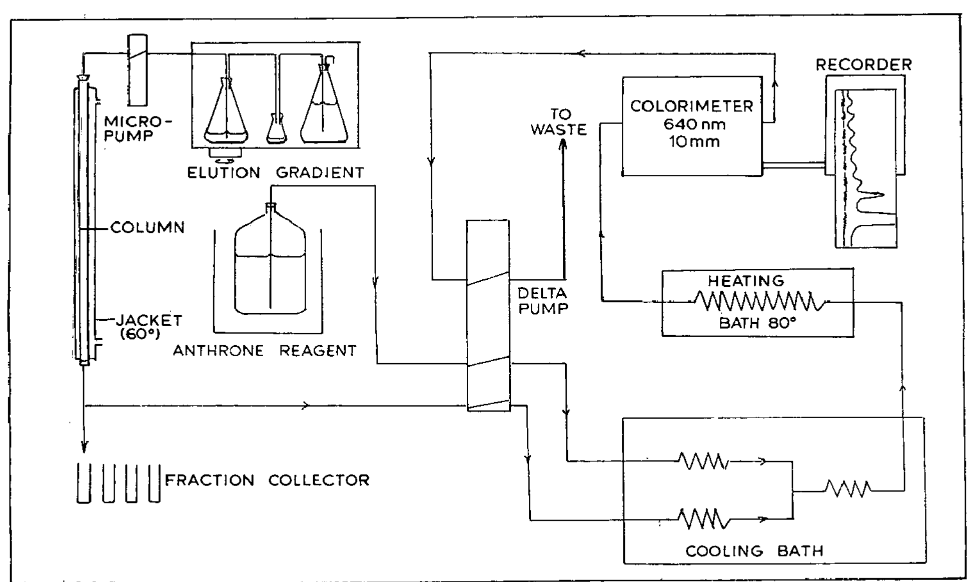

Fig. 1. Chromatography and analyser flow diagram.

Chromatographic column

The column used was a Whatman precision column 45 cm long × 1.5 cm diam. fitted with a water jacket. The Teflon end fittings were removed and replaced by silicone rubber bungs bored to take 1-mm-bore Teflon tubing (Fig. 2). The temperature of the column was maintained at 60° by connecting the water jacket in series with the bath heating the gradient elution device. The column was packed to a depth of 43 cm with Whatman No. CF12 cellulose powder. Prior to use, the fines were removed from the cellulose by stirring 50 g of the powder with 1 l of distilled water

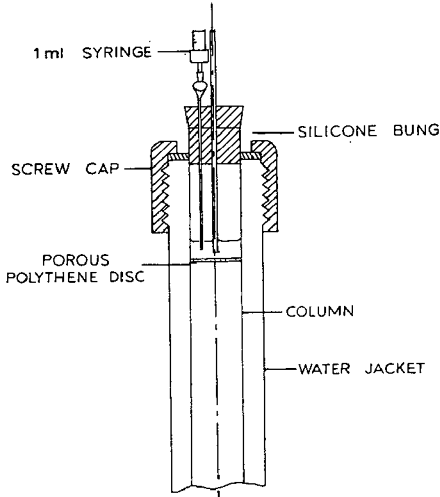

Fig. 2. Chromatography column upper end assembly.

and allowing to settle. The supernatant liquid along with the fines was then poured off and this procedure repeated five times.

The method of packing the column is important for good separation characteristics. The column was filled with a slurry of 50 g (less fines) of cellulose in 800 ml of water at 90° and allowed to settle under gravity with no flow. The packing was consolidated by sucking the surplus water through the outlet with a 10-ml hypodermic syringe. This procedure was repeated until the cellulose was packed to the required depth. When correctly packed, the column should be capable of maintaining a flow rate of 30–40 ml/h and 0.2 ml of universal pH indicator should separate into bands, and run evenly down the column, using hot water as eluent.

The water was displaced by percolating at least 500 ml of water–ethanol–*n*-butanol (24.5:23:52.5) through the column.

Microgranular cellulose did not show any improvement of separation, tended to pack too tightly and to have a reduced flow rate.

Regeneration

After each run it was necessary to wash the column by running hot de-aerated water through it overnight and then to displace the water by 500 ml of the eluting solvent (water–ethanol–*n*-butanol, 24.5:23:52.5).

Analyser

The effluent from the column was split in the ratio 3:1, 7 ml/h being fed to the analyser system and 21 ml/h flowing to a fraction collector.

The flow to the analyser was controlled at 7 ml/h by a Watson-Marlow delta pump fitted with 2 mm I.D. M.S. (high-grade PVC, Esco Rubber Ltd.) pumping tubes.

The eluent was pumped firstly through a chilling coil made from 2 m of Teflon tubing maintained at less than 4° using a Churchill chiller circulator (or ice) to a glass

'T' piece where it met the chilled anthrone reagent flowing at 14 ml/h (effluent to reagent ratio, 1:2.0). The reaction mixture was then fed through a chilled mixing coil made from 2 m of Teflon tubing and then into a Teflon reaction coil of 7-ml capacity (≏ 14 m Teflon tubing) immersed in a water bath at 80°. This coil volume gives a reaction time of 20 min. The absorbance of the effluent stream was measured in a Vitatron colorimeter (Fisons Ltd.) using a flow through cell of 10 mm light path and 0.08 ml capacity and filter No. 641 (640 nm) and then recorded with a Vitatron lin/log integrating recorder. The reaction mixture was then pumped through the delta pump to waste, using a $\frac{1}{4}$ in. I.D. Viton tube.

Detection reagent

The reagent used for colour development was 0.1% anthrone in 85% sulphuric acid freshly prepared at the beginning of each run. The anthrone reagent stock bottle was kept below 4° and the reagent stream was pumped at 14 ml/h through the delta pump using 1/8 in. I.D. Viton tubing (Watson-Marlow), then through a cooling coil of 2 m length to the 'T' piece, where it was mixed with the column effluent.

Preparation of sample

The beer, wort or syrup was dried under vacuum in a rotary evaporator or alternatively by freeze drying and 0.1–0.2 g of the residue was dissolved in the minimum amount of degassed water. 90% dimethyl sulphoxide was found to be a better solvent for some syrups.

The viscous solution obtained was layered onto the top of the column by injecting through the silicone rubber bung using a 1-ml hypodermic syringe (Fig. 2).

Quantitation of results

n-Butanol which is contained in the column eluent interferes with the reaction between anthrone and carbohydrate. It has been shown[21] that this interference can be reduced by reaction at 60° instead of the more usual 100° (refs. 22–24).

YADAV *et al.*[25] showed that if the acid concentration of the anthrone reagent is reduced from 95% to 85% a constant molar extinction, dependent only on the glucose equivalent of the sugar, is obtained. This modification was adopted and it was found, using 0.1% anthrone in 85% sulphuric acid, that the most reproducible results were obtained when the mixture was heated at 80° for 20 min.

The ratio of reagent to column eluent is important and should be kept to 2.0:1 (Fig. 3).

Some batches of commercial *n*-butanol gave different absorbance values when used in the column eluent; for this reason it is necessary to check each batch of *n*-butanol and redistill when required.

Calibration

The fractions corresponding to each peak were collected together and evaporated to dryness under vacuum in a rotary evaporator. The solids obtained were dissolved in hot water treated with charcoal, precipitated with excess ethanol, filtered and dried at 95° under vacuum. Aliquots (1 mg) of each oligosaccharide were then accurately weighed on a micro balance and dissolved in a range of volumes (4–10 ml) of a mixture of ethanol, water and *n*-butanol corresponding to the column

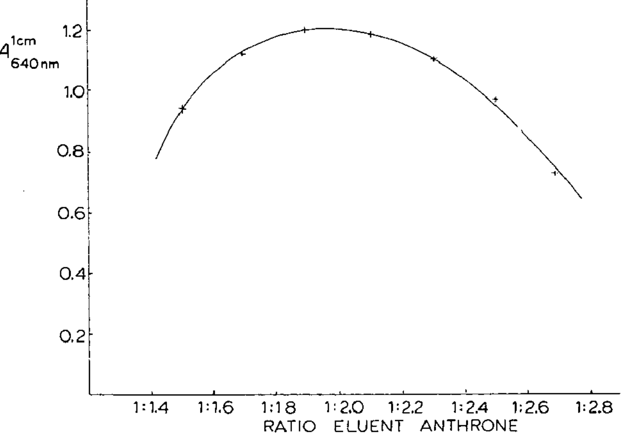

Fig. 3. Variation of absorbance with the ratio of eluent to anthrone reagent.

eluent which would elute that oligosaccharide. These solutions were then pumped through the analyser and the area corresponding to 1 mg was found both in counts and by planimeter (Table I).

TABLE I

ANALYSER CALIBRATION

Peak area corresponding to 1 mg of each oligosaccharide. Results are the average of three determinations. Recorder 6 cm/h and minimum count rate.

	Peak area (counts/mg)	*Peak area (sq. cm/mg)*
Glucose	359 ± 26	41.6 ± 0.9
Fructose	420 ± 24	48.0 ± 1.6
Sucrose	406 ± 24	46.1 ± 0.9
Maltose	333 ± 18	37.5 ± 0.9
Maltotriose	320 ± 28	36.4 ± 1.5
Maltotetraose	275 ± 30	31.1 ± 1.4
Five glucose units	280 ± 15	31.4 ± 0.7
Six glucose units	267 ± 23	29.0 ± 1.1
Seven glucose units	274 ± 11	31.4 ± 0.1
Eight glucose units	293 ± 17	34.7 ± 0.9
Nine glucose units	304 ± 11	34.7 ± 0.3
Ten glucose units	251 ± 23	28.7 ± 1.3
Eleven glucose units	303 ± 17	34.5 ± 1.4
Twelve glucose units	278 ± 8	31.9 ± 0.9
Thirteen glucose units	293 ± 10	32.3 ± 0.9
Fourteen glucose units	209 ± 8	24.7 ± 0.4
Fifteen glucose units	223 ± 29	24.2 ± 1.3

Calculation of results

$$\frac{\text{Flow rate off column}}{\text{Flow rate to analyser}} \times \frac{\text{Area (counts)}}{\text{Area (counts) per mg}} \times \frac{100}{\text{wt. of sample (mg) put on column}} = \%\ \text{oligosaccharide in sample.}$$

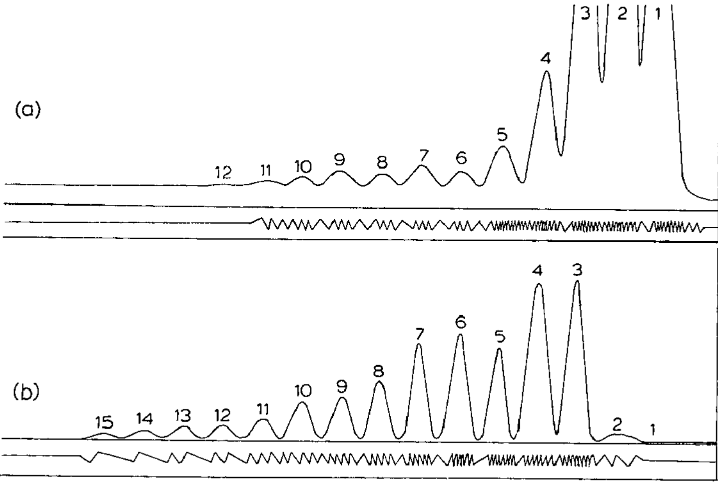

Fig. 4. Typical recorder traces obtained for (a) wort and (b) beer oligosaccharides. Peak numbers also represent glucose units.

RESULTS AND CONCLUSIONS

Up to fifteen homologous glucose oligomers can be determined in wort, beer and brewing syrups in a 24-h run and some typical chromatograms are shown in Fig. 4. The fermentable sugars glucose, fructose, sucrose and maltose are present in

TABLE II

TYPICAL ANALYSIS OF THE CARBOHYDRATES IN WORT AND BEER

	Wort, gravity 1033		*Beer extract, gravity 1013*	
	% w/w of solids	*% w/v of sample*	*% w/w of solids*	*% w/v of sample*
Fructose	0.73	0.06	—	—
Glucose	6.42	0.53	—	—
Sucrose	4.12	0.34	—	—
Maltose	53.58	4.42	3.08	0.10
Maltotriose	18.91	1.56	6.76	0.22
Maltotetraose	3.03	0.25	7.08	0.23
Five glucose units	2.06	0.17	2.77	0.09
Six glucose units	0.85	0.07	3.88	0.11
Seven glucose units	1.58	0.13	4.00	0.13
Eight glucose units	1.70	0.14	4.31	0.14
Nine glucose units	1.33	0.11	3.69	0.12
Ten glucose units	0.24	0.02	2.77	0.09
Eleven glucose units	0.12	0.01	2.46	0.08
Twelve glucose units	0.12	0.01	1.54	0.05
Thirteen glucose units	0.24	0.02	1.23	0.04
Fourteen glucose units	—	—	0.61	0.02
Fifteen glucose units	—	—	0.31	0.01
Total	95.03	7.84	44.5	1.43

wort in too great an amount to permit determination by the analyser and these sugars are best determined by GLC or an alternative method.

The quantitative analysis of wort and beer is shown in Table II. The lower fermentable sugars were determined using GLC (ref. 17).

The oligosaccharides are separated on the basis of number of glucose units in the molecule, without regard to whether the linkages are α-1,4 or α-1,6. If information is required about the relative proportions of α-1,4 and α-1,6 branching, the collected fractions corresponding to each peak can be combined and treated with pure α and β amylase (α-1,4 bonds) or pullanase (α-1,6 bonds) and the fractions recycled to examine the relative amounts of the resulting carbohydrate residues.

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CHROM. 4703

THIN-LAYER QUANTITATIVE CHROMATOGRAPHY OF ARABINOSE, RIBOSE AND XYLOSE IN THE PRESENCE OF OTHER SUGARS

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SUMMARY

We describe a rapid and unequivocally distinct separation of arabinose, ribose and xylose from all sugars, except fucose, which are normally found in biological fluids. The method can be applied quantitatively, with reasonable accuracy, to amounts of up to 80 μg of each aldopentose.

INTRODUCTION

Having found that arabinose, ribose and xylose are invariably present in the urine of fasting normal human subjects we sought for a simple and rapid way to measure the amounts of these sugars. We considered that the development of a procedure which could be applied in a clinical laboratory might prove of value in studying diseases involving connective tissue since it is now well-established that D-xylose and L-arabinose occur as components of glycoproteins associated with this tissue.

The three aldopentoses are not readily separated on paper, if at all, by the published procedures[1–3] and many hours of development, with concomitant spread of the spots, are required, so that quantitative analyses become difficult. Thin layers of Silica Gel G do not allow good separation between arabinose, xylose and mannose[4–6] and analogous overlapping takes place with crystalline cellulose[7].

This paper describes a slight modification of the thin-layer system of STAHL AND KALTENBACH[8] using Kieselguhr G made up with 0.02 M sodium acetate; two developments of 55 min each with suitable proportions of ethyl acetate, propan-2-ol and water give wide spatial separation of the three aldopentoses from one another and from all the other sugars found in normal urine with the exception of fucose. The latter, as far as we can see, occurs only in minor amounts in normal fasting urine but its presence can be detected by a qualitative differential spray reagent. We have not been able to separate xylose from fucose. The clear-cut separation of the aldopentose spots enables quantitative measurements to be made.

Using the unpleasant mixture of ethyl acetate–pyridine–water, it is possible on

cellulose to show separations which are qualitatively satisfactory but the resolution is not sufficient for quantitative purposes.

Our procedure has now been successfully used by colleagues in other laboratories.

EXPERIMENTAL AND RESULTS

Solvents

Alcohols were distilled over potassium hydroxide and silver oxide. Ethyl acetate was distilled after keeping over anhydrous potassium carbonate. Glacial acetic acid was distilled over potassium permanganate.

Sugars

Commercial samples were recrystallised, D-xylose and D-ribose from propan-2-ol and L-arabinose from aqueous propan-2-ol. Stock solutions (5 mg/ml) were prepared in water saturated with benzoic acid.

Reagents

The reagents were commercial samples and usually did not require further purification. 4-Methoxyaniline (*p*-anisidine) hydrochloride was prepared in the laboratory.

Chromatographic plates

Kieselguhr G (Merck) (one part) blended for 1 min with 0.02 *M* sodium acetate (three parts) was spread at a thickness of 0.5 mm. The plates were allowed to dry at room temperature for 24 h and were stored at the humidity and temperature of the laboratory.

Application of sugar solutions

Streaks, about 1.5 cm long, were applied uniformly from a 5-μl graduated pipette. Amounts of 1, 2 and 3 μl were typically used. Water was dried off in a current of warm air.

Development

The tanks were lined with filter paper as a routine but no previous vapour saturation was found to be necessary. The developing solvent, slightly modified in proportions from that of STAHL AND KALTENBACH[8] consisted in ethyl acetate–propan-2-ol–water (4:1:0.5). At room temperature the solvent was allowed to run the full length of the plate (20 cm in 55 min). If fructose was present, or if quantitative results were required, the solvent was evaporated in a current of air (15 min) and a second development was done to separate arabinose from the hexulose.

The developing solvent lasts for 48 h.

Spray reagents and revelation of sugars

Application was made using the Shandon Laboratory Spray Gun.

Attempts to reveal the sugar spots. Sprays commonly used on paper, cellulose powder or silica, when applied to the buffered kieselguhr, despite attempts to over-

come the buffer by increasing the acidity of the reagents, etc., generally proved quite unsatisfactory. Reagents containing aniline salts or salts of 4-aminobenzoic acid[9] gave very faint colours. Naphthoresorcinol[4-6] gave generally blue colours with a poor background. A range of indicators of suitable pK values with boric acid failed completely to show up any sugars. Acid phloroglucinol likewise proved unsatisfactory. However, a new reagent proved to give excellent qualitatively differential results but it was impossible to extract the colours without immediate fading; however, a fairly permanent staining took place with PRIDHAM's[10] p-anisidine spray (see below).

New qualitative spray. Stannous chloride added to the reagent used by BELL[9] for assay of hexoses and pentoses in aqueous solution gave intense colours as follows: pentoses, cerise; aldohexoses, brown-yellow; hexuloses, yellow; 6-deoxyaldohexoses, yellow or yellow-pink. The reagent consisted in 4-aminobenzoic acid (2 g), 3-carboxy-4-hydroxybenzenesulphonic acid (3 g) and $SnCl_2$ (1 g) in 100 ml of 80% aqueous acetic acid. The solution was filtered before use. Air-dried plates were sprayed and, without further drying, were heated for 15 min at 100°.

Quantitative spray. PRIDHAM's[10] reagent, devised for paper work, gave a stable colour which could be extracted from the kieselguhr. It consisted in 4-methoxyaniline (p-anisidine) hydrochloride (1 g) dissolved in MeOH (5 ml) containing sodium dithionite (100 mg) and butan-1-ol (95 ml). The air-dried plate was sprayed in four directions, allowed to dry in a current of air and then heated at 130° for 15 min when all the classes of sugar showed as brownish spots. (This spray gives selective colours when used on paper.)

R_F and R_G values. Typical values are given in Table I.

TABLE I

R_F AND R_G VALUES FOR EIGHT MONOSACCHARIDES

Sugar	*Typical R_F values × 100*	*Typical R_G values × 100*
Rhamnose	78	536
Ribose	66	460
Fucose[a]	47	324
Xylose[a]	45	312
Arabinose	32	224
Fructose	25	170
Glucose	14	100
Galactose	11	80

[a] Fucose and xylose do not separate completely.

Quantitative measurements

Because the sugar spots were so widely separated it was possible completely to remove the coloured areas of the kieselguhr from the glass surface *in toto*. Equal areas of the kieselguhr which contained the stain were carefully transferred to centrifuge tubes; at the same time equal areas which contained no sugars were treated likewise to serve as blanks. To each sample was added 4 ml of 95% aqueous methanol containing 1 g of $SnCl_2$ per 100 ml. The tubes were stoppered and vigorously shaken for 10 min and the solid then packed on the centrifuge. The extinctions of the supernatant

solutions were then measured at 395 nm (which is the λ_{max}), against the "blank" supernatant, on an SP500 spectrophotometer. The colour was stable for about 3 h and the three aldopentoses gave almost identical extinctions in the range of 5 to 80 μg. Typical results are given in Table II.

TABLE II

TYPICAL EXTINCTIONS FOR THREE ALDOPENTOSES STAINED BY PRIDHAM'S REAGENT

Amount (μg)	*Extinctions*		
	Arabinose	*Xylose*	*Ribose*
5	0.045	0.045	0.040
10	0.12	0.12	0.09
20	0.24	0.25	0.23
40	0.58	0.58	0.60
80	1.15	1.15	1.16

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CHROM. 4691

QUANTITATIVE RECOVERY OF SUGARS FROM SILICA GEL THIN LAYERS

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SUMMARY

Simple equipment and techniques were used for quantitative thin-layer chromatography and for recovery of sugars by elution. Although sugar recovery was not complete and varied quantitatively with the type of silica, it was consistent with a single silica and could be measured with good precision. The mean recovery of D-glucose was 95.2% with a relative standard deviation of 0.0074. The extent of losses of many sugars was quite similar. The losses occurred rapidly in conjunction with the drying of sugars on silica gel.

INTRODUCTION

The usefulness of thin-layer chromatography (TLC) for quantitative work will not be fully realized without further development of rapid and simple techniques that maintain good precision. This was the objective sought here. Although this report is limited to the recovery of sugars after TLC on silica gel, the techniques have broader applications. The precision reported here for sugar recoveries could be equaled or exceeded with other substances because the limitation of precision was due more to the sugar analyses than to TLC. This study does not include a comparison of mobile phases for chromatographic separations of sugars.

A review of elution techniques in quantitative TLC, with particular attention to precision, has been written by COURT[1].

METHODS

The basic procedure used in this work for the collection and elution of samples was described by MILLETT *et al.*[2]. Several simplifications of this procedure as well as special techniques for sugars were developed and are described.

* Maintained at Madison, Wisc., in cooperation with the University of Wisconsin.

Plate preparation

Thin-layer plates (20 cm × 20 cm) were prepared with a spreader that provided an even-layer wet thickness of 250 μ. Unless specified otherwise, the adsorbent was SilicAR-7*, a silica without binder. The dried plates were divided into lanes with a plate scriber (a rigid plastic sheet, 24 cm × 7 cm × 0.6 cm, notched on one long edge). The scriber formed alternate 2.2-cm and 1.4-cm lanes on the plate with separations of 1.5 mm where adsorbent was removed. To ensure uniform lane lengths, excess silica was removed from the top edge of the plate. Guides for the starting line were provided by dotting the edges of the wide lanes at a distance of 3 cm from the bottom edge of the plate.

Bureau of Standards D-glucose was used for all glucose measurements. Other sugars were commercial samples, some of which were purified by preparative TLC. Samples were applied from a 2-μl micropipette** to the 2.2-cm lanes in a series of three or four applications across each lane. The sample then approximated a streak $^1/_2$ cm or less broad. A single reference was applied to the center of each narrow (guide) lane at the starting point.

A few precautions were taken to assure uniform delivery from micropipettes. A single micropipette was used for most of the determinations and was stored in distilled water. It was occasionally rinsed with dichromate cleaning solution. Before sample application, the sidewall of the filled micropipette was held against a piece of filter paper and rolled to remove sample clinging on the outside of the micropipette. The pipette was then examined to ensure that it was completely full.

Chromatography

The mobile phase for most chromatograms was a mixture of ethyl acetate–acetone–glacial acetic acid (6:3:1). Plates were placed in an unequilibrated chamber a few minutes after the mobile phase was added. Chambers were kept in small insulated boxes at room temperature (about 23°). Plates were not removed until 5 to 10 min after the slowest lane had completely developed, usually a period of 70 to 100 min. The occasional fast lane was noted because spot migration was slightly increased in these cases.

Detection

Aniline phthalate solution (800 ml of butanol, 40 ml of water, 8 ml of aniline, 14.7 g of phthalic acid) and heat were used for detection of spots on the developed guide strips. The solution was streaked down the centers of guide strips with a 0.1-ml syringe fitted with a very fine-bore, flexible plastic needle. A simple wood slide and guide similar to that described by McKibbins *et al.*[3] was used to apply about 0.013 ml of reagent to a guide strip.

To heat narrow zones on the guide lanes, a resistance wire inside a small-bore glass tube was fixed to a holder that allowed the glass tube to be laid directly on the silica. Spots appeared after 20 sec of electrical heating.

The positions of developed samples were then located by reference to adjacent guide strips. Ordinarily an area extending 1.5 cm on each side of the assumed spot center was marked for removal (about 7 cm^2).

* Mallinckrodt Chemical Works, 3600 N. 2nd. Street, St. Louis, Mo. 63160.
** "Microcap", Drummond Scientific Co., 524 N. 61st. Street, Philadelphia, Pa. 10151.

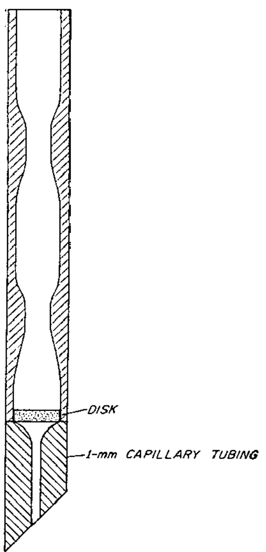

Fig. 1. Pickup tube for removing silica from thin-layer plates and for subsequent elution of adsorbed samples.

Removal of samples from chromatograms

Pickup tubes (Fig. 1) for removing silica were prepared from 5-mm-bore glass tubing as follows: A medium-grade, fritted glass disk was cautiously sealed inside the tubing and about 1 cm of 1-mm-bore capillary tubing was sealed on just below the disk, leaving a minimum volume between disk and capillary. The capillary tubing was cut off with a diagonal cut and was fire-polished. The 5-mm tubing was slightly constricted at 1 cm and at 3 cm above the disk and was cut off at 4.5 cm above the disk. The small fritted glass disk was not easily sealed within the glass tubing without excessive fusion. Its final porosity when tested with water should permit emptying the pickup tube (about 0.8 ml of water) in 8 to 40 sec under a net pressure of about 580 mm of mercury.

A 2.5- to 3-cm length of thin-walled polyolefin tubing that snugly fit into the pickup tube was pulled down at one end to about 1.5-mm diameter and was cut off diagonally. This plastic tip when inserted into the pickup tube easily picked up the adsorbent with suction.

A satisfactory pickup tube similar to that used by MILLETT *et al.*[2], but constructed from polyethylene tubing, is easily made with an integral tip; however, its asbestos mat requires more preparation time than does cleaning the counterpart fritted glass disk by backwashing. The fritted glass disk should be flushed soon after use. If it becomes clogged, it can be restored by soaking in alkali. Silicas with binder are slightly more difficult to remove from the pickup tubes and from thin-layer plates than are silicas without added binder.

Elution

After a sample was collected, the pickup tube was tapped to settle the bed of

silica into the bottom of the tube, and a few small drops of water were added slowly to wash the sides of the tube. A small-bore, flexible plastic needle attached to a small syringe was especially satisfactory for adding the water. After the first 0.05 to 0.1 ml of water had seeped into the silica, the tube was filled with water from the syringe. Elution time was often shortened to 10 to 15 min by a small amount of pressure.

Each eluate was collected in a 0.1-ml micropipette (Microcap). This step was facilitated by clipping pickup tubes in a vertical position onto a wooden holder. The horizontal base of the holder had narrow slots in which the 0.1-ml pipettes rested.

After filling, each 0.1-ml collection pipette was emptied into exactly 0.9 ml of water in a screwcap vial. A micropipette holder (Drummond) was used to blow out the 0.1-ml pipette and to suck up some of the sample solution for two or three rinsings. The micropipette holder consisted of 4 cm of glass tubing with a punctured rubber plug (to hold the micropipette) at one end and a punctured rubber medicine-dropper bulb at the other end.

Spectrophotometric measurement

For a series of samples, the 1.0 ml volume per sample was sufficient for rinsing and for finally filling a 0.25-ml syringe. The analysis of the 0.25-ml aliquot by dehydration with 2.0 ml of sulfuric acid followed by spectrophotometric measurement has been described[4]. For this work, there were two minor changes: (1) Reagent grade concentrated sulfuric acid was used without adjusting its concentration to 95.0% and, (2) the 70° water bath for reaction tubes was replaced by a heating block. A 30-min heating period was used as before.

Because this analysis can easily detect contamination such as cellulosic dust, a few simple but necessary precautions were taken. It was most convenient to dispense the concentrated sulfuric acid from a 9-lb. reagent bottle into a 100-ml glass-stoppered bottle by means of a glass hand pump* screwed onto the reagent bottle. The delivery tip of the pump was protected by a glass vial when not in use and was rinsed with water before using. If acid is poured from a reagent bottle, care must be taken not to collect drips from the side of the neck. The open ends of the reaction tubes (16-mm × 150-mm test tubes) were cleaned in dichromate cleaning solution, rinsed, and covered with clean glass vials; during this procedure handling of the lip areas was always avoided. The tubes were continually covered and protected from dust during use and during storage. The covered tubes were dried in a test-tube rack by laying the rack on its side in a clean oven. Excessive dust within the oven can cause erratic blanks.

For each spectrophotometric run, three blanks of 0.25 ml of water in 2.0 ml of H_2SO_4 were prepared. The absorbance of reagent blanks, depending on the acid, varied from day to day from about 0.012 to 0.022 at 320 nm, but the range within a set of three was usually within 0.004 when the reaction tubes were clean.

Plate blanks were obtained by removing silica samples from blank lanes. These blanks were found to vary from 0.001 to 0.008 with an overall average of 0.004, which was consistent enough to be used without measurement at each run. This blank may change for different silica gels and possibly for different batches of the same silica. SPENCER AND BEGGS[5] found that the blank due to very small silica particles can be reduced by membrane filtration. The blank is here reduced by dilution and by fil-

* "Repipet", Cole-Parmer Instrument Co., 7425 North Oak Park Avenue, Chicago, Ill. 60648.

tration through the fritted glass (or asbestos mat). To increase the sensitivity of this method, it would be necessary to further reduce blanks by some means such as membrane filtration and perhaps also by special extraction of the silica and purification of the solvents used for development.

Standard 2-μl samples were applied onto 4-mm squares of Whatman No. 1 filter paper in screwcap vials. These samples were diluted with 0.9 ml of water, and the net absorbance from the reaction of a 0.25-ml aliquot with 2.0 ml of H_2SO_4 was corrected to a sample volume of 1.0 ml. The preparation of the paper squares included a vacuuming with the tip of a medicine dropper attached to a vacuum line. After removing cellulosic dust in this way, the residual paper blanks varied from 0.001 to 0.006. An average of 0.003 was taken as a uniform paper blank.

The recovery of a sample removed from a thin-layer plate is expressed as a percentage of its mean standard by comparing net absorbances after corrections for blanks.

RESULTS AND DISCUSSION

Application of samples

FAIRBAIRN[6] discussed the critical importance of sample application in quantitative chromatography and reported the results obtained with several devices. He stated his best results were obtained with a mechanical applicator. Here it was found that the precision of the 2-μl micropipette was considerably better than the 3.3% relative standard deviation reported by FAIRBAIRN and the 1.5% reported by JORK[7]. In a set of nine replicate glucose standards, applied and measured as described, the relative standard deviation was 0.55% at a mean absorbance of 0.872 (about 65 μg of glucose). In measurements on 6 different days, a total of 21 samples had a mean absorbance of 0.811 and a relative standard deviation of 0.59%.

Precision of sugar recovery

The recoveries of glucose samples applied to silica gel layers, but not chromatographed, were measured by removing and eluting about 7 cm^2 of silica including the sample. The mean recovery of 25 of these samples in seven runs was 98.1% of the mean standard sample. The relative standard deviation of these recoveries was 0.0091. This includes variation between and within runs.

The chromatographic step was similarly tested by comparison between chromatographed and standard samples. The mean recovery of 23 samples from six runs was 95.2% with a relative standard deviation of 0.0079.

An efficient determination consists of three replicate chromatographed samples (when an average plate blank and an average percentage recovery are known). The chromatographic data were recalculated to find the day-to-day variability between means of three samples. This variability had a relative standard deviation of 0.0074 at the 95.2% mean recovery value (Table I).

Variability between samples within a single run was computed from chromatographed (mean recovery, 95.2%) and unchromatographed (mean recovery, 98.1%) samples. This variability (Table I, line 3) was less than that between chromatographic runs on different days (line 4). The additional error between days was in the chromatographic step because the standards did not show the same effect. Although the smaller

TABLE I

VARIABILITY OF GLUCOSE DETERMINATIONS

Samples	*Data points*	*Standard deviation*	*Relative standard deviation*
Standards	9 (1 run)	0.0048[a]	0.0055
Standards	21 (6 runs)	0.0048[b]	0.0059
Applied to silica	48 (13 runs)	0.54[c]	0.0056
Chromatographed	23 (6 runs)	0.75[d]	0.0079
Chromatographed	23 (6 runs)	0.71[e]	0.0074

[a] Based on a mean absorbance of 0.872.
[b] Based on 21 samples with a mean absorbance of 0.811.
[c] Variability between samples within a single run; based on percentage recovery.
[d] Includes variability within and between runs, and variability around an average recovery of 95.2%.
[e] Computed variability of the means of three samples between runs; overall mean recovery, 95.2%.

error might be used by measuring recoveries of standards and unknowns in a single run, the 0.71 standard deviation between means of three samples is considered the more practical result. Thus, after correction for expected loss on the plate, the mean recovery value of three 60-μg samples of glucose had a 95% probability of being within 3% of the applied amount.

The glucose samples used for determination of recovery had a net absorbance of about 0.8. A considerable part of the variability, that part due to reagent and plate blanks, was independent of sample size and consequently caused greater errors for smaller samples. This was shown by mean percentage recoveries of 20 μg of glucose in three runs that had standard deviations of 2.7 (three samples) and 2.5 and 3.6 (five samples each) compared with the 0.534 standard deviation within a day found for 60-μg samples.

The attainable precision of recovery from silica layers is probably better than that found here with 60-μg glucose samples. The similarity of the variability of standards (Table I, lines 1 and 2) and the variability within chromatographic runs (line 3) is an indication that limitations were imposed by factors not in the chromatographic step. Other colorimetric measurements, reduction of blank values, or classes of compounds other than carbohydrates may result in less variability, especially with small sample sizes. The precision reported here is very similar to the 0.74 to 0.91% relative standard deviation obtained by MILLETT *et al.*[2] for 25 to 100 μg of furoic acid recovered after TLC.

Quantitative analysis

Although glucose samples were not completely recoverable after chromatography, the uniformity of recoveries permitted using a correction factor, 1.05, for estimating the amount of glucose applied to the plate. The reliability of this factor was only well established with 60 μg of glucose, but the results in Fig. 2 suggest that it could be used for a wide range of sample sizes. The 95.2% average recovery of glucose was confirmed by recovery of the same percentage of radioactivity after TLC of a

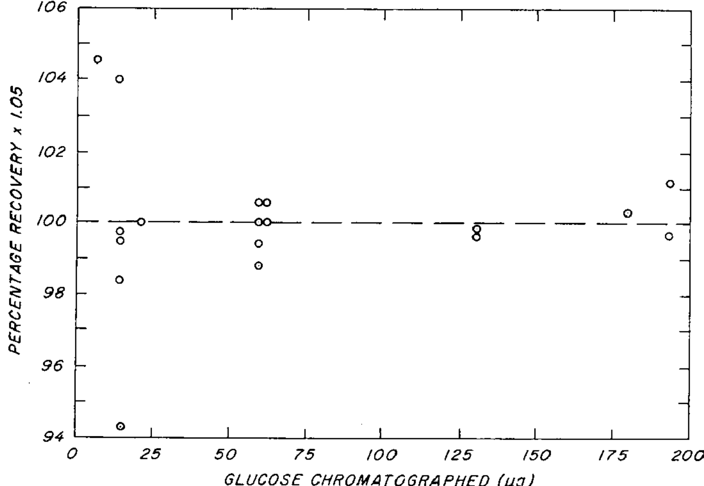

Fig. 2. Variability of corrected glucose recoveries (means of three samples) after thin-layer chromatography on silica gel.

single sample of [^{14}C]glucose. The [^{14}C]glucose used for this test was previously chromatographed by TLC and recovered.

The scatter of results in Fig. 2 at 10 to 20 μg of glucose is evidence of the larger relative errors with these smaller amounts of glucose. To decrease this error, it is practical to increase the sample by two or three applications from a 2-μl micropipette. The larger samples in Fig. 2 were applied in this way.

Insufficient data were collected to determine correction factors for other sugars with the same reliability as for glucose. However, the means of a few determinations were within the limits of error around the 95.2% recovery of D-glucose (L-arabinose 94.8%, D-xylose 96.1%, D-galactose 94.7%, D-mannose 94.4%, sucrose 96.9%).

About 1 h was required for application, detection, pickup, and elution of three replicates. A total time of 3.5 h included also 90 min for development and 45 min for the analytical steps after elution.

Nonrecoverable sugar

There was evidence that the losses of sugar occurred very abruptly at the moment of drying of samples on silica. The recovery of xylose from unchromatographed layers of Silica Gel H was found to be 94% after a few minutes of drying, 91% after 1 h, and 84% after 17 h. However, when a plate was put into a closed chamber at 100% relative humidity immediately after sample application, the recovery was 99% after 1 h in the chamber. There was visual evidence of material left at the starting point of sprayed plates and also at the first position of migration when double development was used. COXON[8] has also reported spots left at the start of a chromatogram after TLC of glucose.

Direct evidence of strong adsorption of glucose was obtained from a spectrophotometric measurement which showed about 1% recovery of hexose in the water eluate of five start areas after TLC of a total 600 μg of glucose. The possibility of extra spots must be considered during qualitative interpretation of multiple-developed

chromatograms and argues against the use of multiple development for quantitative purposes.

Properties of the adsorbent

Initial work with Silica Gel H led to the finding of losses as mentioned and to the testing of other silicas. A comparison based on the percentage recovery of xylose after chromatography gave the following recoveries: Silica Gel H 93.1%, Silica Gel GF 94.6%, and SilicAR-7 96.0%. When xylose was applied, the recoveries without chromatography were the following: Kieselguhr 97.7%, Silica Gel GF 94.3%, Silica Gel H 90.3%, and SilicAR-7 96.6%. Other tests with glucose gave variable results that indicated Silica Gel H gave almost as good recoveries as SilicAR-7 after chromatography but less recovery without chromatography. SilicAR-7 was chosen for further work because recoveries from it were high and consistent. In any case, it appears that different brands of silica gel may give different recoveries, and that different batches of a single brand may possibly give different recoveries.

Other adsorbent factors tested with SilicAR-7 were found of lesser importance than the kind of silica. The drying time of the applied sample or of the chromatographed plate was not critical up to 75 min, the length of the test, which is more than sufficient time for the detection and removal of samples from two or three plates.

Activation of plates at 110° or 150° did not change percentage recoveries of glucose from that found for a plate dried at room temperature, although development time was shorter and separations were improved on the drier plates.

The area of developed spots was estimated by removal of 1- and 2-cm bands of silica. All of the expected glucose and 97% of the expected xylose samples were contained within 2-cm bands. About 80% of the glucose and 70% of the xylose were contained within 1-cm bands. The glucose spot center migrated about 3 cm and the xylose spot center about 7 cm. About 60-μg samples were used for these tests. Because of potential error in spot location, a practical lower limit of separation of spot centers for quantitative determinations would be 3 cm in the system described. With care, 2.5-cm separation could give useful data.

The solvent system used in this work spread galacturonic acid over a large area. However, glucurone that migrated 13.5 cm was contained in a 2-cm band and 4-O-methylglucuronic acid that migrated about 11.5 cm appeared to be equally well concentrated.

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Trade names are included for the benefit of the reader and do not imply any endorsement or preferential treatment of a product by the U.S. Department of Agriculture.

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CHROM. 4679

THIN-LAYER CHROMATOGRAPHIC ANALYSIS OF ENZYMIC HYDROLYSATES OF AGAR

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SUMMARY

The most suitable solvent systems for the analysis, by cellulose thin-layer chromatography of the neutral and charged oligosaccharides formed by the enzymic hydrolysis of agar have been evaluated. As the mixture of oligosaccharides is not adequately resolved by one or two dimensional thin-layer chromatography to enable identification of the individual components, the neutral and charged sugars are first separated from each other on DEAE-Sephadex A-25 (Cl^-). The solvent system for the optimum separation of the neutral sugars depends on the degree of polymerisation of the oligosaccharides under investigation. A good separation of the charged oligosaccharides is obtained in the solvent system butan-1-ol–acetic acid–water (4:1:2).

INTRODUCTION

Agar is a family of polysaccharides obtained from the agarophytes of the Rhodophyceae (red seaweeds). The agarose component of agar is essentially neutral and has been shown to have a structure in which 3-linked β-D-galactopyranose residues and 4-linked 3,6-anhydro-α-L-galactopyranose residues alternate[1]. This repeating sequence is masked in agaropectin, the charged polysaccharide complex of agar. D-Galactose residues can be replaced by the pyruvic acid ketal, 4,6-O-(1'-carboxyethylidene)-D-galactose[2] and the L-galactose residues can be replaced with sulphated galactoses[3]. D-Glucuronic acid has also been reported to be present[4]. In both agarose and agaropectin some of the D-galactose units can be replaced with 6-O-methyl-D-galactose[5]. The extent of masking varies from species to species.

All known enzyme systems which degrade agar do so by cleaving the β-1,4-linkage between the D-galactose and 3,6-anhydro-L-galactose residues[6-8]. The basic neutral unit formed is the disaccharide neoagarobiose[6], the other neutral oligosaccharides formed are multiples of this. The R_{Gal} values of these oligosaccharides as reported by various workers[6-8] together with the R_{Gal} values of the di- and tetrasaccharide in which one of the D-galactose units is replaced by 6-O-methyl-D-galactose[9] are shown in Table I. The enzymic hydrolysate of agar also contains charged oligosaccharides which so far have not been adequately resolved and identified.

TABLE I

R_{Gal} VALUES FOR NEUTRAL OLIGOSACCHARIDES OBTAINED BY ENZYMOLYSIS OF AGAR

Method used: (I) Ascending paper chromatography in solvent system D[6]; (II) Descending paper chromatography in solvent system E[7]; (III) Cellulose TLC in solvent system E (double developed)[8,9].

Oligosaccharide	*R_{Gal} values*		
	I	*II*	*III*
6^1-O-Methyl-neoagarobiose	—	—	1.70
Neoagarobiose	1.30	1.35	1.30
6^3-O-Methyl-neoagarotetraose	—	—	1.05
Neoagarotetraose	0.62	0.75	0.80
Neoagarohexaose	0.34	0.32	0.46
Neoagaro-octaose	0.15	0.12	0.25

In an investigation into the mode of action of bacterial agarases we needed to develop solvent systems which would separate neutral oligosaccharides with a wide range of D.P.*. A chromatographic method of separating the charged oligosaccharides had also to be found.

EXPERIMENTAL

Materials and methods

Cellulose layer. Microcystalline cellulose (Camag D.S.O., 15 g in 80 ml of distilled water) was layered to a thickness of 250 μ on glass plates and then air dried.

Indicator reagent. The modified naphthoresorcinol reagent[7] was used. This is the most sensitive indicator for oligosaccharides containing 3,6-anhydro-L-galactose. The spray reagent consists of two parts ethanolic sulphuric acid (375 ml of ethanol plus 100 ml of concentrated sulphuric acid) and one part naphthoresorcinol solution (0.2% in ethanol). No heat was applied to the plate after spraying as this tends to char the cellulose. The oligosaccharide spots are more distinct under UV light.

Solvents. All solvents used were reagent grade obtained from Fisher Ltd. The solvent systems used for cellulose TLC analysis during the course of this investigation were as follows: (A) Butan-1-ol–ethanol–water (3:1:1); (B) Butan-1-ol–ethanol–water (3:2:2); (C) Butan-1-ol–ethanol–water (1:1:1); (D) Butan-1-ol–acetic acid–water (4:1:2); (E) Butan-1-ol–pyridine–water (2:1:1). Solvent system D must be made up daily in order to obtain reproducible results.

Column chromatography. DEAE-Sephadex A-25 (Pharmacia) was prepared in the chloride form by washing successively with hydrochloric acid (0.5 *N*), sodium hydroxide (0.5 *N*), hydrochloric acid (0.5 *N*) and then extensively with distilled water.

Agar. The following agars were used: (a) Difco Bacto Agar; (b) *Gelidium cartilagineum* agar.

Preparation of agaropectin components. Previous studies[10] have shown that successive extraction of the commercial agar at room temperature and at 50° yields two charged polysaccharide complexes. The charged polysaccharides in each eluant being obtained free of neutral agarose by precipitation with cetyl pyridinium chloride.

* D.P. = degree of polymerisation.

In the text these polysaccharides will be referred to as agaropectin (20°) and agaropectin (50°).

Enzyme. The purified extracellular agarase from *Pseudomonas atlantica*[11] was used to hydrolyse the polysaccharides.

RESULTS AND DISCUSSION

Neutral oligosaccharides

In this study we found that solvent system B has several advantages over solvent system E which has been used previously for the separation of neutral oligosaccharides obtained by the enzymic hydrolysis of agarose. Only one development is necessary and the sensitivity with the naphthoresorcinol spray reagent under UV light is markedly increased as there is no background absorption due to pyridine. This is important when monitoring an enzymic reaction in which the quantity of an intermediate might be very small. The R_{Gal} values for the oligosaccharides with D.P.'s between 2 and 8 are shown in Table II.

Solvent system A gives an excellent separation of the di- and tetrasaccharides but was unsuitable for higher oligosaccharides (Table II). This was therefore the solvent of choice for the preparative TLC of these two oligosaccharides.

TABLE II

COMPARISON OF THE R_{Gal} VALUES OF NEUTRAL OLIGOSACCHARIDES ON CELLULOSE TLC USING DIFFERENT SOLVENT SYSTEMS

Oligosaccharide	*D.P.*[a]	*Solvent A*	*Solvent B*	*Solvent C*
Neoagarobiose	2	1.30	1.11	—
Neoagarotetraose	4	0.51	0.87	—
Neoagarohexaose	6	0.19	0.65	—
Neoagaro-octaose	8	0.05	0.41	0.57
	10	—	—	0.42
	12	—	—	0.30
	14	—	—	0.18

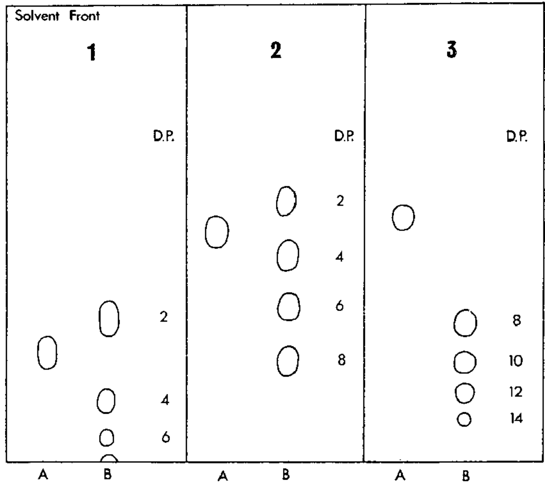
[a] D.P. = degree of polymerisation.

Neutral oligosaccharides with D.P.'s 8 to 14 are well separated in solvent system C (Table II). Multiple developments in this solvent made preparative cellulose TLC possible for these higher oligosaccharides. Fig. 1 compares the separation of the neutral oligosaccharides in solvents A, B and C.

These solvent systems have been found to be extremely valuable in monitoring how various agarases degrade neutral high molecular weight oligosaccharides which have been prepared by the partial enzymolysis of agarose, and purified by preparative thin-layer chromatography using the most suitable solvent system.

Charged oligosaccharides

The mixture of neutral and charged sugars obtained by the enzymic hydrolysis of complete agar and various agaropectin fractions is not resolved by one dimensional TLC as the charged oligosaccharides have the same R_{Gal} values as the neutral oligo-



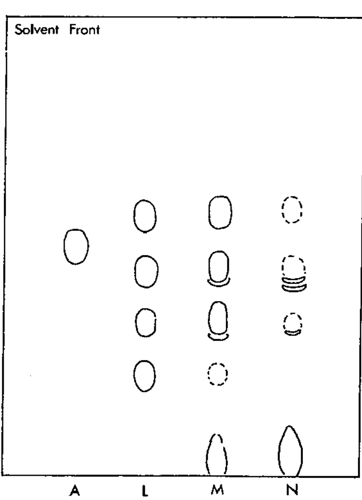


Fig. 1. Separation on cellulose TLC of the neutral oligosaccharides formed by the enzymic degradation of *Gelideum cartilagineum* agar. A comparison of the separation of various D.P. ranges in different solvents. A in each case is galactose and B the neutral oligosaccharides. Plate 1 was developed in solvent system A, plate 2 in solvent system B and plate 3 in solvent system C.

Fig. 2. Comparison using solvent system B of the enzymic hydrolysate of agarose (L), agaropectin (50°) (M) and agaropectin (20°) (N). A is the reference sugar galactose.

saccharides in some solvents and in others the charged sugars run as poorly defined spots. Fig. 2 compares the enzymic hydrolysates of two agaropectin fractions and the hydrolysate of agarose using solvent B. The "crescents" due to the charged sugars in a neutral solvent can be reduced by using the basic solvent E but the effect is not eliminated.

The mixture of neutral and charged sugars can be partially resolved by two dimensional TLC. The homologous series of neutral oligosaccharides lie along the diagonal and are well separated from the charged sugars. This procedure is not useful in characterising the charged oligosaccharides, which lie off the diagonal, as they often streak and their absolute positions are difficult to reproduce (Fig. 3).

TABLE III

COMPARISON OF THE R_{Gal} VALUES OF NEUTRAL AND CHARGED OLIGOSACCHARIDES USING SOLVENT D

Neutral oligosaccharides D.P.	R_{Gal}	*Charged oligosaccharide**	R_{Gal}
2	1.10	a	0.91
4	0.80	b	0.64
6	0.51	c	0.38
8	0.32	d	0.23
10	0.18		

* a, b, c, d refers to oligosaccharides of unknown structure which contain a pyruvate group as the only charged group. The designation a, b, c, d is also used in Fig. 4.

A good separation of the charged oligosaccharides on cellulose TLC was only obtained after completely separating them from the neutral oligosaccharides. A convenient method of carrying out this separation was by column chromatography on DEAE-Sephadex A-25 (Cl^-). The neutral oligosaccharides are eluted from the gel with distilled water and the charged oligosaccharides with a solution of sodium chloride (2.0 *M*). The charged oligosaccharides were desalted on Sephadex G-25 before being examined by cellulose TLC.

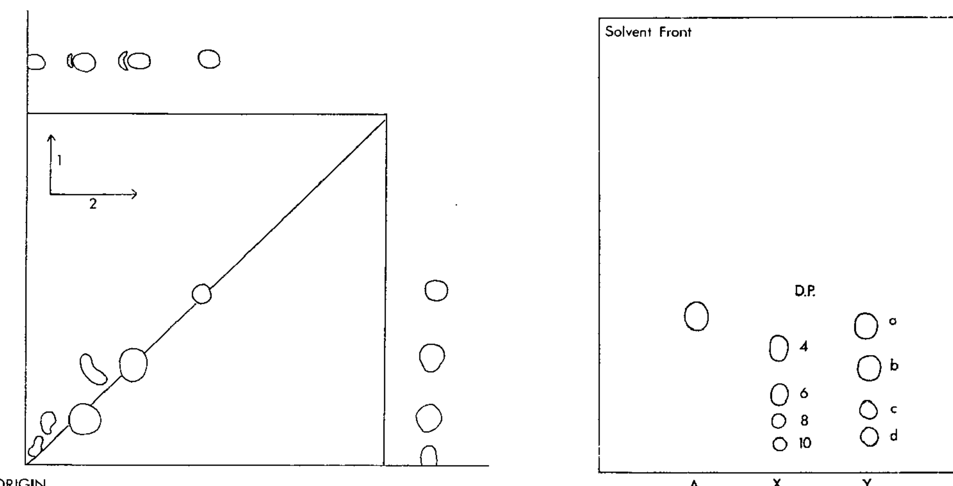


Fig. 3. Two dimensional separation of the complete hydrolysate of Difco Bacto Agar. Direction 1 solvent system E and direction 2 solvent system B.

Fig. 4. Comparison of the separation in solvent D of the charged oligosaccharides (Y) and the neutral oligosaccharides (X) formed by the enzymic degradation of *Gelideum cartilagineum* agar or Difco Bacto Agar. A is the reference sugar galactose.

Poor resolution of the charged oligosaccharides from Difco Bacto Agar and *Gelidium cartilagineum* agar was obtained in neutral and basic solvents, but an excellent separation was obtained in solvent system D (Fig. 4). The R_{Gal} values of the principle charged sugars are shown in Table III. Chemical analysis of these oligosaccharides have indicated that they contain a combined pyruvic acid molecule as the only charged group. The sulphated oligosaccharides formed by the enzymolysis of agar are of high molecular weight presumably because the agarose cannot cleave a β-1,4-linkage close to a sulphated galactose unit[12].

In solvent system D the neutral oligosaccharides have similar R_{Gal} values to the charged oligosaccharides, Table III, and hence it is not possible to study the complete enzymic hydrolysate of agar in this solvent as the higher yield of neutral oligosaccharides tends to mask the presence of the charged oligosaccharides.

CONCLUSION

The most suitable solvent system for the separation of the neutral series of oligosaccharides depends on their D.P. For the di- and tetrasaccharide the best

solvent is solvent A. Oligosaccharides with a D.P. between 2 and 8 are separated in solvent B and the range of D.P. from 8 to 14 in solvent C.

The charged oligosaccharides can only be examined after prior separation from the neutrals on DEAE-Sephadex. Solvent D gives a good separation of the charged oligosaccharides containing a pyruvic acid molecule. The sulphated oligosaccharides are of higher molecular weight and to date no solvent system is known which adequately separates them.

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CHROM. 4689

ZUR ANALYTIK PFLANZLICHER GLYKO- UND PHOSPHOLIPOIDE UND IHRER FETTSÄUREN

I. EINE NEUE DÜNNSCHICHTCHROMATOGRAPHISCHE METHODE ZUR TRENNUNG PFLANZLICHER LIPOIDE UND QUANTITATIVEN BESTIMMUNG IHRER FETTSÄURE-ZUSAMMENSETZUNG

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SUMMARY

By means of a new solvent system (acetone–benzene–water (91:30:8)) for one-dimensional thin-layer chromatography on silica gel the complete separation of plant glycolipids (monogalactosyldiglyceride, digalactosyldiglyceride, sulfolipid), phospholipids (cardiolipin, phosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol, phosphatidylcholine) and of neutral lipids is achieved. By transmethylation of the singular lipid zones from the thin-layer plate with sodium methylate in test tubes the fatty acids of all the lipids out of 2–4 mg of total lipids can be determined and compared quantitatively within a short time.

EINLEITUNG UND ALLGEMEINE METHODIK

Auf dem Gebiet pflanzlicher Lipoide hat sich in neuerer Zeit das Interesse vor allem auf die Gruppe der Glykolipoide (Mono- und Digalactosyldiglycerid sowie Sulfolipid) konzentriert. Diese Lipoide, zusammen mit dem Phosphatidylglycerin charakterische Inhaltsstoffe von assimilierenden Geweben[1–9], stehen offenbar mit Photosynthese-Prozessen in engem Zusammenhang.

Für die schnelle Routine-Analyse dieser Lipoide ist die Dünnschichtchromatographie (DC) die Methode der Wahl. Jedoch gelingt es mit keinem der bisher verwendeten Laufmittelsysteme (meist Mischungen von Chloroform, Methanol und Wasser bzw. Diisobutylketon und Wasser, z.T. unter Zusatz von Essigsäure oder NH_4OH)[10–18], die oben erwähnten Glyko- und Phospholipoide sowie die Neutrallipoide durch eindimensionale DC vollständig zu trennen. Einige "Lipoidpaare" bleiben in diesen Systemen völlig ungetrennt. Zumeist wird deshalb die zweidimensionale DC mit zwei verschiedenen Laufmittelsystemen benutzt. Diese Methode hat aber abgesehen von dem grösseren Zeitaufwand und der damit verbundenen grösseren Oxydationsgefahr für die Lipoide den Nachteil, dass man in einem Arbeitsgang stets nur einen Extrakt und auch nur sehr geringe Mengen analysieren kann.

Wir berichten daher im Folgenden über ein neues Laufmittelsystem für die eindimensionale DC, das alle oben erwähnten Glyko- und Phospholipoide vollständig voneinander trennt. Ausserdem werden die Neutral-Lipoide von den genannten Lipoiden abgetrennt und ihrerseits nochmals in zwei Gruppen unterteilt. Durch direkte Transmethylierung der aus dem Kieselgel eluierten Lipoide mit Natriummethylat und anschliessender Gaschromatographie (GC) der erhaltenen Fettsäuremethylester kann in verhältnismässig kurzer Zeit für jedes Lipoid die Fettsäure-Zusammensetzung ermittelt werden. Diese Methode wurde so ausgearbeitet, dass ein quantitativer Vergleich der einzelnen Lipoide hinsichtlich ihrer Fettsäuren möglich ist.

EXPERIMENTELLER TEIL

Lipoid-Extrakte

Die Extrakte können auf die übliche Weise durch Extraktion von frischem oder getrocknetem Pflanzenmaterial mit organischen Lösungsmitteln (Äther, Chloroform, Methanol u.a.) hergestellt werden. Es empfiehlt sich aber, eventuell vorhandenes Wasser durch Trocknung der Extrakte im Exsikkator zu entfernen.

Dünnschicht-Platten

Die Platten (12 × 20 cm) werden mit einer Suspension von Kieselgel HF_{254} (Merck) in Wasser (pro Platte 3.5 g Kieselgel und 9 ml Wasser) bestrichen und nach 30 Min. Trocknen an der Luft 2 Std. bei 130° aktiviert. Wichtig für gute Trennungen sind optimal aktivierte Platten. Nach dem Abkühlen werden deshalb die noch warmen Platten in einen Exsikkator gestellt und im Vakuum über frischem P_2O_5 aufbewahrt. Das P_2O_5 ist öfters zu erneuern. KOH *(rotulis)* eignet sich nicht als Trocknungsmittel.

Auftragsmenge und Auftragen der Lipoide

Bei Bedarf wird eine Platte dem Exsikkator entnommen. Dann werden möglichst rasch die aufzutrennenden Lipoide (gelöst in Chloroform–Methanol o.ä.) mit einem weichen Pinsel strichförmig auf die Platten aufgetragen. Pro Platte lassen sich etwa 2–4 mg Gesamtlipoide auftrennen.

Laufmittel

Aceton p.A.–Benzol p.A.–Wasser (91:30:8). Die DC-Kammern werden durch Einstellen von Filtrierpapier gesättigt. Zur Erreichung optimaler Trennungen empfiehlt es sich, nur p.A. Lösungsmittel zu verwenden und diese zusätzlich über eine Kolonne zu destillieren.

Laufzeit: *ca.* 30 Min/16 cm bei 23°.

Anfärben der Lipoide

Nach der Chromatographie werden die Platten im kalten Luftstrom getrocknet und mit alkalischer 0.003%-iger Lösung von Rhodamin 6 G besprüht. Dieses Reagenz wird unmittelbar vor dem Besprühen durch Mischen gleicher Volumina von wässriger 8%-iger NaOH und wässriger 0.006%-iger Rhodamin 6 G-Lösung hergestellt[1]. Die Platten werden solange besprüht, bis die ganze Schicht gleichmässig durchfeuchtet ist. Dann werden die Platten sofort unter dem UV-Licht (366 nm) betrachtet. Die Lipoidzonen treten als hellgelbe Flecken auf blauviolettem Untergrund hervor.

Eluieren der einzelnen Lipoid-Zonen

Die einzelnen Lipoid-Zonen einer besprühten und noch feuchten Platte werden mit einer Nadel umrandet. Dann wird die Platte im kalten Luftstrom vollständig getrocknet, die markierten Kieselgel-Zonen mit einem Spatel ausgekratzt und das Kieselgelmaterial in je ein Reagenzglas gegeben.

Darstellung der Fettsäuremethylester

(a) Herstellung der 2 N Natriummethylat-Standardlösung. 4.6 g metallisches Natrium werden in 100 ml Methanol p.A. in einem 250 ml-Erlenmeyerkolben gelöst. Nach vollständiger Lösung wird die Natriummethylat-Lösung mit Methanol p.A. bis auf 100 ml aufgefüllt und gut verschlossen im Kühlschrank aufbewahrt. Die Lösung ist so mehrere Monate haltbar.

(b) Transmethylierung der Lipoid-Zonen. Das in einem Reagenzglas befindliche Kieselgel-Material einer Lipoid-Zone (s.o.) wird mit 2.5 ml Methanol p.A. versetzt und einige Minuten gut geschüttelt. Dann werden 2.5 ml 2 *N* Natriummethylatlösung zugegeben und diese Mischung unter häufigem Umschütteln 20 Min. bei Zimmertemperatur stehen gelassen. Danach wird durch Zugabe von 1 ml 37%-iger HCl angesäuert, diese Mischung nach kurzem Umschütteln mit 20 ml Wasser verdünnt und durch Einstellen in kaltes Wasser auf Zimmertemperatur abgekühlt. Die Mischung wird in einen 250 ml Schütteltrichter überführt und das Reagenzglas mehrfach mit Wasser und abschliessend einmal mit 1 ml Methanol nachgespült, so dass sich etwa 70 ml im Schütteltrichter befinden. Diese Menge wird dreimal mit je 30 ml Petroläther–Äther (1:1) extrahiert und die vereinigten Oberphasen einmal mit Wasser gewaschen. Die Oberphase wird in einen 250 ml Erlenmeyerkolben überführt, das Lösungsmittel im Stickstoffstrom vollständig entfernt, der Rückstand mit 3×5 ml Petroläther–Äther (1:1) in ein Reagenzglas überführt, wiederum das Lösungsmittel im N_2-Strom entfernt und der Rückstand mit 3×0.5 ml Chloroform quantitativ in ein 2 ml-Reagenzglas überführt. Erst unmittelbar vor der GC wird dieses Lösungsmittel nochmals vorsichtig im N_2-Strom entfernt, der Rückstand in genau 0.05 ml Chloroform p.A. gelöst und aliquote Mengen dieser Lösung gaschromatographisch analysiert.

Diese Methode der Transmethylierung erwies sich in Reihenversuchen als sehr gut reproduzierbar. Gegenüber der zweistündigen Verseifungsmethode mit methanolischer 2 *N*-KOH und anderen Verfahren ergaben sich keine Unterschiede.

Gaschromatographie

Modell Packard, Säule: 3 m × 4 mm, Säulenmaterial: 20% Reoplex 400 auf Chromosorb WS (45–60 mesh), Säulentemperatur: 190°, Gasdurchfluss: 80 ml Argon/Min, Einspritzmenge: 0.01 mg.

Quantitativer GC-Vergleich der Fettsäuren aller Lipoide aus einem Gesamtlipoid-Extrakt

Bei Auftrennung von *ca.* 2–4 mg eines Gesamtlipoid-Extrakts über eine Dünnschichtplatte (s.o., s. auch Fig. 1A) erhält man nach dem Eluieren aller Lipoid-Zonen aus der Platte und nach der Transmethylierung dieser Zonen unter gleichen Bedingungen die Fettsäuren aller Lipoide in den ursprünglich vorhandenen absoluten Mengen.

Wenn man nun die Fettsäuremethylester immer in der gleichen Lösungsmittel-

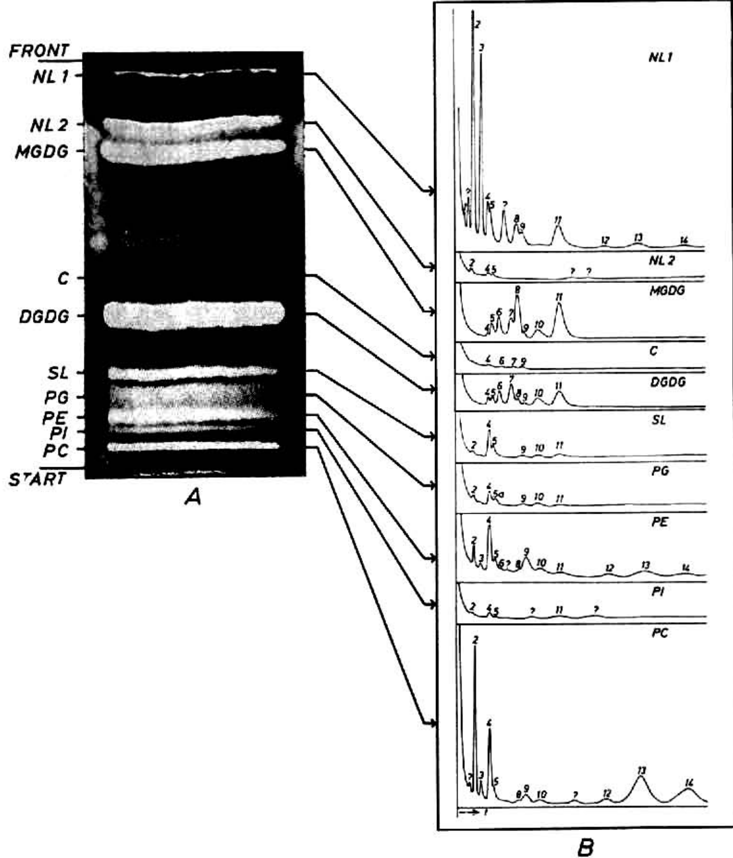

Fig. 1. (A) DC-Trennung der Lipoide von *Euglena gracilis*. Laufmittel: Aceton–Benzol–Wasser (91:30:8). (B) Fettsäure-Zusammensetzung der Lipoide von *Euglena gracilis* (Züchtung der Algen in organischem Medium 5 Tage im Dunkeln, danach 4 Tage im Tageslicht bei *ca.* 800 lux). Abkürzungen: NL 1: Neutrallipoide (vorwiegend Di- und Triglyceride); NL 2: Neutrallipoide (vorwiegend Monoglyceride und Sterin-Ester; MGDG: Monogalactosyldiglycerid; DGDG: Digalactosyldiglycerid; C: Cardiolipin; SL: Sulfolipid; PG: Phosphatidylglycerin; PE: Phos-phatidyläthanolamin; PI: Phosphatidylinositol; PC: Phosphatidylcholin. (B) 1: C_{12}; 2: C_{14}; 3: C_{15}(?); 4: C_{16}; 5: 7-C_{16}(1=); 5a: *trans*-3-C_{16} (1=); 6: C_{16} (2=); 7: C_{16} (3=); 8: C_{16} (4=) + C_{18}; 9: C_{18} (1=); 10: C_{18} (2=); 11: C_{18} (3=); 12: C_{18} (4=); 13: C_{20} (4=); 14: C_{20} (5=).

menge (z.B. 0.05 ml Chloroform, wie oben angegeben) löst, und jeweils gleiche Mengen (z.B. je 6 μl) durch denselben Gaschromatographen bei gleichbleibender Apparat-Einstellung analysiert, erhält man (s. Fig. 1 B) einen quantitativen Vergleich der Fettsäuren aller Lipoide eines Extraktes (unter der Voraussetzung, dass der Gaschromatograph linear auf die verschiedenen Substanzmengen anspricht). Proben, die bei der gewählten Standard-Einstellung des Gaschromatographen wegen der zu geringen Fettsäuremengen nur sehr niedrige GC-Peaks ergeben, können dann anschliessend bei höherer Empfindlichkeit des Apparates nochmals gaschromatographiert werden.

Wie in Fig. 1 B am Beispiel eines Lipoid-Extraktes aus *Euglena gracilis* gezeigt wird, haben die einzelnen Lipoide in Übereinstimmung mit den Literaturangaben[1-9] unterschiedliche, aber für die jeweiligen Lipoide eine charakteristische Fettsäure-Zusammensetzung: Die Zone NL 1 (überwiegend Di- und Triglyceride) sowie PC und PE enthalten die Hauptmenge der gesättigten und der C_{18}- und C_{20}-Polyenfettsäuren. MGDG und DGDG besitzen vorwiegend höher ungesättigte C_{16}- und C_{18}-Fettsäuren, SL ist durch die C_{16}-Fettsäure und PG durch die *trans*-3-C_{16}(1 =)-Fettsäure charakterisiert. PI, C und NL 2 (vorwiegend Sterin-Ester und Monoglyceride) spielen mit ihrem geringen Gehalt an Fettsäuren nur eine untergeordnete Rolle.

DANK

Unser Dank gilt der Deutschen Forschungsgemeinschaft für die Unterstützung dieser Arbeit mit Sachbeihilfen.

ZUSAMMENFASSUNG

Mit Hilfe eines neuen Laufmittelsystems, Aceton–Benzol–Wasser (91:30:8) gelingt mit eindimensionaler Kieselgel-Dünnschichtchromatographie die vollständige Trennung pflanzlicher Glykolipoide (Monogalactosyldiglycerid, Digalactosyldiglycerid, Sulfolipid), Phospholipoide (Cardiolipin, Phosphatidylglycerin, Phosphatidylaethanolamin, Phosphatidylinositol, Phosphatidylcholin) sowie der Neutrallipoide. Durch Transmethylierung der einzelnen Lipoidzonen aus der DC-Platte mit Natrium-Methylat können die Fettsäuren aller Lipoide aus etwa 2–4 mg Gesamtlipoid-Extrakt in relativ kurzer Zeit gaschromatographisch bestimmt und quantitativ miteinander verglichen werden.

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THIN-LAYER CHROMATOGRAPHY AND ULTRAVIOLET SPECTROPHOTOMETRY OF MIXTURES OF SULFONAMIDES

PRACTICAL APPLICATIONS

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SUMMARY

A method is proposed for the analysis of mixed sulfonamides by thin-layer chromatography and ultraviolet spectrophotometry. Solutions of sample mixtures are spotted on fluorescent Silica Gel H plates and developed in chloroform–methanol (88:12). The developed spots are delineated under short-wave ultraviolet light, scraped from the plate and extracted with 0.1 *N* NaOH. The centrifuged extracts are read with a recording spectrophotometer. A new method of calculation is introduced which permits to calculate the recovery of each chromatogram and to derive from it the recoveries of the individual compounds.

INTRODUCTION

Thin-layer chromatography, in addition to being very widely used for identification purposes, has been occasionally employed for the quantitative estimation of components of mixtures. A review of quantitative TLC methods was made by SPENCER AND BEGGS[1] and, in the field of mixed sulfonamides, by CIERI[2] in a recent publication. The procedure usually followed is to scrape and extract the developed spots and subsequently analyze the extracts colorimetrically or spectrophotometrically. Reference standards, in quantities nearly equal to those of the spotted compounds or covering the expected range, are similarly chromatographed, extracted and, if necessary, reacted.

Because of the empirical approach followed, absolute recoveries are not generally calculated and consequently the recovery of the spotted compounds is not known. There have still been indications[1] that these are never completely recovered from TLC plates. A rather extensive study of the absolute recoveries of five sulfonamides from developed TLC plates has been reported[2], using spectrophotometry as the determinative step. The recoveries of the sulfonamides, extracted in acidic alcohol or 0.1 *N* NaOH, varied from 86.4 to 99.7% but were most commonly in the 89–93%

range. The recoveries of different sulfonamides in each chromatogram were generally very close with the exception of sulfacetamide, which often had lower recoveries than the others. The percent recoveries were not substantially affected by changes in the quantity of sulfonamide per spot in the considered range of 100–200 μg per spot, but remained at a rather constant level. The losses were attributed to scattering of particles of the adsorbent containing sulfonamides during or after spotting and to some adsorptive retention of each compound by the chromatographic medium.

The results obtained in such study[2] and in a previous investigation[3] strongly suggest the possibility that a constant quantity of a compound is retained by a unit area (or perhaps unit volume) of adsorbent. Under given development conditions the concentration of a compound per unit area of a developed spot is also reasonably constant, regardless of the quantities spotted, since the sizes of such spots vary proportionately with the quantities of the compounds. The developed spots of different compounds may, however, have different concentrations of compound per unit area, as was found in a previous work[3], and thus produce different retention losses.

An accurate estimation of the losses related to adsorption cannot be made since, due to the possibility that small quantities of the compounds may detach from the plate, the amount spotted in each instance is not known with great precision. The relative extent of such losses, for compounds chromatographed under specified conditions, can be, however, reasonably well established by comparing their recoveries in each of several chromatograms. The study of the five sulfonamides previously investigated[2] was then continued for the purpose of obtaining more conclusive information on their relative recoveries. The developed spots, each containing a sulfonamide in the 80–120 μg range, were extracted with 0.1 *N* NaOH.

After the completion of the additional recovery study a method was developed for the analysis of mixed sulfonamides by TLC and UV spectrophotometry. The proposed method contains an outstanding innovation in that it permits to calculate the recovery of each sample chromatogram and to derive from it the recoveries of the different sulfonamides comprising the chromatogram. The recovery of a sample chromatogram is obtained from the ratio of the sum of the absorbances, all referred to a given volume, of the extracts of the sulfonamide spots to the absorbance of a dilution of the unchromatographed sample solution containing, in the reference volume, the amount of total sulfonamides theoretically delivered by the micropipet used. The calculated recovery of each chromatogram is then corrected, when necessary, in such a way that the recoveries of the individual sulfonamides are related by the same ratios that were found to characterize the recoveries of the chromatographed standard sulfonamides. The introduction of such a method of calculation, it is hoped, will eliminate or at least reduce considerably the error associated with the variability of the volume delivered by the micropipet and of the losses occurring during spotting. Seven synthetic mixtures of known composition and four commercial preparations have then been analyzed by the proposed method.

EXPERIMENTAL

Apparatus, reagents and TLC plates

See previous article[2].

UV spectrophotometry

Read UV absorbance of all solutions with a recording spectrophotometer from 350 to 220 mμ in matched 0.5- or 1-cm cells. Use the same set of cells throughout the experiment.

Reference solutions

Following the procedure outlined in the previous article[2], prepare a reference solution containing about 10 μg/ml 0.1 *N* NaOH for each of the following compounds: sulfacetamide (SC), sulfathiazole (SZ), sulfadiazine (SD), sulfamerazine (SM) and sulfamethazine (SH). Read UV absorbances, calculate the absorbance, at the maximum near 255 mμ, of a solution containing 10.00 μg/ml and designate as ${}_MA_x$, where the letter x identifies a particular sulfonamide.

Recovery of the chromatographed standard sulfonamides

Weigh accurately about 100 mg each of the five sulfonamides listed above, in a 100-ml volumetric flask. Add 5 ml of alcohol and 2 ml of strong ammonia water, swirl well to dissolve compounds, fill to mark with alcohol and mix. Similarly prepare two solutions containing respectively about 80 and 120 mg of each of the five sulfonamides in 100 ml.

Analyze each solution as follows. Pipet a 2-ml aliquot into a 100-ml volumetric flask, fill the flask to mark with alcohol and mix. Pipet 10 ml of the diluted solution to a small beaker, evaporate to dryness on a steam bath, transfer residue to a 100-ml volumetric flask with small portions of 0.1 *N* NaOH, fill to mark with 0.1 *N* NaOH and mix. Read UV absorbances and designate as ${}_gA_t$ the reading at the maximum near 255 mμ, the letter g identifying the wavelength of maximum of this solution.

Spot five 100-μl aliquots of the undiluted solution, each over an area about 4 cm wide, and develop in chloroform–methanol (88:12) until the solvent front reaches the top of the plate. Delineate the developed spots, transfer each to a 10-ml volumetric flask and read UV absorbances with 0.1 *N* NaOH in the reference cells, as described in the previous article[2]. Scrape also two or more blank spots, extract with 10 ml 0.1 *N* NaOH and read UV absorbances of the centrifuged extracts. Record the absorbances of each sulfonamide spot at the maximum near 255 mμ and also at wavelength g (see above), subtract an average absorbance blank, and designate the blank corrected readings as ${}_MA_{sx}$ and ${}_gA_{sx}$, the letter x identifying a particular sulfonamide. Calculate the percent recovery ($\%R_x$) of each sulfonamide by the formula:

$$\%R_x = (1000)({}_MA_{sx})/({}_MA_x)(W_x)(V_p),$$

where W_x indicates the weighed amount of the sulfonamide in mg and V_p the average volume in ml delivered by the micropipet. Average the percent recoveries of the five sulfonamides, in each chromatogram, and designate as $\%R_{ms}$. Calculate, also for each chromatogram, the average percent recovery ($\%R_{mc}$) by the formula:

$$\%R_{mc} = 10 \sum_{x=1}^{n} ({}_gA_{sx})/n({}_gA_t)\ (V_p),$$

where n equals the number of sulfonamide spots, five in this instance. The other terms have the same meaning as previously explained in this section.

Analysis of synthetic mixtures

Prepare several three- or four-component synthetic mixtures, each simulating a commercial preparation, as described below.

Designate as L_x the labeled amount of a sulfonamide in the commercial preparation, the letter x identifying a particular sulfonamide. Calculate then the e_x value of each component, to two decimal figures, by the formula $e_x = L_x/L_m$, where L_m indicates the labeled amount of the sulfonamide (or sulfonamides) whose quantity is the lowest in the mixture. Transfer to a volumetric flask accurately weighed amounts of the sulfonamides such that the quantity of each, in mg per ml of the resulting solution, equals its e_x value or does not differ from it by more than 20%. Designate as W_x the weighed amount of a sulfonamide in mg, as W_t the sum of the W_x values, as V_s the labeled volume of the flask in ml and as e_t the sum of the e_x values. Prepare also some synthetic mixtures that do not have a commercial equivalent; in this case call L_x the intended quantity of a sulfonamide in a mixture.

Analyze each mixture as follows. Add to flask 5 ml of alcohol and 2 ml of strong ammonia water, fill to mark with alcohol and mix. Pipet 5 ml of the solution to a glass-stoppered flask and add to it an accurately measured volume of alcohol such that the volume of the resulting solution in ml equals ten times the e_t value. After mixing, pipet 2 ml of the solution to a small beaker and evaporate to dryness on a steam bath. Transfer the residue to a 100-ml volumetric flask with small portions of 0.1 N NaOH, fill flask to mark with 0.1 N NaOH and mix. Read absorbances and designate the reading at maximum near 255 mμ as ${}_gA_t$, the letter g indicating the wavelength of maximum of this solution. Read also the absorbances, at wavelength g, of the reference sulfonamides, calculate the absorbance of each at a concentration of 10.00 μg/ml and designate as ${}_gA_x$. Calculate the total sulfonamide content (S_t) in mg by the formula:

$$S_t = ({}_gA_{st})\ (V_s)\ (e_t) / \sum_{x=1}^{n} [(e_x/e_t)\ ({}_gA_x)]$$

where n indicates the number of sulfonamides in the mixture. To obtain the percent total sulfonamide content multiply S_t by $100/W_t$.

Spot three 100-μl aliquots of the undiluted solution, develop plate and delineate spots under UV light. Transfer each spot to a volumetric flask estimated to produce, when filled to mark, an absorbance in the 0.4–0.9 range and designate as V_x the labeled volume of a flask containing a given sulfonamide spot. Add to flask 0.1 N NaOH to half of its volume, swirl well for 1 min, fill to mark with 0.1 N NaOH, mix and centrifuge. Extract also, and similarly centrifuge, two or more blank spots in each of the volumes used for the extraction of the sulfonamide spots. Read absorbances, with 0.1 N NaOH in the reference cells, and designate as ${}_MA_{sx}$ and ${}_gA_{sx}$, respectively, the blank corrected readings of the extract of a sulfonamide spot, at its maximum and at wavelength g. For each chromatogram determine the recovery value (R_v) by the formula:

$$R_v = \sum_{x=1}^{n} ({}_gA_{sx})\ (V_x)/(10)\ (e_t)\ ({}_gA_t),$$

where n indicates the number of spots in the chromatogram. Calculate then the content in mg of each sulfonamide (S_x) by the formula:

$S_x = (_MA_{sx})(V_s)/(_MA_x)(R_v + k)$*

The term k equals 0 if the mixture does not contain sulfacetamide. If this compound is present in the mixture, $k = (1 - n)/100\,n$ when calculating the recovery value of sulfacetamide and $k = 1/100\,n$ when calculating the recovery value of any of the others. Multiply the S_x values by $100/W_x$ to obtain the percentage of each sulfonamide relative to the amount weighed.

Analysis of commercial tablets

From the label declarations calculate the e_x and e_t values of each sample. Weigh twenty or more tablets, calculate the average weight of a tablet and grind to uniform powder. Weigh a portion of the powder into a volumetric flask and analyze as in the preceding section. Calculate the S_t and S_x values and multiply by the ratio of the average tablet weight to the amount of sample weighed, to obtain the average content per tablet of the total and individual sulfonamides. Determine the percentage of total and individual sulfonamides relative to the label declarations.

TABLE I

RECOVERY OF STANDARD SULFONAMIDES

Aliquot	*Content of spot*	$\%R_{SC}$	$\%R_{SZ}$	$\%R_{SD}$	$\%R_{SM}$	$\%R_{SH}$	$\%R_{ms}$	$\%R_{mc}$
1	100 µg	92.6	93.3	92.4	94.3	94.4	93.4	93.8
2		92.6	91.6	95.8	94.5	93.3	93.6	93.8
3		92.6	91.9	94.6	94.0	93.9	93.4	93.6
4		91.8	90.1	93.6	92.7	93.9	92.4	93.0
5		91.2	91.0	93.1	92.7	93.6	92.3	93.0
Av. (5)		92.2	91.6	93.9	93.6	93.8	93.0	93.4
1	120 µg	90.6	92.0	92.5	92.4	91.0	91.7	92.2
2		90.7	94.6	92.7	93.9	90.6	92.5	92.5
3		90.5	94.9	90.5	92.4	91.4	91.9	92.2
4		89.8	94.9	90.1	92.6	91.9	91.9	92.3
5		88.7	91.3	90.5	90.5	88.0	89.8	90.3
Av. (5)		90.1	93.5	91.3	92.4	90.6	91.6	91.9
1	80 µg	91.1	91.9	94.9	93.0	94.0	93.0	93.0
2		90.8	91.2	93.7	92.9	93.3	92.4	92.5
3		91.5	90.8	92.5	91.1	93.0	91.8	91.8
4		90.7	90.8	88.9	89.2	90.6	90.0	90.1
5		91.4	94.5	91.9	93.3	92.3	92.7	92.0
Av. (5)		91.1	91.8	92.4	91.9	92.6	92.0	91.9
Av. (15)		91.1	92.3	92.5	92.6	92.3	92.2	92.4

* The correction factor k is introduced to account for the lower observed recoveries of sulfacetamide. The assumption is made, based on the results of the recovery study, that the recoveries of sulfathiazole, sulfadiazine, sulfamerazine and sulfamethazine are equal in a chromatogram and that the recovery of sulfacetamide is 0.01 lower than that of any of the others. Thus calling R_{sc} the recovery of sulfacetamide and R_0 the recovery of any of the other sulfonamides the following relationships can be set up:

$R_{sc} = R_0 - 0.01$ and $R_{sc} + (n - 1)R_0 = nR_v$

After proper substitutions these formulas are obtained:

$R_{sc} = R_v + (1 - n)/100\,n$ and $R_0 = R_v + 1/100\,n$.

TABLE II

ANALYSIS OF SYNTHETIC MIXTURES OF SULFONAMIDES

Compound	*Individual sulfonamides by quantitative TLC*									
	Amount weighed (mg)	*Aliquot No. 1*			*Aliquot No. 2*			*Aliquot No. 3*		
		Found (mg)	*% of amount weighed*	R_v	*Found (mg)*	*% of amount weighed*	R_v	*Found (mg)*	*% of amount weighed*	R_v
Sulfadiazine	109.1	105.6	96.8		107.5	98.5		108.2	99.2	
Sulfamerazine	93.4	91.3	97.8	(0.9180)	92.6	99.1	(0.8860)	92.0	98.5	(0.9003)
Sulfamethazine	102.1	104.0	101.9		100.5	98.4		100.4	98.3	
Total	304.6	300.9	98.8		300.6	98.7		300.6	98.7	
Sulfathiazole	101.0	101.0	100.0		99.7	98.7		102.1	101.1	
Sulfadiazine	97.2	97.7	100.5	(0.9397)	97.8	100.6	(0.9524)	94.5	97.2	(0.9442)
Sulfamerazine	91.4	90.7	99.2		90.9	99.5		92.2	100.9	
Total	289.6	289.4	99.9		288.4	99.6		288.8	99.7	
Sulfacetamide	99.6	99.3	99.7		100.6	101.0		98.8	99.2	
Sulfadiazine	108.5	110.5	101.8	(0.9094)	108.0	99.5	(0.9169)	107.9	99.4	(0.9219)
Sulfamerazine	97.5	94.9	97.3		96.3	98.8		97.9	100.4	
Total	305.6	304.7	99.7		304.9	99.8		304.6	99.7	
Sulfacetamide	213.5	214.9	100.7		214.2	100.3		212.3	99.4	
Sulfadiazine	200.0	199.3	99.6	(0.9450)	199.1	99.5	(0.9508)	200.3	100.1	(0.9682)
Sulfamerazine	102.5	105.7	103.1		102.7	100.2		105.5	102.9	
Sulfamethazine	101.6	101.6	100.0		104.7	103.0		103.4	101.8	
Total	617.6	621.5	100.6		620.7	100.5		621.5	100.6	
Sulfacetamide	194.3	188.3	96.9		191.1	98.4		191.7	98.7	
Sulfadiazine	105.2	107.8	102.5	(0.9419)	105.8	100.6	(0.9095)	105.7	100.5	(0.9212)
Sulfamerazine	92.9	94.6	101.8		93.3	100.4		93.7	100.9	
Sulfamethazine	98.2	100.3	102.1		101.2	103.0		100.2	102.0	
Total	490.6	491.0	100.1		491.4	100.2		491.3	100.1	
Sulfacetamide	108.0	106.8	98.9		109.2	101.1		107.1	99.2	
Sulfadiazine	106.3	106.6	100.3	(0.9520)	106.3	100.0	(0.9186)	105.7	99.4	(0.9291)
Sulfamerazine	110.0	113.1	102.8		108.5	98.6		112.8	102.5	
Sulfamethazine	93.9	91.0	96.9		94.0	100.1		92.5	98.5	
Total	418.2	417.5	99.8		418.0	100.0		418.1	100.0	
Sulfathiazole	108.0	109.5	101.4		108.9	100.8		107.4	99.4	
Sulfadiazine	106.7	105.7	99.1	(0.9390)	106.1	99.4	(0.9709)	104.4	97.8	(0.9304)
Sulfamerazine	94.4	91.7	97.1		95.4	101.1		95.3	101.0	
Sulfamethazine	106.3	105.7	99.4		101.7	95.7		105.1	98.9	
Total	415.4	412.6	99.3		412.1	99.2		412.2	99.2	

DISCUSSION

The results of Table I confirm the conclusions of the previous investigation[2], which indicated that the recoveries of the different sulfonamides in a chromatogram are nearly equal. The situation arises probably from the fact that the concentration per unit area of spot is almost the same for all the five sulfonamides, when developed under the described conditions. Sulfacetamide has generally the lowest recovery in a chromatogram but, as previously discussed[2], this may be due to partial degradation of this compound during the marking of the spots under UV light rather than to

Average (3)			Total sulfonamides by direct dilution						
			Total amount weighed (mg)	Determination No. 1		Determination No. 2		Average (2)	
Found (mg)	% of amount weighed	R_v		Found (mg)	% of amount weighed	Found (mg)	% of amount weighed	Found (mg)	% of amount weighed
107.1	98.2		304.6	301.5	99.0	300.7	98.7	301.1	98.9
92.0	98.5	(0.9014)							
101.6	99.5								
300.7	98.7								
109.9	99.9		289.6	288.7	99.7	287.2	99.2	288.0	99.4
96.7	99.4	(0.9454)							
91.3	99.9								
288.9	99.7								
99.6	100.0		305.6	305.2	99.9	305.2	99.9	305.2	99.9
108.8	100.3	(0.9161)							
96.4	98.9								
304.8	99.7								
213.8	100.1		617.6	617.1	99.9	624.6	101.1	620.9	100.5
199.6	99.8	(0.9547)							
104.6	102.0								
103.2	101.6								
621.2	100.6								
190.4	98.0		490.6	493.7	100.6	488.7	99.6	491.2	100.1
106.4	101.1	(0.9242)							
93.9	101.1								
100.6	102.4								
491.3	100.1								
107.7	99.7		418.2	420.4	100.5	416.4	99.6	418.4	100.0
106.2	99.9	(0.9332)							
111.5	101.4								
92.5	98.5								
417.9	99.9								
108.6	100.6		415.4	412.6	99.3	410.6	98.8	411.6	99.1
105.4	98.8	(0.9378)							
94.1	99.7								
104.2	98.0								
412.3	99.3								

higher retention by the adsorbent. Even the recoveries of sulfacetamide do not differ substantially from those of the other four sulfonamides; if the fifteen determinations of each are averaged, the recovery of sulfacetamide is about 1% lower than that of any of the others (Table I). The difference is considered significant only because it is the outcome of a constant trend, observed in this as well as in the previous investigation[2].

Another important conclusion drawn from the results of Table I is that, in each chromatogram, the calculated recovery (designated as R_{mc}) very nearly equals the average of the actual recoveries of the individual sulfonamides (designated as R_{ms}),

TABLE III

ANALYSIS OF COMMERCIAL TABLETS OF SULFONAMIDES

Compound	*Individual sulfonamides by quantitative TLC*									
	Label per tablet (mg)	*Aliquot No. 1*			*Aliquot No. 2*			*Aliquot No. 3*		
		Found (mg)	*% of label*	R_v	*Found (mg)*	*% of label*	R_v	*Found (mg)*	*% of label*	R_v
Sulfadiazine	162	158.0	97.5		157.7	97.3		160.7	99.2	
Sulfamerazine	162	162.8	100.5	(0.9283)	165.0	101.8	(0.9084)	161.5	99.7	(0.8959)
Sulfamethazine	162	165.2	102.0		163.2	100.7		163.5	100.9	
Total	486	486.0	100.0		485.9	100.0		485.7	99.9	
Sulfathiazole	32.4	29.3	90.4		29.4	90.7		29.9	92.3	
Sulfadiazine	32.4	30.4	93.8	(0.9286)	30.1	92.9	(0.9216)	29.9	92.3	(0.9117)
Sulfamerazine	32.4	30.2	93.2		30.3	93.5		30.2	93.2	
Total	97.2	89.9	92.5		89.8	92.4		90.0	92.6	
Sulfacetamide	166.5	168.5	101.2		169.1	101.6		167.2	100.4	
Sulfadiazine	166.5	154.9	93.0	(0.8562)	158.8	95.4	(0.8834)	156.2	93.8	(0.8460)
Sulfamerazine	166.5	164.6	98.8		160.7	96.5		165.2	99.2	
Total	499.5	488.0	97.7		488.6	97.8		488.6	97.8	
Sulfacetamide	200	214.6	107.3		211.4	105.7		215.1	107.6	
Sulfadiazine	100	103.0	103.0	(0.9398)	104.4	104.4	(0.9642)	103.6	103.6	(0.9237)
Sulfamerazine	100	104.1	104.1		105.4	105.4		104.9	104.9	
Sulfamethazine	100	98.5	98.5		99.1	99.1		97.0	97.0	
Total	500	520.2	104.0		520.3	104.1		520.6	104.1	

the maximum observed difference between any of such two terms being 0.7%. This justifies the assumption, made for the estimation of the constituents of sample mixtures (Tables II and III), that the calculated recovery indicates the actual recovery of a chromatogram. In these calculations a new term is introduced, designated as R_v, which relates the recovery of a chromatogram to the labeled rather than to the determined volume of the micropipet used. This approach eliminates the need of knowing the volume of the micropipet and consequently allows standardization of the dilution factor. The R_v terms do not then indicate absolute recoveries; such recoveries can, however, be easily obtained, if desired, by multiplying the R_v values by the ratio of the labeled volume to the determined volume of the micropipet.

The total sulfonamide content of the sample mixtures is calculated with the assumption that the quantities of the individual components are related by the theoretical or labeled ratios. This permits estimation of the total sulfonamide content with a maximum possible error of about 1%, even when the quantities of the individual components differ by as much as 10% from those expected from the theoretical ratios, as indicated by an examination of the results of Table II.

The reliability of the method of calculating the individual components is also indicated by the results of Table II. Even when the recovery values (R_v) of chromatograms of the same solution are considerably different, the calculated quantities of each sulfonamide do not differ appreciably. The highest observed error in a single determination of a sulfonamide is 4.3%, but very seldom is such an extreme value reached; if three determinations are averaged, the maximum observed error is reduced

Average (3)			Total sulfonamides by direct dilution						
			Total declrd. mg per tablet	Determination No. 1		Determination No. 2		Average (2)	
Found (mg)	% of label	R_v		Found (mg)	% of label	Found (mg)	% of label	Found (mg)	% of label
158.8	98.0		486	486.9	100.2	484.6	99.7	485.8	99.9
163.1	100.7	(0.9109)							
164.0	101.2								
485.9	100.0								
29.5	91.0		97.2	89.4	92.0	90.7	93.3	90.1	92.6
30.1	92.9	(0.9206)							
30.2	93.2								
89.8	92.4								
168.3	101.1		499.5	487.7	97.6	487.7	97.6	487.7	97.6
156.6	94.1	(0.8619)							
163.5	98.2								
488.4	97.8								
213.7	106.9		500	522.9	104.6	518.2	103.6	520.6	104.1
103.7	103.7	(0.9426)							
104.8	104.8								
98.2	98.2								
520.4	104.1								

to 2.4%. The correction to the recovery value, made when sulfacetamide is present in a mixture, helps also to maintain the margin of error within the indicated limits. Without the correction factor the calculated quantities of sulfacetamide would be slightly lower and those of the other sulfonamides slightly higher than those reported in Table II. It may also be noted at this point that the sum of the calculated individual components generally very nearly equals the total sulfonamide content of the unchromatographed solution.

Although still negligible with 0.5-cm cells, the absorbance blanks were slightly higher than found in the previous investigation[2]. The average absorbance blank was about 0.01 with 0.5-cm cells and about 0.02 with 1-cm cells. This indicates that, while caused mostly by suspended colloidal particles, the absorbance blanks are also partially contributed by variable amounts of impurities present in the adsorbent. Prewashing of the silica gel is still not necessary, as long as the average absorbance blanks are not higher than those here indicated. To limit the possible error caused by the variability of the absorbance blank, the absorbances of the sulfonamide spots should be at least 0.3 in 0.5-cm cells and 0.6 in 1-cm cells.

Future applications of the proposed method do not necessitate, in our opinion, determining again the recoveries of the reference sulfonamides, as long as no changes are made to the specified conditions. A new recovery study of the standards would be needed only if a mixture contains one or more components other than those investigated or if the spotted quantities should be outside the considered range of 80–200 μg.

It is hoped that other types of mixtures can be similarly characterized by the recovery ratios of their constituents and that they can be consequently analyzed by the simple procedure here introduced.

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ISOELECTRIC FOCUSING OF *TRYPANOSOMA BRUCEI* SUBGROUP ANTIGENS IN POLYACRYLAMIDE GEL THIN LAYERS

A METHOD FOR RESOLVING AND CHARACTERISING PROTEIN-CARBOHYDRATE COMPLEXES OF AN ENZYMIC AND IMMUNOLOGICAL NATURE

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SUMMARY

The high resolution afforded by isoelectric focusing in thin layers of polyacrylamide gel has been applied to the separation of trypanosome antigens that previously could not be resolved either by column chromatography or by starch-gel electrophoresis. The above method, in combination with characterisation reactions, showed that a group of precipitating antigens were protein–carbohydrate complexes which appeared to have enzymic activity.

INTRODUCTION

Previous studies of proteins of the *Trypanosoma brucei** subgroup organisms have revealed a group of soluble antigens (4S) which appeared to be heterogeneous by immunodiffusion analysis but could not be separated by starch-gel electrophoresis or column chromatography[1]. In order to resolve and identify the antigens further, a method incorporating isoelectric focusing in polyacrylamide gel thin layers followed by characterisation reactions for proteins, carbohydrates, enzymes and immunological activity has been developed.

The principle of isoelectric focusing as first reported by IKEDA AND SUZUKI[2] had little practical application. It was the work of SVENSSON[3] followed by that of VESTERBERG AND SVENSSON[4] which led to the commercial availability of 'Ampholine' carrier electrolytes (LKB Produkter-AB, Sweden), which are a mixture of relatively low-molecular weight ampholytes. Subsequently, there have been reports of iso-

* The *Trypanosoma brucei* subgroup contains the pathogens causing sleeping sickness in man and nagana in cattle, which are widespread in tropical Africa. The organisms have a single flagellum and are classified with the Kinetoplastida, Zoomastigophorea, Protozoa. (HONIGBERG *et al.*, *J. Protozool.*, 11 (1964) 7).

electric focusing in sucrose density gradients[5–10], including a review of the applications by HAGLAND[11], but only a few reports of isoelectric focusing in polyacrylamide gel. Of these, two were based on the disc electrophoresis method[12,13] using the apparatus described by DAVIS[14], and one a polyacrylamide gel thin-layer method[15]. The applications of the latter method have been widened by using it together with the aforementioned characterisation reactions. This method, as reported in detail below, has been applied to the study of *Trypanosoma brucei* subgroup organisms but further valuable information may be obtained when it is applied to other trypanosome groups or biological material.

MATERIALS AND METHODS

Trypanosoma brucei homogenate

Infected rats were bled at peak parasitaemia, using EDTA as an anticoagulant, and the trypanosomes harvested by differential centrifugation[16] and the use of DEAE-cellulose[17]. The packed trypanosomes so obtained were mixed with an equal volume of glass distilled water and homogenised in an all glass tissue grinder. The protein concentration was estimated from the absorption at 280/260 nm of the clear supernatant obtained after centrifuging for 1 h at 4° and 120,000 $\times g$ in an MSE 'Superspeed 50'. This material was stored, in capillary tubes, at −20° until required.

Preparation of the gel

A 5% polyacrylamide gel containing 2% carrier ampholytes was prepared from the following solutions.

Catalyst stock solution. N,N,N',N'-tetramethylethylenediamine, 1.4 ml; riboflavin, 14.0 mg; and water to 100.0 ml.

Acrylamide stock solution. N,N'-methylenebisacrylamide, 0.8 g; acrylamide, 30.0 g; and water to 100.0 ml.

When kept at 4° and in the dark both solutions could be stored for at least one month.

Gel mixture. The quantities are sufficient for a plate 20 × 10 cm. 6 ml acrylamide solution were mixed with 1.6 ml of catalyst solution and 0.7 ml of carrier ampholytes, water was then added to make 36.0 ml.

The gel mixture was prepared immediately before use by mixing the stock solutions as shown above. The chemicals for the stock solutions were purchased from Koch–Light, U.K. and the carrier ampholytes of either pH range 3–10 or 5–7 were purchased as 40% solutions from LKB Produkter-AB, Sweden. A gel mould was prepared on a level table using grease-free TLC plates (20 × 10 cm). The lower plate was covered by a sheet of Melinex (200 gauge, 'S' type, ICI Products, Great Britain) overlapping at the sides; the Melinex being held in close contact with the plate by a water-film. The upper plate was raised 1 mm above the lower plate by plastic spacer strips. The overlapping edges of Melinex were raised by means of a horizontal glass rod along the length of the plates in such a way that the Melinex inclined downwards and inwards towards the plates. Using a syringe with an 18 gauge needle attached, gel mixture was carefully run down the inclined Melinex plane to replace the air space between the plates. Photopolymerisation was effected using two 10 Watt fluorescent strip lights placed 5 cm above the gel for 1 h. The whole preparation was inverted, the

top glass slid from the Melinex, and the remainder left to cool at 4° for a further hour before use. The Melinex was carefully peeled off to expose the gel surface and left for 5 min in ambient laboratory conditions, to ensure that the gel surface was suitable for sample application.

Isoelectric focusing

A template was placed under the supporting plate (top plate of mould) as a guide for the application of the samples. The samples were applied to the gel over a rectangle of 1.5 cm long by 1.0 cm wide, commencing 1.5 cm from the plate edge, with 1.0 cm left between applications. Most samples contained 100–400 μg protein in not more than 40 μl of fluid. For dilute samples, volumes of up to 75 μl were applied if they were first absorbed onto a 1.5 cm by 1.0 cm piece of cellulose acetate membrane (Schleicher and Schüll, G.F.R.). The volumes of the samples were adjusted to within 5 μl of each other with glass distilled water. To apply a single sample across the gel, either for a small scale preparation or for comparative analysis, a 1.5 cm strip of cellulose acetate membrane was used to ensure that the sample was applied uniformly to the gel. The rear edge of the rectangle of sample was 5 cm from the anode end of the gel when using pH 5–7 carrier ampholytes and 4 cm from the cathode end of the gel when using pH 3–10 carrier ampholytes. Electrical contact was made by inverting the plate so that the gel rested in a horizontal plane across two carbon rod electrodes 17.5 cm apart. Immediately before placing the gel on the electrodes the cathode was moistened with 5% (v/v) ethylenediamine, and the anode with 5% (v/v) phosphoric acid. Isoelectric focusing was performed in a humid chamber at 4° by applying 350 V (20 V/cm) from a constant voltage supply for 16 h. The initial current was 16 mA but dropped during the first hour to about 2 mA for the remainder of the run. At the completion of isoelectric focusing the cellulose acetate membrane, when used, was removed by moistening with a little glass distilled water before carefully peeling off. The pH gradient was determined by cutting 5 mm discs (No. 2 cork borer) at 5 mm intervals along the length of the gel. Each disc was added to 0.75 ml glass distilled water in a 75 × 10 mm test tube. The pH was measured using a 5 mm diameter glass electrode and a Pye 'Dynacap' pH meter, after equilibrating the samples for 3 h at room temperature. Readings were taken on the expanded scale to an accuracy of 0.02 pH unit. Discs of gel removed from between two samples served as ready reference marks.

Handling of the gel

It was not necessary to remove the gel from the glass plate when all the samples were to be examined in the same manner, but treatment of parts of the gel by different identification techniques was normally required. This necessitated the cutting of strips of gel and their removal from the glass plate. Strips of Melinex, 2 cm wide, were laid over the gel using the original template to indicate where the samples had been run, leaving a small gap, sufficient to admit a scalpel blade, between the strips of Melinex. The gel was cut and the strips, adhering to the Melinex, were cautiously peeled from the glass plate.

Protein staining

A method similar to that described by URIEL[18] for staining protein in agar gel

was found to be suitable for polyacrylamide gel using sulphosalicylic acid[19] as fixative.

Fixing solution. 5% w/v sulphosalicylic acid

Staining solution. Ponceau S (G.T. Gurr Ltd.), 0.1 g; acetic acid, 1.0 *M*, 45 ml; sodium acetate, 0.1 *M*, 45 ml; glycerol, 10 ml.

Destaining solution. Acetic acid, glacial, 20 ml; glycerol, 125 ml; water to 1000 ml.

Except where indicated May and Baker Reagent Grade chemicals were used throughout.

The carrier ampholytes, being aminocarboxylic acids, normally interfered with the staining procedure for proteins and were removed in the present case during fixation of the gel. Sulphosalicylic acid was found to give fixation comparable to trichloroacetic acid and has the advantage of not being so corrosive to the skin. Each strip of gel was fixed for 48 h in four changes of 150 ml of sulphosalicylic acid and stained for 1 h in 0.1% Ponceau S. The gel was readily removed from the Melinex after it had been fixed for about 1 h. A near colourless background was obtained after a few changes of destaining solution.

Carbohydrate staining

The use of Schiff's reagent (sulphited fuchsin) with periodic acid as oxidant has been utilised by McMANNUS[20] and HOTCHKISS[21] for the histochemical characterisation of carbohydrate. This reaction has been used to demonstrate the presence of carbohydrate following the isoelectric focusing of trypanosome homogenate in polyacrylamide gel.

Fixing solution. Acetic acid, glacial, 2 ml; ethyl alcohol, 50 ml; water to 100 ml.

Schiff's reagent. This was prepared according to DE TOMASI's method[22].

Periodic acid solution. 1% w/v periodic acid in 0.2 *M* sodium acetate.

Sulphurous acid solution. 10% sodium metabisulphite, 25 ml; hydrochloric acid, 2 *N*, 25 ml; water to 500 ml.

Glycerinated sulphurous acid solution. 400 ml sulphurous acid solution and 100 ml glycerol.

The strip of gel, attached to the Melinex, was fixed for 3–6 h, during which time it shrank to about half-size and freed itself from the Melinex. Following fixation the gel was rinsed briefly in 0.2 *M* sodium acetate and oxidised in the periodic acid solution for 15 min. Excess periodic acid was removed by washing in running water for 30 min after which time the gel had returned to its original size. Schiff's reagent was diluted with an equal volume of glass distilled water and applied for 10 min. This was followed by three washes of 3 min each in freshly prepared sulphurous acid solution and a final wash in glycerinated sulphurous acid solution for 1 h after which the gel could be stored in the same solution. The results, particularly if there were any faint bands present, were photographed within 24 h. There was no apparent further loss in depth of colour after the first 48 h, and in most instances, after several weeks. As an alternative after photographing, the gels were washed overnight in one change of 5% sulphosalicylic acid and then counterstained for protein. Although a satisfactory result was obtained there was a general loss in intensity of staining compared with the direct method.

Immunological analysis

The gel was transferred to another supporting plate and a frame formed around it so that an area of 17.5 × 2.5 cm was left on one long side of the gel while the other sides were in contact with the frame. The frame was formed from glass strips 3 mm thick cemented into position with a 1.5% agar solution in normal saline containing 0.02% sodium azide. The same agar solution, at 50°, was poured into the enclosed area to a depth of approximately 1.5 cm. When the agar had set a trough was cut, 2.5 mm wide, parallel to and 1.5 cm from the gel strip. The trough was filled with rabbit anti-trypanosome serum and allowed to develop in a humid chamber at room temperature. When the antiserum had diffused into the agar the trough was filled with normal saline containing 0.02% sodium azide and the system left for seven days. The soluble protein was then removed in three changes of normal saline over 48 h and the gels allowed to stain overnight in 0.01% Ponceau S. The slight background colour was removed with protein destaining solution.

Enzyme location

The positions of trypanosome enzymes have been located histochemically as formazan deposits following starch-gel electrophoresis[23]. The same principle has been applied following isoelectric focusing in polyacrylamide gel.

Substrate solution. Phenazine methosulphate (PMS), 1.0 mg; tetrazolium salt, (MTT), 1.5 mg; NAD/NADP, 1.0 mg; magnesium chloride, 0.1 *M*, 0.75 ml; substrate, 0.2 *M*, pH 7.0–7.2, 0.05 ml.

Agar solution. Agar, 78.0 mg; Tris buffer, 0.2 *M* (pH 8.0), 7.0 ml.

Stop bath. Methyl alcohol, 45 ml; acetic acid, glacial, 10 ml; water, 45 ml.

For this procedure, which was completed as soon after the run as possible, the gel was not removed from the glass plate. Strips of glass, 3 mm thick, were used to form a frame around the gel and cemented in position as before. The agar solution was cooled to 50°, mixed with the substrate solution and immediately poured evenly over the gel. Once the agar had set the overlayered gel was incubated in the dark at 37° for 30 min. The enzyme appeared as a blue band. The reaction was stopped, to control nonspecific background colour, by immersing in the stop bath solution for 1 h in the dark.

Photography of results

All results were photographed on Kodak VP 120 film using transmitted light and a good diffusing screen. A green filter enhanced the contrast.

RESULTS AND DISCUSSION

The total soluble trypanosome proteins were resolved within the pH range 4.0–8.6 (Fig. 1a). A group of bands within the pH range 5.5–6.1, which stained for protein, also stained for carbohydrate (Fig. 1b). Similar p*I* values were obtained for this group after isoelectric focusing using narrow range, pH 5–7, carrier ampholytes (Figs. 2a and 2b). Immunodiffusion analysis, using homologous antiserum, gave a common precipitin line covering all components of the group within the range pH 5.5–6.1 (Fig. 3). This suggests that some, or all, of the antigens of this group may have a common antigenic determinant. None of the NAD/NADP specific dehydro-

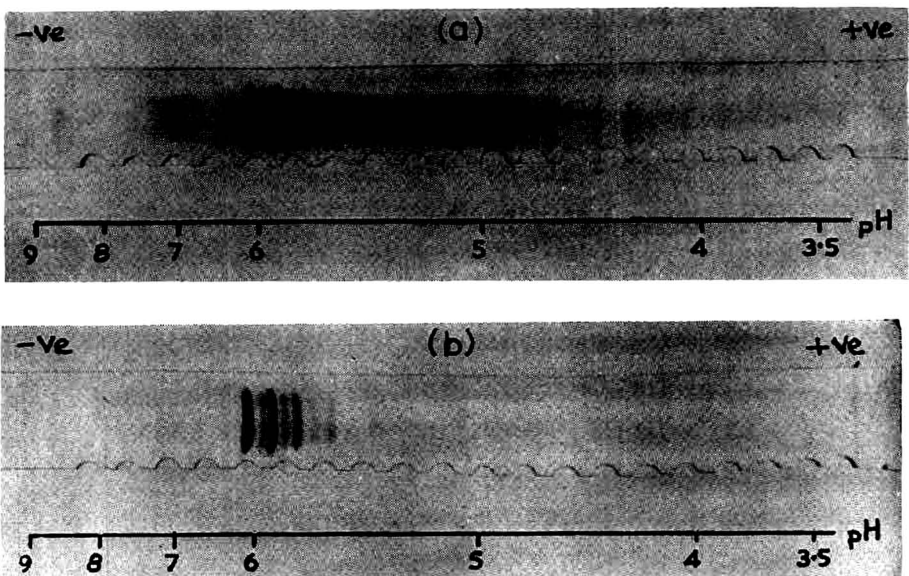

Fig. 1. Isoelectric focusing of trypanosome homogenate using pH 3–10 carrier ampholytes. (a) Stained for protein with Ponceau S; (b) stained for carbohydrate by periodic acid—Schiffs' reaction.

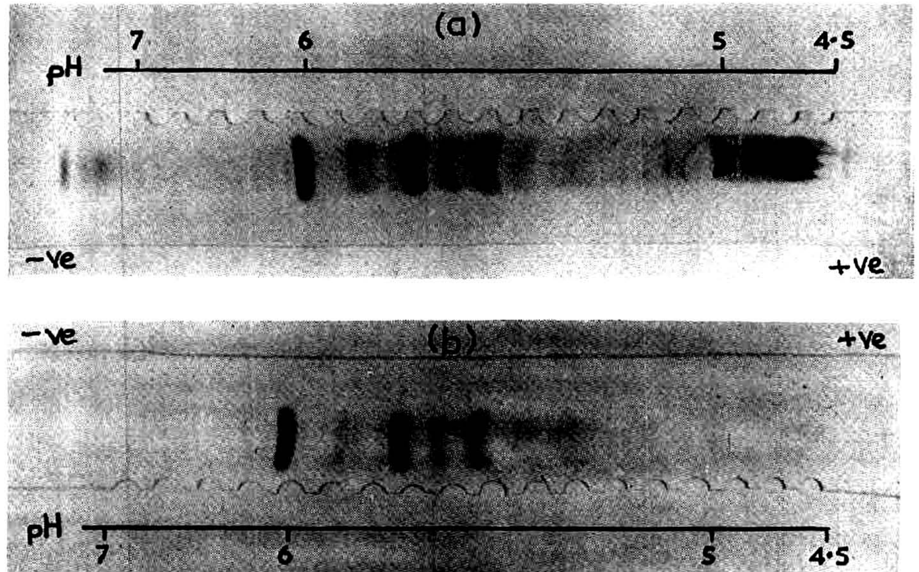

Fig. 2. Isoelectric focusing of trypanosome homogenate using pH 5–7 carrier ampholytes. (a) Stained for protein with Ponceau S; (b) stained for carbohydrate by periodic acid—Schiffs' reaction.

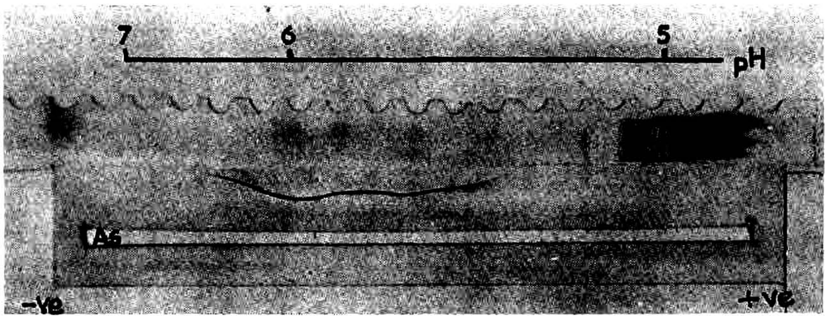

Fig. 3. Immunodiffusion pattern from trypanosome homogenate *vs*. rabbit anti-trypanosome serum (As) after isoelectric focusing of the homogenate using pH 5–7 carrier ampholytes.

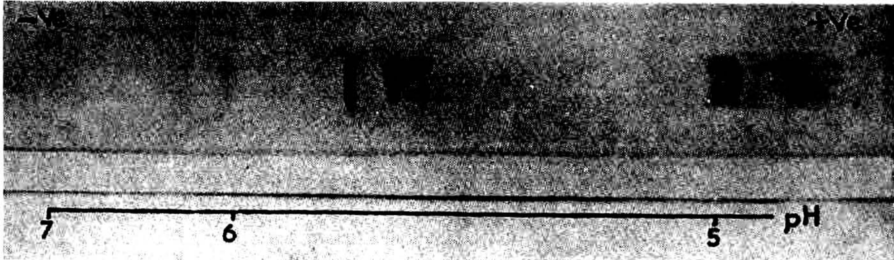

Fig. 4. Location of Ochoa's enzyme in the gel as a formazan deposit following isoelectric focusing of the trypanosome homogenate using pH 5–7 carrier ampholytes.

genases of the glycolytic pathway, known to be present in *Trypanosoma brucei* organisms[24], were located within the range of these protein–carbohydrate complexes. However, not only could Ochoa's malate (decarboxylating) enzyme be located within this group but it would appear to be an isoenzyme (Fig. 4). It was possible to stain for nucleoprotein and lipoprotein following isoelectric focusing but neither were detected in this sample.

Isoelectric focusing in polyacrylamide gel thin layers provides a method of separating complex mixtures containing as little as 100 μg protein, with high sensitivity and high resolution. This method is very favourable as samples, which are incorporated in the gel for the disc method, are not exposed to adverse conditions during polymerisation nor are they present to inhibit polymerisation. Any hydrostatic or electro-osmotic effects are eliminated as the gel is run in a horizontal plane and in direct contact with the electrodes.

The results provided by this method have given a new perspective to the study of trypanosome antigens and antigenic variations and will be discussed elsewhere.

NOTE ADDED IN PROOF

The above method has been applied on a micro-scale to study samples of tsetse fly haemolymph. The gel, 0.75 mm thick, was supported on a 7.5 × 2.5 cm microscope slide and a 10 μl sample containing about 100 μl protein applied to an area 1.0 cm long by 0.5 cm wide. Isoelectric focusing was performed using similar conditions to

those described above after which the pH gradient was determined by eluting 2.5 mm discs, cut at 5.0 mm between centres, in 0.5 ml glass distilled water.

ACKNOWLEDGEMENTS

The author wishes to thank Drs. A. R. NJOGU, P. J. WALKER and B. A. ALLSOPP for helpful discussions and the Director, EATRO, for permission to publish this paper.

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CHROM. 4626

SEPARATION AND IDENTIFICATION OF ALCOHOLS AS N,N-DIMETHYL-*p*-AMINOBENZENEAZOBENZOATES BY PAPER AND THIN-LAYER CHROMATOGRAPHY

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SUMMARY

Crystalline esters of alcohols were prepared by means of N,N-dimethyl-*p*-aminobenzeneazobenzoyl chloride as reagent, and suitable conditions were found for the separation of these esters by means of paper and thin-layer chromatography. A detection reagent can be dispensed with as N,N-dimethyl-*p*-aminobenzeneazobenzoates are highly coloured substances. Less than 0.5 μg can be determined in a spot.

INTRODUCTION

For the identification and separation of low and medium molecular weight alcohols by paper (PC) and thin-layer (TLC) chromatography, esters of benzoic acid[1–8], nitrated or substituted in different ways, are used most frequently. For the identification of low molecular weight alcohols, 2,4-dinitrobenzyl bromide can be used as the agent for etherification[9]. N,N-dimethyl-*p*-aminobenzeneazobenzoyl chloride, because of its reactivity and detectability, has also proved to be very suitable for the preparation of esters. Its preparation is relatively simple, and the synthesis of the esters is rapid. The esters obtained are crystalline, bright red substances with sharp characteristic melting points which can be used for their identification by classical methods[10].

EXPERIMENTAL

Preparation of N,N-dimethyl-p-aminobenzeneazobenzoates

In contrast to the classical identification procedure where perfectly pure esters are required, the impure reaction mixture can be applied directly to the paper or thin layer for identification by chromatography. The preparation of esters is, therefore, greatly simplified.

1 mg of N,N-dimethyl-*p*-aminobenzeneazobenzoyl chloride, 2–5 μl of alcohol, 1 drop of pyridine and 0.3–0.5 ml of benzene are placed in a micro test tube. The mixture is heated to dissolve the esterification reagent, and then kept boiling for an appropriate time. In medium and high molecular weight alcohols, the reaction mix-

ture is heated for several minutes or the micro test tube is sealed and placed in a boiling water bath for 5–10 min. Immediately after cooling, the reaction mixture can be applied to the chromatogram without further purification. The high reactivity of the reagent, even without addition of pyridine, is very evident in the preparation of esters of low molecular weight alcohols when merely mixing the given components without heating produces a sufficient quantity of ester within seconds.

Paper chromatography

Chromatography was carried out on Whatman No. 2 paper and under various experimental conditions. Mobile phases were saturated with a stationary phase in all cases. The paper was impregnated by passing it through a solution of the stationary phase. The paper was allowed to hang for about 20 sec with the start end uppermost, the drops of surplus solution were wiped off with filter paper, and the paper was then hung with the start end downwards until the solvent had evaporated completely. In this way the stationary phase was distributed more uniformly throughout the length of the paper. The time for drying the paper after impregnation and for the application of the esters to the start is arranged so that the chromatogram can be placed in the chromatography chamber, previously saturated with vapours of the solvents used, in the 15th minute after beginning the impregnation. These working conditions must be strictly observed in order to achieve good reproducibility of the R_F values; this applies in other cases as well, but especially when working with dimethylformamide[11].

For chromatographic separation of esters a whole series of combinations of polar stationary phases was tested with non-polar solvents in mobile phases, and so were several reversed-phase combinations. The following systems were found most suitable:

S_1: a 60% solution of dimethylformamide in methanol/petroleum (boiling range 60–75°)

TABLE I

R_F AND R_M VALUES OBTAINED BY PC OF N,N-DIMETHYL-*p*-AMINOBENZENEAZOBENZOATES OF ALIPHATIC C_1–C_{10} ALCOHOLS

Alcohol	*System*							
	S_1		S_2		S_3		S_4	
	R_F	R_M	R_F	R_M	R_F	R_M	R_F	R_M
Methyl	0.31	0.35	0.48	0.04	0.82	−0.66		
Ethyl	0.45	0.09	0.61	−0.19	0.76	−0.50		
Propyl	0.57	−0.12	0.68	−0.33	0.65	−0.27		
Isopropyl	0.55				0.67			
Butyl	0.68	−0.33	0.75	−0.48	0.55	−0.09		
Isobutyl	0.65				0.58			
Amyl	0.78	−0.55	0.79	−0.58	0.43	0.12	0.67	−0.31
Isoamyl	0.75							
Hexyl			0.84	−0.72	0.32	0.33	0.57	−0.12
Heptyl					0.23[a]		0.47[a]	
Octyl			0.88	−0.87	0.16	0.72	0.38	0.21
Nonyl			0.89	−0.91	0.10	0.95	0.29	0.39
Decyl					0.07	1.13	0.22	0.55

[a] Calculated values.

S_2: a 20% solution of dimethylformamide in methanol/cyclohexane–benzene (10:1)

S_3: a 10% solution of paraffin oil in pentane/dimethylformamide–water (4:1)

S_4: a 10% solution of paraffin oil in pentane/dimethylformamide–methanol–water (8:2:1)

2–5 µl of a benzene or dimethylformamide solution of the ester was applied to the start; each sample contained 2–10 µg of ester.

The results are given in Table I and in Figs. 1–3.

The dependence of the R_M values on the number of C atoms in the alcohol molecule is demonstrated in Fig. 4.

Thin-layer chromatography

Silica Gel G thin layers were used for the separation of the esters; in some cases they were impregnated with a stationary phase. The plates (12 × 22 cm) were activated for 30 min at 120°.

The impregnation was made in a chromatography chamber by allowing a solution of the stationary phase to ascend the plate by capillary action. The volatile solvent was then allowed to evaporate into the air from the layer at laboratory temperature. In all cases, and especially when working with an impregnated layer, it is necessary to ensure that the chromatography chamber is completely saturated with developing solvent vapours. The esters were applied to the plate in the form of benzene solutions of the same concentration as indicated in PC. The following solvent systems were used:

S_5: a 40% solution of dimethylformamide in methane/cyclohexane–benzene (25:1)

S_6: a 10% solution of paraffin oil in pentane/dimethylformamide–water (4:1)

S_7: a 10% solution of paraffin oil in pentane/dimethylformamide–methanol–water (4:1:1)

S_8: a 10% solution of paraffin oil in pentane/dimethylformamide–methanol–water (8:2:1)

S_9: a 10% solution of paraffin oil in pentane/dimethylformamide–water (3:1)

S_{10}: cyclohexane–ethyl acetate (4:1)

S_{11}: hexane–ethyl acetate (4:1)

S_{12}: cyclohexane–methyl ethyl ketone (4:1)

The results are shown in Table II and Fig. 5.

Detection

As N,N-dimethyl-*p*-aminobenzeneazobenzoates are coloured substances, a special detection reagent is not necessary. However, when only low concentrations of sample have been applied to the paper or layer, the intensity of spots can be increased by spraying the chromatogram with 0.01 *N* H_2SO_4.

Sensitivity determination

The following amounts of the esters of C_2 and C_9 alcohols were gradually applied to the paper: 0.2; 0.4; 2; 4 µg and the limit of detection was determined. The development and the method of increasing the detection sensitivity were as described above.

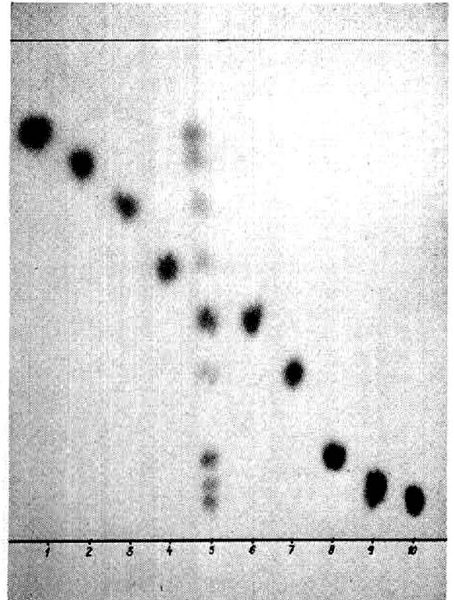
TABLE II

R_F AND R_M VALUES OBTAINED BY TLC OF N,N-DIMETHYL-*p*-AMINOBENZENEAZOBENZOATES

Alcohol	*System*															
	S_5		S_6		S_7		S_8		S_9		S_{10}		S_{11}		S_{12}	
	R_F	R_M	R_F	R_M	R_F	R_M	R_F	R_M	R_F	R_M	R_F	R_M	R_F	R_M	R_F	R_M
Methyl	0.32	+0.33			0.80	−0.60					0.42	+0.14	0.33	+0.31		
Ethyl	0.38	+0.21	0.82	−0.66	0.79	−0.58					0.47	+0.05	0.37	+0.23		
Propyl	0.44	+0.11	0.74	−0.45	0.74	−0.45					0.50	0.00	0.41	+0.16		
Butyl	0.51	−0.02	0.64	−0.25	0.68	−0.33					0.55	−0.09	0.44	+0.11		
Amyl	0.59	−0.16	0.53	−0.05	0.61	−0.19					0.58	−0.14	0.50	0.00		
Hexyl	0.67	−0.31	0.42	+0.14	0.54	−0.07					0.61	−0.19	0.60	−0.18		
Octyl							0.66	−0.29								
Nonyl							0.59	−0.16								
Decyl							0.53	−0.05								
Lauryl									0.45	+0.09					0.73	−0.43
Cetyl									0.41	+0.16					0.80	−0.60
Benzyl									0.64	−0.25					0.46	+0.07
Phenyl ethyl									0.63	−0.23					0.47	+0.05
Anisyl									0.70	· 0.37					0.38	+0.21
Cinnamyl									0.61	−0.19					0.48	+0.04

RESULTS AND DISCUSSION

The chromatographic behaviour of N,N-dimethyl-*p*-aminobenzeneazobenzoates of aliphatic alcohols is similar to that of other esters of substituted benzoic acids. The whole series of esters of the C_1–C_{10} alcohols cannot be completely separated in a single solvent system; that ideal state is most nearly approached by the separation in system S_3 (10% solution of paraffin oil/dimethylformamide–water, 4:1) on Whatman No. 2 paper (Fig. 1), where all the esters are separated well except the lowest and highest homologues, *i.e.* the esters of the C_1 and C_2 alcohols and C_9 and C_{10} alcohols are not well separated. If a good separation of the methyl alcohol and ethyl alcohol



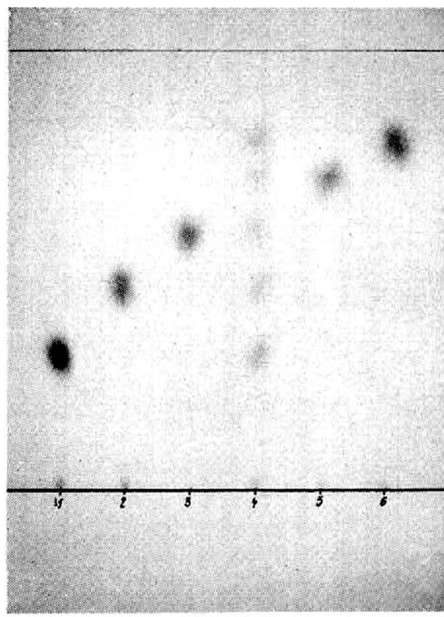


Fig. 1. System, S_3; Whatman No. 2 paper. N,N-Dimethyl-*p*-aminobenzeneazobenzoates of alcohols: 1 = methyl; 2 = ethyl, 3 = *n*-propyl, 4 = *n*-butyl; 5 = mixture C_1–C_{10}; 6 = *n*-amyl; 7 = *n*-hexyl; 8 = *n*-octyl; 9 = *n*-nonyl; 10 = *n*-decyl.

Fig. 2. System, S_1; Whatman No. 2 paper. N,N-Dimethyl-*p*-aminobenzeneazobenzoates of alcohols: 1 = methyl; 2 = ethyl; 3 = *n*-propyl; 4 = mixture C_1–C_5; 5 = *n*-butyl; 6 = *n*-amyl.

esters is of importance, the S_1 (Fig. 2) and S_2 systems are the most suitable for paper, and the S_5, S_{11}, or S_{10} systems for thin layers. If we want to separate the higher alcohols completely, the most suitable system for paper (Fig. 3) is the S_4 system: paraffin oil/dimethylformamide–methanol–water (8:2:1), or for thin layers, the S_9 system: paraffin oil/dimethylformamide–water (3:1), and S_{10} or S_{12} system. From the course of the separation on paper (Figs. 1 and 3) it is evident that an increase in the amount of dimethylformamide in the mobile phase would make a separation of esters of alcohols higher than C_{10} possible. If pure dimethylformamide is used as the mobile phase, the hexylalcohol ester moves practically with the front.

In all the systems mentioned a linear dependence was found between R_M values

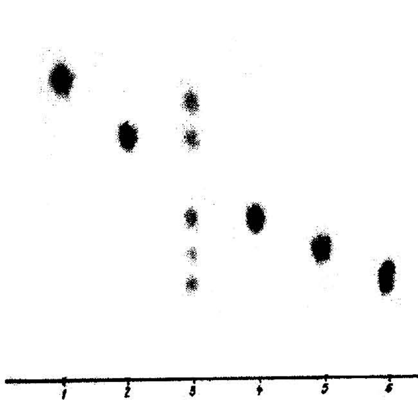


Fig. 3. System, S_4; Whatman No. 2 paper. N,N-Dimethyl-*p*-aminobenzeneazobenzoates of alcohols: 1 = *n*-amyl; 2 = *n*-hexyl; 3 = mixture C_5–C_{10}; 4 = *n*-octyl; 5 = *n*-nonyl; 6 = *n*-decyl.

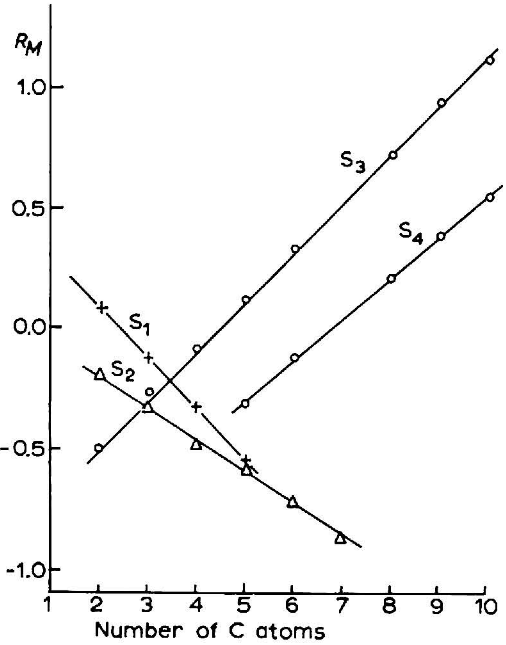


Fig. 4. Dependence between R_M values and the number of carbon atoms in the alcohol; R_M values obtained by PC of the esters.

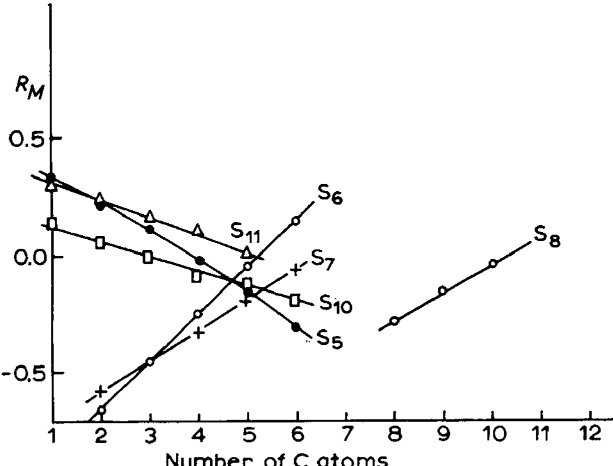


Fig. 5. Dependence between R_M values and the number of carbon atoms in the alcohol; R_M values obtained by TLC of the esters.

and the number of carbons in the alcohol molecule (Figs. 4 and 5). It is thus possible to identify the alcohols on this basis. The R_F values of heptyl N,N-dimethyl-*p*-aminobenzeneazobenzoate was calculated from this relationship, as the substance was not available (see Table I). In fair agreement with previous experience, isomeric alcohols or respectively their esters, were not separated, and were therefore not identifiable in this way.

The reagent, N,N-dimethyl-*p*-aminobenzeneazobenzoyl chloride, does not interfere with the identification in either its normal or hydrolysed form.

The sensitivity of the method described is high, and can be increased by spraying the chromatogram with 0.01 N sulphuric acid. From the series of known quantities of esters of ethyl and nonyl alcohol, in the range of 0.2–4 μg, it is evident that as little as 0.4 μg of ester in a spot can be easily detected. With the nonyl alcohol, resp. its ester, as little as 0.2 μg in a spot can be seen. Two alcohols differing by one carbon atom can be identified using this method, even in the ratio of 1:1000. However, the ester of the alcohol occurring as the insignificant quantity should have a higher R_F value than the alcohol in excess; this can easily be arranged with the solvent systems given.

EVALUATION OF THE METHOD

The method most used for the chromatographic identification of alcohols is their separation in the form of 3,5-dinitrobenzoates. This method, described below, was therefore chosen for comparison with the results obtained in the present work.

If we examine the various stages of both methods, the advantages and disadvantages pertaining to each operation can be evaluated.

The reagent and its preparation

The stability of 3,5-dinitrobenzoyl chloride as exposed to the air in a reagent

bottle with a ground glass stopper is rather low, and on storage under these conditions it rapidly loses its efficiency. Immediately after the preparation it should be distributed in small quantities into ampoules, and sealed. On the other hand, N,N-dimethyl-*p*-aminobenzeneazobenzoyl chloride is very stable. Even after long-term storage (several weeks) in an open vessel in the laboratory atmosphere no change in its reactivity was observed.

While the preparation of 3,5-dinitrobenzoyl chloride, and especially, its purification by means of distillation is very difficult, and even risky, the preparation of N,N-dimethyl-*p*-aminobenzoyl chloride is relatively simple[10].

Ester preparation

The use of a coloured reagent for the preparation of esters for the chromatographic microidentification of alcohols is advantageous. In the work described, long heating in sealed ampoules is unnecessary. In the lower and medium molecular weight alcohols the reaction rate is fairly high, and it is sufficient to heat the reaction mixture for the preparation of the respective esters for only a relatively short time. The presence of a small quantity of water in the reaction mixture is harmless, so that lower alcohols can also be identified in aqueous solutions.

Chromatography

When both the methods are compared from the viewpoint of the chromatographic separation both on paper and on thin layers, it was found that the behaviour of both the groups of esters is about the same. A great advantage of the new derivatives, however, is their colour, making a detection reagent unnecessary. The sensitivity of the new method is slightly higher. If we consider that 0.4 μg of ester can be easily seen, it means that the concentration of the alcohol concerned can be approximately five times lower. Coupled with the sensitivity is also the possibility of identifying very low quantities of one alcohol in admixture with a much higher concentration of a neighbouring homologue, even in a ratio as high as 1:1,000.

Time taken for the analysis

In the case of a micropreparation, the time for the identification of the alcohols according to the method described is considerably lower than that with the method using 3,5-dinitrobenzoyl chloride. The time consumption for coloured ester preparations is neglibgile in comparison with 3,5-dinitrobenzoates. The time for the identification of the alcohols is substantially reduced in the case of the coloured esters; with 3,5-dinitrobenzoates it takes a considerable time. Another advantage of the coloured esters is the possibility of a substantial reduction of chromatogram development time owing to the fact that the separation process is plainly visible.

Possibility of quantitative analysis

As the esters of N,N-dimethyl-*p*-aminobenzeneazobenzoic acid are essentially azodyes, at low concentrations it should be possible to carry out quantitative chromatographic analysis, providing the colour–concentration relation obeys the Lambert–Beer law. This problem is now being investigated.

If we consider all the known methods of separation and identification of alcohols by means of paper and thin-layer chromatography, we come to the con-

clusion that the newly suggested reagent, N,N-dimethyl-*p*-aminobenzeneazobenzoyl chloride has a number of advantages over all the methods used hitherto. From preliminary tests, its high reactivity can also be used for the chromatographic separation and identification of other classes of substances, such as glycols, cellosolves, phenols, amines, etc., where it reacts with the hydroxy or amino groups.

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CHROM. 4729

PAPIERIONOPHORETISCHE VERFOLGUNG DER DARSTELLUNG GEMISCHTER CYANO—THIOCYANATOCHROMATE(III)

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(Eingegangen am 17. März 1970)

SUMMARY

Paper ionophoretic investigation of the composition of a mixture of cyano–thiocyanato-chromates(III)

When $K_3[Cr(SCN)_6]$ reacts with KCN in boiling acetonitrile a mixture of all complex ions $[Cr(SCN)_{6-x}(CN)_x]^{3-}$ ($x = 0, 1, \ldots 6$) is formed. This reaction can be observed by high-voltage paper ionophoresis. The R_B-values give a first evidence of the composition of the complex ions.

EINLEITUNG

Die Chemie des Cr(III) in wässriger Lösung wird durch die starke Tendenz zur Komplexbildung bestimmt. Gemischte Aquo–Thiocyanatochromate(III)[1,2] sowie gemischte Aquo–Cyanochromate(III)[3–6] sind bereits dargestellt und untersucht worden. Gemischte Cyano–Thiocyanatochromate(III) wurden jedoch—soweit bekannt—bisher in der Literatur noch nicht beschrieben.

In der vorliegenden Arbeit werden die Bedingungen der Umsetzung von $K_3[Cr(SCN)_6]$ mit KCN in Acetonitril papierionophoretisch verfolgt, um Unterlagen für die Trennung der gebildeten Gemischtligandkomplexe in der neu entwickelten Trennschlauchappatur[7] zu gewinnen. Im Gegensatz zur Darstellung der Komplexionen kann ihre papierionophoretische Trennung in wässriger Lösung durchgeführt werden, da sie bei 0° kinetisch stabil sind und keine Hydrolyse eintritt. Zur ersten Charakterisierung der Komplexionen dienen die ermittelten R_B-Werte. In einer späteren Arbeit wird beschrieben, wie die Komplexionen in grösseren Mengen isoliert und durch Elementaranalysen sowie UV-Spektren charakterisiert worden sind.

EXPERIMENTELLER TEIL

Grundlagen

Bei der Papierionophorese gilt für den rein elektrophoretischen Wanderungs-

weg, korrigiert um den Einfluss von Elektroosmose und Sog, folgende Beziehung:

$$\frac{s}{z \cdot e_0} = \frac{u \cdot E \cdot t}{z \cdot e_0}$$

Hierin bedeuten:

s = rein elektrophoretischer Wanderungsweg [cm]
z = Ladung des betrachteten Ions
e_0 = Elementarladung
u = Ionenbeweglichkeit auf dem Träger [$cm^2 \cdot sec^{-1} \cdot V^{-1}$]
E = elektrische Feldstärke [$V \cdot cm^{-1}$]
t = Zeit.

Für ein- und denselben Papierstreifen sind t und E konstant, so dass der Weg s direkt proportional der Ionenbeweglichkeit u ist. JOKL[8,9] ermittelte mit der Hochspannungspapierionophorese die R_B-Werte mehrerer Komplexionen mit $(C_2H_5)_4N^+$ als Bezugsion:

$$R_{B_i} = \frac{s_i/z_i}{s_B/z_B} = \frac{u_i/z_i}{u_B/z_B} = \frac{a}{\sqrt{M_i}} + b$$

a, b = apparative Konstanten
M_i = Molekulargewicht des Ions i.

Chemikalien und Arbeitsmethoden

Über die benutzten Komplexsalze wurde bereits berichtet[10]. Alle anderen Substanzen waren pro analysi-Präparate der Fa. Merck. Die Hochspannungspapierionophorese-Apparatur wurde mehrfach beschrieben[11–13]. Die Trennbedingungen für das vorliegende Problem sind:

Lösungsmittel:	Wasser
Leitelektrolyt:	0.5 M CH_3COOK/0.5 M CH_3COOH
Spannung:	2.9 kV
Stromstärke:	30 mA pro Streifen
Elektrodenabstand:	90 cm
Trennzeit:	5/4 h
Kühltemperatur:	−4°
Trägermaterial:	Filterpapier 2043 Bmgl der Fa. Schleicher & Schüll.

Die Identifizierung der Zonen von $[Cr(SCN)_6]^{3-}$ und $[Cr(CN)_6]^{3-}$ erfolgt durch Laufstreckenvergleich. Hierzu werden die Komplexsalze $K_3[Cr(SCN)_6]$ und $K_3[Cr(CN)_6]$ zusammen auf die obere Hälfte der Ionophoresestreifen aufgetragen.

Bei den verwendeten Mengen (3–5 μl Komplexgemisch der Konzentration 0.5 Mol/l) sind die Zonen nur unter der UV-Lampe zu erkennen. Zur Dokumentation werden sie daher mit Tusche nachgezogen.

Darstellung

$[Cr(CN)_6]^{3-}$ als sehr stabiles Komplexion reagiert nicht mit KSCN. In Lösungen von Acetonitril, Formamid, Dimethylformamid und Dimethylsulfoxid mit verschiedenen Gehalten an $K_3[Cr(CN)_6]$ und KSCN, die mehrere Tage im Bombenrohr auf 100° erhitzt werden, ist auf den Ionopherogrammen nur die eine Zone von $[Cr(CN)_6]^{3-}$ zu erkennen.

Dagegen entstehen unter gleichen Bedingungen bei der Umsetzung von $K_3[Cr(SCN)_6]$ mit KCN Reaktionsprodukte. Dies erkennt man an der Farbänderung der violetten $K_3[Cr(SCN)_6]$-Lösungen.

Bei einem Unterschuss von KCN ($[Cr(SCN)_6]^{3-}$:KCN = 1:3) bilden sich nach 1 h Reaktionszeit in den Lösungsmitteln Formamid, Dimethylformamid und Dimethylsulfoxid neben gemischten Cyano–Thiocyanatochromaten(III) noch Solvatkomplexe. Die Solvatkomplexionen können Ladungszahlen zwischen 3+ und 2– tragen. Ihre Zonen sind daher auf den Pherogrammen weit hinter der langsamsten Zone der gemischten Cyano–Thiocyanatochromate(III) zu finden. Lediglich in Acetonitril als Lösungsmittel sind auf den Ionopherogrammen keine Solvatkomplexe zu erkennen.

Die Reaktion verläuft heterogen. Das in Acetonitril schwerlösliche KCN setzt sich mit dem leichtlöslichen violetten $K_3[Cr(SCN)_6]$ um. Die Lösung ändert dabei laufend ihre Farbe von Violett über Rot nach Orange. Ausserdem entsteht ein gelber Niederschlag. Die Gesamtheit der in Acetonitril leichtlöslichen Gemischtligandkomplexe nebst $K_3[Cr(SCN)_6]$ wird im folgenden als Komplexgruppe a, die der schwerlöslichen gelben Komplexe als Komplexgruppe b bezeichnet.

Zur eindeutigen und reproduzierbaren Festlegung des Reaktionsverlaufes in Acetonitril werden variiert: Reaktionstemperatur, Molverhältnisse, Reaktionsdauer. Hierzu werden jedesmal 10 ml einer Acetonitrillösung, welche die Reaktionspartner $K_3[Cr(SCN)_6]$ und KCN enthält, in einem 20 ml Rundkolben unter Rückfluss gerührt. Nach Abschrecken des Reaktionsgemisches auf 0° wird der gelbe Niederschlag abfiltriert, mehrmals mit Acetonitril gewaschen und das Filtrat im Vakuum vorsichtig eingedampft. 160 mg des gelben Niederschlages (0.5 mMol bezogen auf $K_3[Cr(CN)_6]$ bzw. 260 mg des Filtratrückstandes (0.5 mMol bezogen auf $K_3[Cr(SCN)_4]$) werden je in 1 ml Wasser aufgenommen und papierionophoretisch untersucht.

Temperatureinfluss. Bei Temperaturen $< 70°$ sind Reaktionszeiten von über 4 h notwendig. Dabei wird die Reaktionslösung und der Niederschlag braun. Diese braunen Verbindungen, wahrscheinlich Polymerisationsprodukte, beeinträchtigen die Ausbeute und stören die Trennung der Komplexe.

Die günstigste Reaktionstemperatur ist 81° (Siedepunkt des Acetonitrils). Hier dauert es erfahrungsgemäss 3 h, bis das in Acetonitril schwerlösliche KCN in der festen Phase nicht mehr nachzuweisen ist.

Fig. 1 zeigt die Ionopherogramme sämtlicher entstehender Gemischtligandkomplexe.

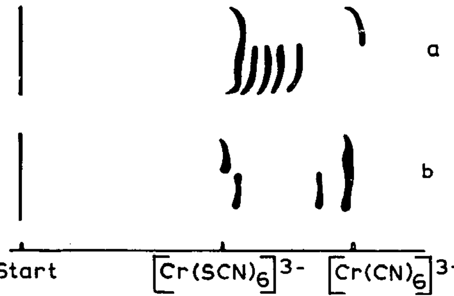

Fig. 1. Ionopherogramm sämtlicher Gemischtligandkomplexe. (a) Komplexgruppe a, (b) Komplexgruppe b; Reaktionstemperatur 81°; CN^-: $[Cr(SCN)_6]^{3-}$ = 4:1; Reaktionszeit 1 h.

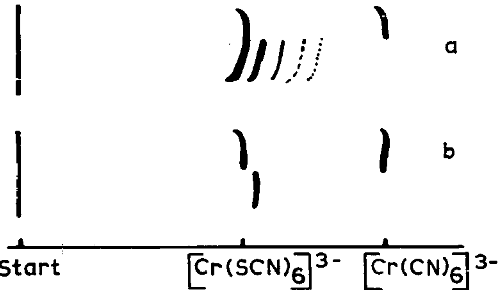

Fig. 2. Ionopherogramm der Gemischtligandkomplexe. (a) Komplexgruppe a, (b) Komplexgruppe b; Reaktionstemperatur 81°; CN^-: $[Cr(SCN)_6]^{3-}$ $\leqq$ 2:1; Reaktionszeit 3 h.

Molverhältnisse der Reaktionspartner. Es werden 2.5 mMol $K_3[Cr(SCN)_6]$ in 10 ml Acetonitril gelöst und mit verschiedenen KCN-Mengen (1.25–25 mMol KCN) 3 h bei 81° unter Rückfluss gerührt. Nur bei diesem Konzentrationsverhältnis ist die Abtrennung der schwerlöslichen Komplexe der Gruppe b von den leichtlöslichen der Gruppe a quantitativ.

Bei einem Verhältnis CN^-:$[Cr(SCN)_6]^{3-}$ $\leqq$ 2:1 bildet sich neben den Komplexionen der Gruppe a die erste schwerlösliche Verbindung der Komplexgruppe b. Ihre Zone liegt auf dem Pherogramm an der gleichen Stelle wie die des ersten Komplexions der Komplexgruppe a (Fig. 2).

Bei dem Verhältnis CN^-:$[Cr(SCN)_6]^{3-}$ > 2:1 entstehen zwei weitere (gelbe) Komplexionen der Komplexgruppe b (Fig. 3). Liegt CN^- gegenüber $[Cr(SCN)_6]^{3-}$ in neunfachem Überschuss vor, bilden sich ausschliesslich die drei Komplexionen der Komplexgruppe b.

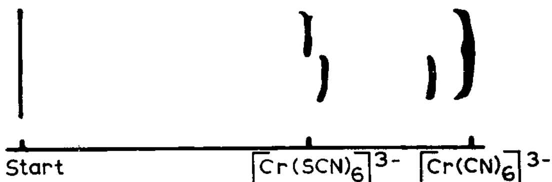

Fig. 3. Ionopherogramm der Komplexgruppe b. Reaktionstemperatur 81°; CN^-: $[Cr(SCN)_6]^{3-}$ > 2:1; Reaktionszeit 3 h.

Reaktionsdauer. 1.3 g (2.5 mMol) $K_3[Cr(SCN)_6]$ werden mit 0.5 g (7.5 mMol) KCN in 10 ml Acetonitril bei 81° unter Rückfluss gerührt. Viertelstündlich wird die Reaktion abgebrochen und das Reaktionsgemisch bei 0° eingefroren. Fig. 4 zeigt die Ionopherogramme der Reaktionsgemische bei verschiedenen Reaktionszeiten. Nach Fig. 4 treten die beiden schnellwandernden Komplexionen der Komplexgruppe b erst auf, wenn der fünfte von Komplexgruppe a gebildet ist. Ausserdem erkennt man hier das gleichzeitige Auftreten des ersten Komplexions der Komplexgruppe a und des ersten Komplexions der Komplexgruppe b. Sie wandern beide gleich schnell.

Wie die papierionophoretischen Untersuchungen zeigen, sind die günstigsten Reaktionsbedingungen zur Darstellung des Gemisches sämtlicher Gemischtligandkomplexe die in Fig. 1 angegebenen. Unter diesen Bedingungen liegen die einzelnen Komplexe in etwa gleicher Menge vor.

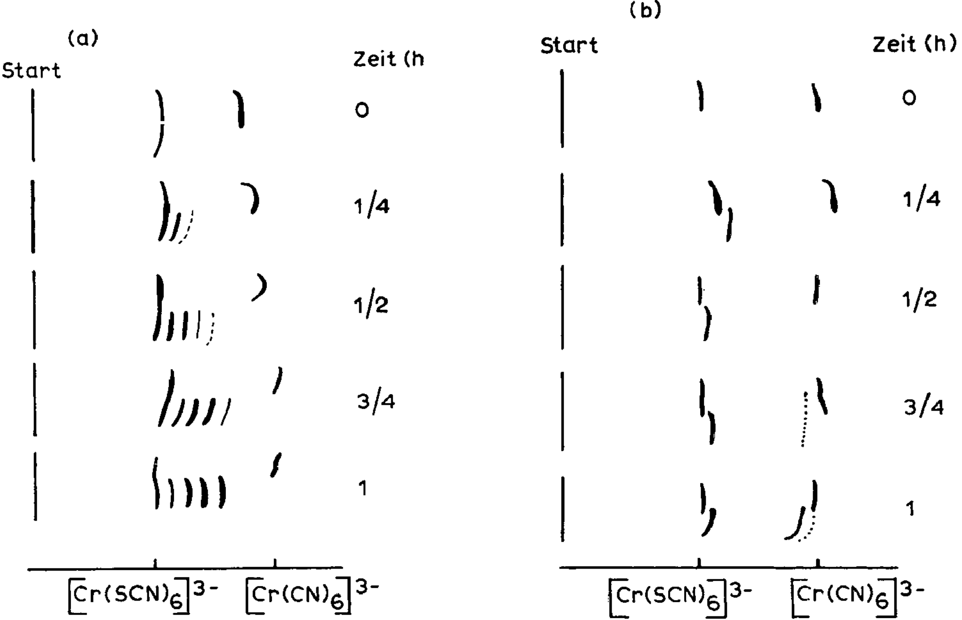

Fig. 4. Ionopherogramme der Gemischtligandkomplexe bei verschiedenen Reaktionszeiten. Reaktionstemperatur 81°; CN^-: $[Cr(SCN)_6]^{3-}$ = 3:1; (a) Komplexgruppe a, (b) Komplexgruppe b.

R_B-Werte

Auf Grund der Ergebnisse der Hochspannungspapierionophorese verläuft die Reaktion ausgehend von $[Cr(SCN)_6]^{3-}$ durch sukzessiven Ligandenaustausch SCN^- gegen CN^- über sämtliche Gemischtligandkomplexe bis zum $[Cr(CN)_6]^{3-}$. Die R_B-Werte der einzelnen Komplexe in Abhängigkeit von $M^{-\frac{1}{2}}$ (M = angenommenes Molekulargewicht) liegen in guter Näherung auf einer Geraden, so dass die angegebene Zusammensetzung der Komplexionen gerechtfertigt ist (Fig. 5).

Die experimentelle Bestimmung der R_B-Werte der einzelnen Komplexionen erfolgt durch Ermittlung der rein elektrophoretischen Wanderungswege nach KLAM-

TABELLE I

R_B-WERTE DER KOMPLEXIONEN

	M	$M^{-1/2} \cdot 10^2$	R_B	$\pm \Delta R_B$
Komplexgruppe a				
$[Cr(SCN)_6]^{3-}$	400	5.000	1.00	—
$[Cr(SCN)_5(CN)]^{3-}$	368	5.213	1.06	0.01
$[Cr(SCN)_4(CN)_2]^{3-}$	336	5.455	1.14	0.01
$[Cr(SCN)_3(CN)_3]^{3-}$	304	5.735	1.23	0.02
$[Cr(SCN)_2(CN)_4]^{3-}$	272	6.063	1.31	0.02
Komplexgruppe b				
$[Cr(SCN)(CN)_5]^{3-}$	240	6.455	1.40	0.02
$[Cr(CN)_6]^{3-}$	208	6.934	1.55	0.03
X^{n-}			(1.06)	

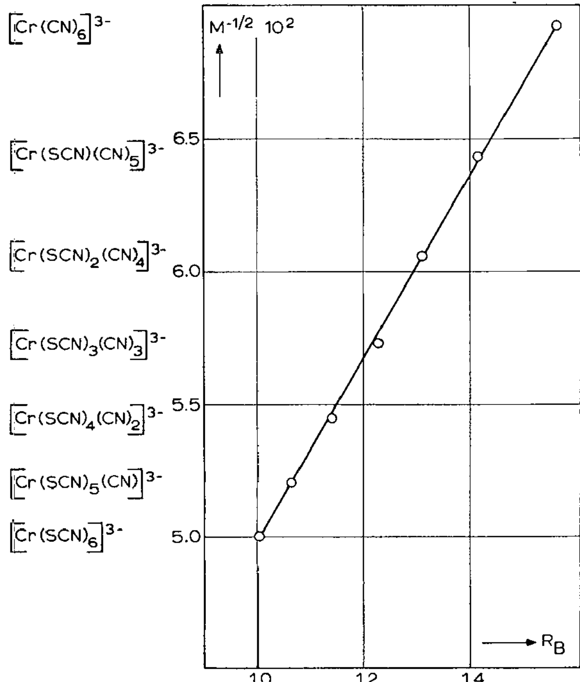

Fig. 5. R_B-Werte als Funktion von $M^{-1/2}$ ($M^{-1/2} \cdot 10^2$).

BERG *et al.*[14] mit Stärke als neutraler Indikatorsubstanz. Hierzu werden Komplexgruppe a bzw. Komplexgruppe b (Darstellung nach den Reaktionsbedingungen der Fig. 1, wie oben beschrieben), der Hochspannungspapierionophorese unterworfen. Als Bezugsion dient $[Cr(SCN)_6]^{3-}$. Die Ergebnisse von Fig. 5 sind in der Tabelle I aufgeführt. Die angegeben R_B-Werte stellen den Mittelwert aus zehn Bestimmungen dar. Für das nicht zur Reihe gehörende gelbe, als Kaliumsalz in Acetonitril schwerlösliche Komplexion X^{n-}, das gleichschnell mit dem Komplexion $[Cr(SCN)_5(CH)]^{3-}$ wandert, kann kein R_B-Wert angegeben werden, da seine Ladung nicht bekannt ist. Der in Klammern angezeigte Wert geht von der Annahme einer Ionenladung von 3— aus.

ZUSAMMENFASSUNG

Gemische sämtlicher Komplexionen $[Cr(SCN)_{6-x}(CN)_x]^{3-}$ ($x = 0, 1, \ldots 6$) entstehen bei der Umsetzung von $K_3[Cr(SCN)_6]$ mit KCN in siedendem Acetonitril. Die Reaktion wird mit Hilfe der Hochspannungspapierionophorese verfolgt. Die ermittelten R_B-Werte stellen einen ersten Beleg für die Zusammensetzung der Komplexionen dar.

Für die Bereitstellung von Mitteln danken wir der deutschen Forschungsgemeinschaft.

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APPARATUR ZUR GELIONOPHORESE IN NICHTWÄSSRIGEN LÖSUNGSMITTELN

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SUMMARY

Apparatus for gel ionophoresis in non-aqueous solvents

A simple ionophoretic technique has been developed using the method of moving boundaries. Stabilisation of the separating media can be accomplished by gel-forming cellulose acetate. The gelling agents hold out promise of use on a wider scale for ionophoresis in non-aqueous solvents. The method is verified by separating $[Cr(SCN)_6]^{3-}$ and $[Cr(CN)_6]^{3-}$ in formamide.

EINLEITUNG

Die Trennung von chemisch sehr ähnlichen, kinetisch stabilen Komplexionen lässt sich mit ionophoretischen Methoden sowohl in analytischem als auch in präparativem Masstab gut durchführen. Hier sind besonders die Arbeiten von BLASIUS und Mitarbeitern[1–5] sowie PREETZ und Mitarbeitern[6–10] zu nennen.

Lassen sich Solvolysereaktionen mit den Bestandteilen des Wassers, d.h. H_2O und OH^-, nicht verhindern, so kann Herstellung und Trennung der Komplexe in nichtwässrigen Lösungsmitteln erfolgen. Dazu wird ein ionophoretisches Verfahren entwickelt, das nach der Methode der wandernden Grenzflächen arbeitet und durch eine Stabilisierung des Trennmediums mit einem Gel eine Trennung in analytischen Mengen erlaubt. Das Gel besteht aus Celluloseacetat als Gelbildner und Formamid als Lösungsmittel. Die Erprobung des Verfahrens erfolgt an dem Gemisch der Komplexionen $[Cr(SCN)_6]^{3-}$ und $[Cr(CN)_6]^{3-}$. Es dient als Grundlage für die in einer späteren Mitteilung beschriebenen Trennung der hydrolyseempfindlichen Gemischtligandkomplexe $[Cr(SCN)_{6-x}(CN)_x]^{3-}$ ($x = 0, 1 \ldots 6$).

GRUNDLAGEN

KOHLRAUSCH[11] entdeckte als erster die Fähigkeit von Elektrolytgemischen zur Ausbildung von Grenzflächen bei Stromdurchtritt. KENDALL und Mitarbeiter trennten Ionengemische nach der Methode der wandernden Grenzflächen[12–18].

Prinzip[19]

In einem Trennrohr befindet sich das Anionengemisch A_1^-, A_2^-, A_3^-. Es wird von Anionen schneller Beweglichkeit an der Vorderfront und Anionen langsamer Beweglichkeit an der Rückfront begrenzt. Für die Beweglichkeit der vorliegenden Anionen gilt:

$$u_l < u_1 < u_2 < u_3 < u_s$$

wobei

u_l = Beweglichkeit des langsamsten Ions an der Rückfront $[\mathrm{cm^2 \cdot sec^{-1} \cdot V^{-1}}]$

u_s = Beweglichkeit des schnellsten Ions an der Vorderfront

Das gemeinsame Gegenion is M^+.

Bei Anlegen einer Spannung setzt die ionophoretische Wanderung ein. Vorder- und Rückfront werden sofort scharf ausgebildet, während sich die Anionen A_1^-, A_2^-, A_3^- langsam entmischen. Bei vollständiger Trennung entstehen neue Grenzflächen, die mit konstanter Geschwindigkeit v wandern. Es gilt:

$$u_l E_l = u_1 E_1 = u_2 E_2 = u_3 E_3 = u_s E_s = v$$

wobei

E = elektrische Feldstärke $[\mathrm{V \cdot cm^{-1}}]$

v = Geschwindigkeit $[\mathrm{cm \cdot sec^{-1}}]$

Demnach verringert sich die Feldstärke stufenförmig zur Anode hin.

Störende Effekte, wie Diffusion und Konvektion, die die Trennschärfe herabsetzen können, werden ausgeglichen. Ionen mit grösserer Beweglichkeit eilen voraus, bis sie am Ort kleinerer Feldstärke die Geschwindigkeit v erreichen. Ionen mit geringer Beweglichkeit bleiben zurück, bis sie im Bereich ihrer Zone eine ausreichend hohe Feldstärke antreffen, die sie mit der Geschwindigkeit v wandern lässt.

Die Konzentrationen der einzelnen Zonen gehorchen im stationären Zustand der Kohlrauschbeziehung:

$$\frac{c_i}{n_i} = \text{const.}$$

wobei

c_i = Konzentration

n_i = Überführungszahl

Gelionophorese

Die Gelionophorese verbindet Eigenschaften der trägerfreien und trägerhaltigen Ionophorese. Die homogene Phase bleibt bei der Stabilisierung des Systems erhalten. Konvektion und Diffusion werden jedoch gegenüber der freien Ionophorese herabgesetzt. Adsorption und Chemisorption können den Trenneffekt beeinflussen.

Bisher wurden gelionophoretische Trennungen nur in wässrigem Medium durchgeführt. In einer Monographie[20] sind die gebräuchlichsten Gelbildner zusammengefasst. Für die Gelionophorese in nichtwässrigen Lösungsmitteln muss der Gelbildner folgende Bedingungen erfüllen:

(a) Gelierfähigkeit mit dem betreffenden organischen Lösungsmittel,

(b) Indifferenz gegenüber den Trennsubstanzen,

(c) Starke Temperaturabhängigkeit der Viskosität des Gels.

Ionophorese in nichtwässrigen Lösungsmitteln

Die Gesetze über die Wanderung elektrisch geladener Teilchen gelten grundsätzlich auch in nichtwässrigen Lösungsmitteln. Für gleichartige Ionen in verschiedenen Lösungsmitteln gilt die Waldensche Regel:

$$\Lambda_0 \cdot \eta = \text{const.}$$

wobei

Λ_0 = Äquivalentleitfähigkeit bei unendlicher Verdünnung
η = Viskosität

Ionen mit grossem Radius, die offenbar keine Solvathülle besitzen, erfüllen diese Bedingung hinreichend genau.

Abweichungen von der Waldenschen Regel treten auf, wenn ein Komplexsalz in verschiedenen Lösungsmitteln eine unterschiedliche Primärdissoziation erfährt. Nur die effektive Beweglichkeit ist wirksam:

$$u_{\text{eff}} = u_0 \cdot \alpha$$

wobei

u_{eff} = effektive Beweglichkeit
u_0 = Ionenbeweglichkeit bei vollständiger Primärdissoziation
α = Dissoziationsgrad

Eine unterschiedliche Dissoziation kann somit eine völlige Umkehr der Ionenfolge gleichartiger Komplexionen hervorrufen. Ein in Wasser langsam wanderndes Ion bildet in einem anderen Lösungsmittel die schnellstwandernde Zone. Diese Umkehr erfolgt bei der Trennung[21] von $[OsCl_6]^{2-}$ und $[OsBr_6]^{2-}$ in H_2O bzw. NH_3.

EXPERIMENTELLES

Über die benutzten Chemikalien wurde bereits berichtet[5].

Apparatur

Die Trenneinheit besteht aus zwei Elektrodentrögen, die mit einem Plastikschlauch aus PVC (Länge 1500 mm) verbunden sind. Sie ist mit dem Gel gefüllt und steht in einer Kühlwanne (Fig. 1). Zur Abführung der Jouleschen Wärme wird eine Kühlsole (Wasser–Glycerin, 3:1) von 0° durch die Wanne gepumpt. In einer breiteren Kühlwanne können auch mehrere Trenneinheiten untergebracht werden. Bedingt durch die Wandstärke (0.75 mm) und den Innendurchmesser (3 mm) des Schlauches

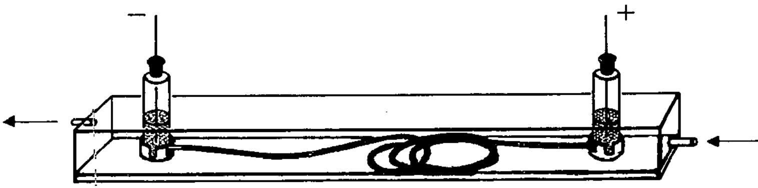

Fig. 1. Trennschlauchapparatur.

entsteht ein Temperaturgefälle, das Dichte- und Viskositätsunterschiede bewirkt. Die Stabilisierung durch das Gel fängt diese Störungen grössenteils auf.

Ein geeigneter Gelbildner für Formamid als Lösungsmittel ist Celluloseacetat F 900 der Farbenfabriken Bayer mit 2.5 veresterten Gruppen pro Baustein (56.1% CH_3COOH). Das Gel wird durch Erhitzen eines Gemisches aus 2.5 Gew.-% Celluloseacetat und 97.5 Gew.-% Formamid auf 130° homogenisiert.

Füllung der Trenneinheit

Während der Füllung dürfen im Trennschlauch keine Luftblasen entstehen. Zur Füllung des Anodentroges und des Schlauches mit der gelierten Elektrolytlösung eines schnellwandernden Anions wird das Gel durch Überdruck in den Schlauch gepresst (Fig. 2). Das Einfüllen von 1–2 ml Trennlösung in den Schlauch geschieht durch einen leichten Unterdruck (Fig. 3). Anschliessend wird der Trennschlauch mit dem Kathodentrog, der die gelierte Elektrolytlösung eines langsam wandernden Anions enthält, verbunden.

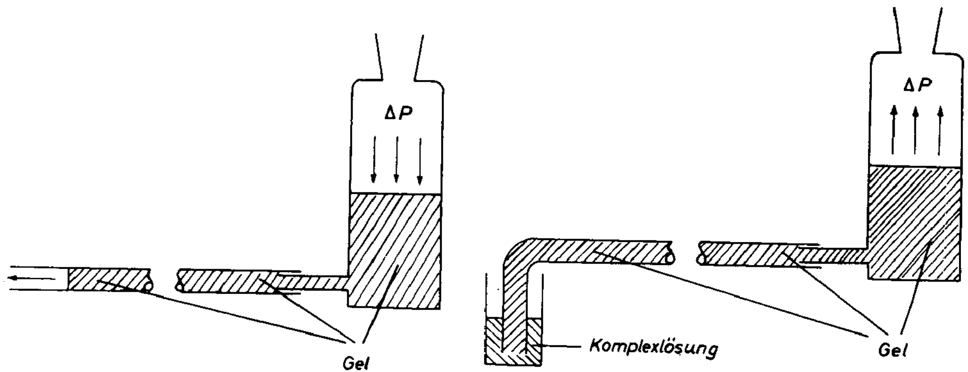

Fig. 2. Füllung des Trennschlauches I.

Fig. 3. Füllung des Trennschlauches II.

Auswertung

Während der Trennung ordnen sich die Komplexe in scharfen Fronten aneinander. Die Zonenlänge kann bei farbigen Ionen optisch leicht ermittelt werden. Eine Möglichkeit zur Festlegung der Zonen farbloser Komplexe bietet sich durch die Ermittlung der Feldstärkeprofile an.

Nach der Trennung schneidet man den Trennschlauch an den Fronten auseinander. Das Gel wird aus dem Schlauch herausgedrückt und die Komplexe können anschliessend mit einem geeigneten Lösungsmittel aus dem Gel eluiert werden.

MODELLTRENNUNGEN

$K_3[Cr(SCN)_6]$ und $K_3[Cr(CN)_6]$ sind leicht löslich in Formamid und erleiden bei 0° keine Solvolyse. Deshalb ist dieses Lösungsmittel für die Trennung geeignet. Die Trennbedingungen sind in der Tabelle I zusammengefasst. Die Angaben für die Konzentrationen der Trennlösung und des langsamen Elektrolyten sowie für die

TABELLE I

TRENNBEDINGUNGEN

Lösungsmittel	Formamid
Gelbildner	2.5 Gew.-% F 900
Schneller Elektrolyt	0.66 *M* KBr
Trennlösung	Mischung aus 0.2 *M* $K_3[Cr(SCN)_6]$ und 0.2 *M* $K_3[Cr(CN)_6]$
Langsamer Elektrolyt	0.6 *M* CH_3COOK
Spannung	1.2 kV
Mittlere Feldstärke	8 V/cm
Stromstärke	10 mA
Trennzeit	24 h
Kühltemperatur	0°

Stromstärke gelten nur für den Beginn der ionophoretischen Trennung in der Trennschlauchapparatur. Im stationär dynamischen Zustand sind sie abhängig von der Konzentration des schnellwandernden Elektrolyten. Der Überschuss an Acetat ist notwendig, um eine Verarmung des Kathodentrogs zu verhindern.

Fig. 4 zeigt eine schematische Darstellung des Trennschlauches nach 24 h. Zur deutlicheren Unterscheidung der Grenzflächen kann zu der Trennlösung noch ein Zwischenion hinzugegeben werden, dessen Beweglichkeit zwischen denen der zu trennenden Komplexionen liegt. Das Zwischenion zwischen den Zonen der beiden Komplexionen verdeutlicht deren Auftrennung. Die Trennung mit dem Zwischenion SCN^- zeigt Fig. 5.

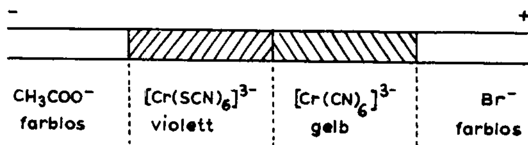

Fig. 4. Trennung von $[Cr(SCN)_6]^{3-}$ und $[Cr(CN)_6]^{3-}$.

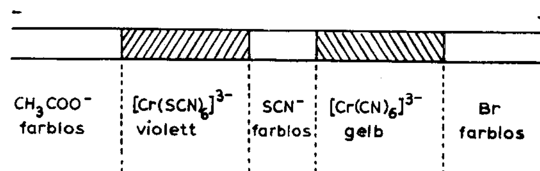

Fig. 5. Trennung von $[Cr(SCN)_6]^{3-}$ und $[Cr(CN)_6]^{3-}$ mit Zwischenion.

DISKUSSION

Die Apparatur arbeitet diskontinuierlich nach dem Prinzip der Ionophorese der wandernden Grenzflächen.

Die Auswahl der Lösungsmittel ist ausschliesslich abhängig vom Trennproblem. Trennungen lassen sich sowohl in Wasser als auch in nichtwässrigen Lösungsmitteln vornehmen. Die Gelionophorese ist unempfindlich gegen kleine Temperaturschwankungen. Die Kühlung kann bis 0° herunter in einem offenen System erfolgen. Die

Konzentration in den Zonen wird durch die Konzentration des vorgegebenen schnellwandernden Anions bestimmt (Kohlrauschbeziehung). Besondere Steuerungsprobleme ergeben sich nicht. Die Trennung ist unempfindlich gegen Spannungsschwankungen. Es ist ohne weiteres möglich, die Spannung während der Trennung zu verändern. Spannungskonstanthalter sind nicht erforderlich. Der Lösungsmittelverbrauch ist ausserordentlich gering. Für eine Trennung werden 40–50 ml Lösungsmittel verbraucht. Dadurch wird auch die Brandgefahr durch Funkenübergänge bei organischen Substanzen gegenstandslos.

Diese Vorteile bedingen einen geringen apparativen Aufwand bei grosser Trennschärfe. Demgegenüber sind die folgenden drei Nachteile zu nennen, von denen die beiden letzteren durch Weiterentwicklung der Trennapparatur ausgeschaltet werden können.

(a) Die getrennten Komplexe müssen vom Gelbildner befreit werden. (b) Die Bedingungen der Kohlrauschbeziehung sind nicht vorgegeben, da die Konzentration an Acetationen im Kathodentrog so hoch gewählt werden muss, dass während der Ionophorese keine Verarmung eintritt. Anderenfalls wird der Stromfluss unterbrochen. (c) Das dritte Problem stellt die Abführung der in den Elektrodentrögen entstehenden Elektrolyseprodukte dar.

In einer verbesserten Apparatur müssen demnach die Elektrodentröge durch ein Diaphragma gegen den Trennschlauch abgegrenzt und laufend mit frischer Elektrolytlösung gespült werden.

Die für die Geldarstellung benutzten Hochpolymeren sind auf der Basis von Cellulose aufgebaut, deren Hydroxogruppen mit verschiedenen Carbonsäuren verestert sind. Die Löslichkeit und damit die Gelierfähigkeit hängt in erster Linie von den freien Hydroxylgruppen ab. Eine geringe Veresterung bewirkt eine grosse Löslichkeit im organischen Lösungsmittel. Ein entsprechendes Gel enthält einen grossen Gewichtsanteil an Gelbildner. Inhomogenitäten im Gel durch Austrocknen sind zu befürchten. Weiterhin bereitet eine spätere Elution mit Wasser Schwierigkeiten, da diese Cellulosecarboxylate etwas wasserlöslich sind. Bei sehr starker Veresterung ist die Löslichkeit der Celluloseacetate auch im organischen Lösungsmittel zu gering. Es bildet sich kein Gel. Als optimaler Gelbildner für Formamid als Lösungsmittel ergibt sich Celluloseacetat mit 2.5–2.6 veresterten Gruppen pro Moleküleinheit. Der Durchsatz während einer Gelionophorese hängt primär von der Löslichkeit der Elektrolyte im Lösungsmittel ab. Formamid löst jedoch viele Salze so gut, dass schon weit unterhalb der Sättigungskonzentration die Joulesche Wärme nicht mehr abgeführt werden kann.

ZUSAMMENFASSUNG

Es wird ein einfaches ionophoretisches Verfahren entwickelt, das nach dem Prinzip der wandernden Grenzflächen arbeitet. Die Stabilisierung der Trennmedien wird durch Gelierung mit Celluloseacetaten erreicht. Diese Gelbildner versprechen einen grösseren Anwendungsbereich für die Ionophorese in nichtwässrigen Lösungsmitteln. Das Verfahren wird anhand einer Modelltrennung von $[Cr(SCN)_6]^{3-}$ und $[Cr(CN)_6]^{3-}$ in Formamid erprobt.

Für die Bereitstellung von Mitteln danken wir der deutschen Forschungsgemeinschaft.

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Notes

CHROM. 4732

Effect of terminal "groups" on gas–liquid chromatographic retention times of long-chain compounds: A case of tail *versus* dog

Among the modified long-chain fatty acids for which GLC retention data have been tabulated for a full range of isomers are *cis* and *trans* octadecenoates[1], methylene-interrupted *cis,cis*-octadecadienoates[2], monomethyl-branched octadecanoates[3], nonynoates[4] and dodecynoates[1], and methyleneoctadecanoates (cyclopropanoids)[5]. Except for the latter class the general rule is that the ultimate or terminal isomer will have a retention time less than that of the penultimate isomer and often similar to that of the next isomer adjacent to the penultimate isomer.

The latter type of behaviour is known to be associated with boiling points. For example, in the *n*-octenes the elution order of isomers is 1, 3, 2 on both a polyester and squalane, corresponding to boiling points of 121.8°, 122.7° and 125.0° (ref. 6). Vapour pressures in isomeric structures are broadly considered to be maximal with the most highly centralised structure. Generally boiling points for higher molecular weight compounds are unknown, but a relationship between boiling point and GLC retention time has been explored definitively for isomeric alkylbenzenes[7]. As part of this study retention times were determined on Apiezon L at 210° and 240°. Thus ratios $t_{210°} \div t_{240°}$ for phenyldodecane isomers eluting in isomer order 6, 5, 4, 3, 2, 1 are 1.91, 1.91, 1.91, 1.93, 1.97 and 2.08. Evidently when a structure is asymmetric, vapour pressure is sensitive to temperature modification.

These observations came to mind in considering the anomalous behaviour of the cyclopropanoids and it was noted that methyl cyclohexylundecanoate[8] seemed peculiarly sensitive to GLC operating temperatures for a nominally fully saturated system (compare insensitivity of apolar methyl-branched acids[3]). On a modified EGA substrate ECL values were: 19.12 at 207° and 18.84 at 178°. On Apiezon L they were 18.00 at 207° and 17.82 at 180°. Other values (Table I) were determined in our laboratory. Presumably this sensitivity would be due to the asymmetry generated by a C_6 ring (terminal group) attached to a C_{11} chain (base chain).

When a variety of structures commonly regarded as terminal C_x "isomers" were re-examined from this novel view point of a C_{x-n} base chain plus a C_n terminal group (Table I) it was noted that the differences (Δ) between the experimental ECL value and the total carbons in the terminal group plus the base chain, for Apiezon L, could be graded in approximate proportion to the base chain carbons divided by the terminal group carbons. The C_{15} ω-alicyclics fit particularly well into the series, and represent data from one laboratory. On polyester liquid phases this group of structures is also an adequate and coherent demonstration of Δ gradient but the bulk of the polyester data are modified somewhat by the polarity of the different functional groups as well as differences among the polyesters employed.

It seems probable that the positive Δ value of $+0.38$ as an indication of molecular asymmetry is adequate to explain the anomalous behaviour of 17,18-methylene-octadecanoate relative to the balance of the octadecanoate-based series of cyclo-

TABLE I

POSSIBLE PHYSICAL BASIS FOR MODIFICATION OF RETENTION TIME BY RELATING TOTAL CARBONS IN A BASE CHAIN TO THE TOTAL CARBONS IN A TERMINAL GROUP WHICH IS ATTACHED TO IT

Ester	*Base chain*	*Terminal group*	*Apiezon L*		*Polar*		*Polyester*[a]
			ECL	*Δ*	*ECL*	*Δ*	
11-Cyclohexylundecanoate[3]	C_{11}	C_6	17.92	+0.92	18.40	+1.40	BDS
11-Cyclohexylnonanoate[11]	C_9	C_6	15.9	+0.9	16.7	+1.7	Reoplex
10-Cyclopentyldecanoate[11]	C_{10}	C_5	15.8	+0.8	16.5	+1.5	Reoplex
9-Cyclobutylundecanoate[11]	C_{11}	C_4	15.6	+0.6	16.1	+1.1	Reoplex
Chaulmoograte[12]	C_{13}	C_5[b]	18.90	+0.90	20.50	+2.50	PEGS
Hydnocarpate[12]	C_{11}	C_5[b]	16.85	+0.85	18.65	+2.65	PEGS
17,18-Methyleneoctadecanoate[5]	C_{16}	C_3	19.38	+0.38	20.04	+1.04	NPGS
11-Dodecynoate[1]	C_{10}	C_2	12.01	+0.01	14.00	+2.00	NPGS
17,18-Octadecenoate[1]	C_{16}	C_2	17.89	−0.11	18.49	+0.49	NPGS
14,17-Octadecadienoate[2]	C_{13}	C_5	17.80	−0.20	19.04	+1.04	NPGS
17-Methyloctadecanoate[3]	C_{16}	C_3	18.56	−0.44	18.60	−0.40	BDS

[a] The lowest polarity polyester is selected if there are data for more than one.
[b] Cyclopentenyl ring.

propanoid isomers. The negative value (−0.44) for 17-methyloctadecanoate, which has a terminal group analogous to the cyclopropanoid ring but of an apolar non-rigid structure, exemplifies the more familiar types of isomeric series where boiling points are not exaggerated by molecular asymmetry.

STROCCHI *et al.*[9] have considered the relationship between molecular configuration in linear chains and GLC retention times. They mention that methyl 9-(2-*n*-propylcyclohexa-3,5-diene)nonanoate (C_{18}) has a centralised structure which should reduce retention time relative to C_{18} straight-chain compounds, yet has an enhanced Apiezon L ECL value of 18.14. The Δ value of +0.14 does not match anticipated levels for a base chain of 9 and a terminal group of 9 (compare cyclohexylundecanoate and nonanoate) and therefore as far as GLC is concerned this structure is essentially a C_{14} chain bridged across carbons 10 and 11 and there is no strong terminal effect. STROCCHI *et al.* are therefore probably correct in considering the conjugated diene as a major factor in increasing retention time, slightly offset by the centralised structure relative to linear C_{18} fatty acids with conjugated dienes.

As a general concept asymmetry due to terminal groups can of course also predict elution order of non-fatty acid compounds. For example 1-cyclohexylnonadecane, 1-cyclopentyleicosane (both $C_{25}H_{50}$) and 2,2-dimethyltricosane ($C_{25}H_{52}$) have respective retention times (mm) on Apiezon L of 80.5, 75.5 and 41.2 (ref. 10). The base chain and terminal group ratios rank in corresponding order of 19:6, 20:5 and 21:4.

Since synthesis of new long-chain compounds is often most facile through attachment of a large group to the ω-position of a base chain, a knowledge of whether Δ were positive or negative could indicate the relative GLC behaviour of adjacent isomers which might be more difficult to synthesise.

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CHROM. 4734

Gas-liquid chromatography of thirty-five amino acids and two amino sugars

The value of gas chromatography for the qualitative and quantitative analyses of mixtures of protein amino acids is well known from the work of GEHRKE and others[1-4]. Either the N-trifluoroacetyl *n*-butyl ester or the trimethylsilyl derivatives[5] can be used to achieve volatility. In connection with current research on the accumulation of peat in the Everglades of Florida, we wished to determine not only the common protein amino acids, but also amino acid degradation products and the unusual amino acids found in muco-substances of bacterial cell walls. Certain amino acid fractions from peat in fact show well over thirty peaks. The purpose of this note is to report that such complex mixtures can be analyzed by GLC and to give specific details of the chromatographic behavior of those non-protein acids whose separation on the same column along with the protein amino acids has not previously been reported.

Experimental

Amino acids used in this work were purchased from Mann Research Laboratories and were found to be chromatographically pure. Reagents used for the derivatization technique were purchased from Regis Chemical Company. A Hewlett-Packard research chromatograph, model No. 5750, equipped with a dual hydrogen flame ionization detector, was used in this study.

The derivatization technique for protein amino acids, introduced by LAMKIN AND GEHRKE[1] in 1965, was used to prepare the protein and non-protein amino acids for gas chromatography as their N-trifluoroacetyl *n*-butyl esters. The procedure for derivatization is a three-step process: (1) a $\frac{1}{2}$-h room temperature esterification with 1.25 *N* HCl in methanol, (2) a $2\frac{1}{2}$-h interesterification at 100° with 1.25 *N* HCl in *n*-butanol, (3) a high temperature sealed tube acylation (150°) for 5 min. Each step must be done under strictly anhydrous conditions. ROACH AND GEHRKE have recently introduced a much faster derivatization procedure which involves a direct esterification with 3 *N* HCl in *n*-butanol[6].

TABLE I

CONDITIONS USED TO EFFECT SEPARATION OF AMINO ACIDS

Column liquid phase	0.325 w/w% EGA	1.0 w/w% OV-17
Column solid support	AW, heat-treated Chromosorb G	HP Chromosorb G
Hydrogen flow, ml/min	40	40
Nitrogen flow, ml/min	60	60
Air flow, ml/min	450	450
Initial temperature, °C	75	115
Post-injection interval, min	5	4
Programmed temperature operation, °C/min	4	6
Final temperature, °C	210	235
Upper limit interval, min	10	7
Detector temperature, °C	250	250
Injection port temperature, °C	200	200
Chart speed, in./min	0.5	0.5

In order to effect separation of the amino acids, two glass columns, 4 mm I.D. were needed; a 1.5-m column packed with 0.325 w/w% ethylene glycol adipate (EGA) on acid-washed (AW), specially heat-treated Chromosorb G (ref. 7), and a 1-m column packed with 1.0 w/w% OV-17 on high-performance (HP) Chromosorb G. Both packings can be obtained from Regis Chemical Company. Because we prefer single-column operation and because we often run the instrument at 10 × 2 (range × attenuation), we found it necessary to condition the EGA column at 210° for 48 h and the OV-17 column at 235° for 24 h.

Various parameters required for operation of the two columns are reported in Table I. Once conditioning is complete, both columns can be put into the same oven. As soon as the EGA column run is completed, the oven is cooled to 115° and the sample is injected onto the OV-17 column. Care must be taken not to subject the EGA column to temperatures above 235° for any extended period of time. Glass injection liners must be used at all times since some of the amino acids degrade on contact with hot metals[4].

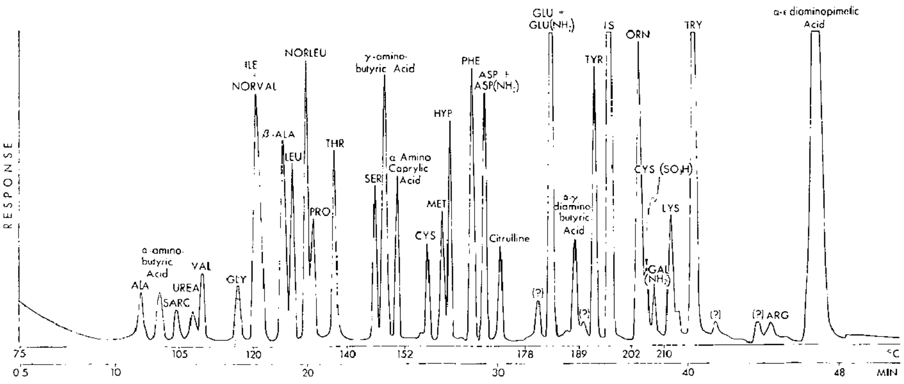

Fig. 1. Separation of amino acid N-TFA *n*-butyl esters. Liquid phase, 0.325 w/w % EGA; support, 80–100 mesh AW heat-treated Chromosorb G; column dimensions, 1.5 m × 4 mm I.D., glass; initial temperature, 75°, 4°/min; 10 × 8.

Results and discussion

The elution times and temperatures for the various amino acids investigated on the EGA and OV-17 columns are reported in Figs. 1 and 2. When thirty-three amino acids were injected on to the EGA column, thirty peaks resulted. The loss of three peaks resulted from the facts that: (1) isoleucine did not separate from norvaline and (2) asparagine and glutamine did not usually separate from their parent acids. A 6 *N* HCl hydrolysis is needed to liberate the acids from peat fractions, and this destroys most of the asparagine and glutamine; hence the problem of incomplete separation does not affect our work. It did seem that when the concentrations of glutamic acid and glutamine were similar, they could be separated. The same was true for asparagine and aspartic acid.

In some cases the resolution of urea and sarcosine was poor, but if a post injection interval of 5 to 10 min was used, they could be separated. The upper limit interval of 10 min was necessary when using the EGA column to effect separation of α,ε-diaminopimelic acid, an amino acid found solely in microorganisms.

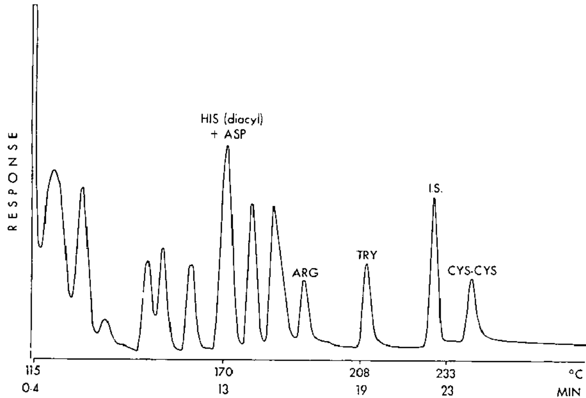

Fig. 2. Separation of amino acid N-TFA *n*-butyl esters. Liquid phase, 1.0 w/w % OV-17; support, 80–100 mesh HP Chromosorb G; column dimensions, 1.0 m × 4 mm I.D., glass; initial temperature, 115°, 6°/min; 10 × 16.

The only problem encountered using the OV-17 column concerned the analysis of histidine. Using the derivatization procedure of GEHRKE *et al.*[7], one obtains histidine as the diacyl derivative, which elutes with aspartic acid. If 7 µl of *n*-butanol are injected on to the column immediately after the sample injection, then the monoacyl derivative is formed on the column and is well separated from any other amino acid[8]. However, we prefer using the diacyl derivative since the quantitation of histidine as its monoacyl derivative is dependent on too many parameters that are hard to control[8]. Knowing the relative molar responses of aspartic acid on OV-17 and EGA columns and its amount from the chromatogram on the EGA column, one can calculate the amount of histidine present from the composite peak on OV-17 (ref. 9). If purely qualitative analysis is being done, then we should prefer forming the monoacyl derivative of histidine. Although tryptophan and arginine can be separated from the other amino acids on the EGA column, which could therefore be used for qualitative purposes, the quantitation of these two amino acids on this column has been poor since apparently there is some interaction with the column packing[8,10]. Therefore for quantitative determination, the OV-17 column must be used.

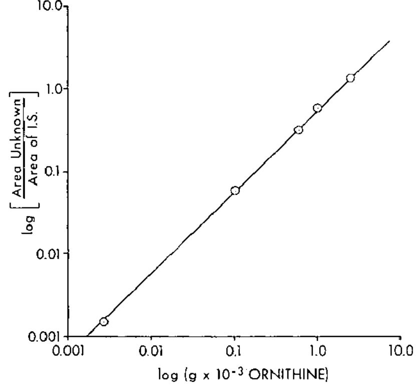

Fig. 3. Standard curve for derivatized concentrations of ornithine.

The excellent quantitative accuracy obtainable with the protein amino acids has already been demonstrated by GEHRKE *et al.*[4,7]. A full statistical study of the accuracy obtainable with the non-protein acids has not yet been carried out by us. However the calibration curve for ornithine, shown in Fig. 3, illustrates the linearity obtained with all the acids. Each point represents three independent experiments. As can be seen the points do fit a straight line. The maximum % standard deviation of any point on the curve is 4.2%.

It is interesting and potentially useful that galactosamine and glucosamine subjected to the same derivatization procedure as the amino acids and injected on to the EGA column gave distinct peaks, that of galactosamine appearing immediately after the elution of cysteic acid (Fig. 1), while that of glucosamine eluted with ornithine. Presumably the derivatives were 3,4,6-tri-O-trifluoroacetyl-N-trifluoroacetyl-1-O-methyl-2-amino-2-deoxy-hexoses.

If one should wish to determine the amino sugars in a mixture containing amino acids, it would be desirable to separate the amino sugars from the amino acids on an ion-exchange column before derivatization and GLC analysis. Although a prior ion-exchange separation is necessary if one is to determine glucosamine in the presence of amino acids, it is of practical convenience to use the same derivatization reagents and procedure for both classes of amino compounds. This possibility has not been previously reported.

The analysis of amino acids from hydrolyzates of peat fractions presents a number of problems to the investigator. The identification of the number of peaks by itself represents a formidable challenge. There are still three or four relatively small peaks in our chromatograms that have not yet been identified, so that further work may well define the chromatographic behavior of a number of additional acids. Using a gas chromatograph–mass spectrometer would of course facilitate the identification of these peaks.

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CHROM. 4731

Separation of the degradation products of 1-benzoyl-5,5-diethylbarbituric acid by means of vapour programmed thin-layer chromatography

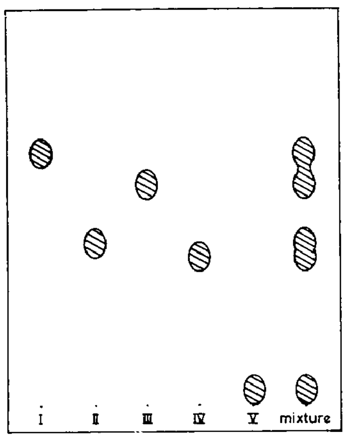
It has previously been established that during the alkaline hydrolysis of N-acyl derivatives of barbituric acid the splitting of the ring occurs near to the acyl radical[1,2], as a result of which the corresponding derivatives of acetylurea (a) and the diamide of malonic acid (b) are formed

Investigation of the alkaline hydrolysis of 1-benzoyl-5,5-diethylbarbituric acid (I) showed that the degradation products included N-benzoyl diethylacetylurea (II), diethylmalonic acid diamide (III), diethylmalonic acid monoamide (IV) and diethylmalonic acid (V).

Attempts were made to separate and identify these compounds by means of TLC but in spite of many variations in the chromatographic systems it was not possible to resolve compounds I and III and II and IV. The best separation possible was that using silica gel and ethanol–isopropanol (75:25) as solvent system with a development distance of 15 cm. The results are shown in Fig. 1.

Recent work by DE ZEEUW[3,4] has shown that the solvent vapour in contact with the adsorbent has considerable influence on the separation process and he designed a special development chamber whereby the method known as vapour programmed TLC can be carried out under reproducible conditions to give a more efficient resolution of compounds than can be achieved with the conventional saturated or unsaturated chambers.

Application of vapour programmed TLC to the five compounds under discussion gave very good separations, as shown in Fig. 2. Reproduction of the separation was extremely good, the R_X values (1-benzoyl-5,5-diethylbarbituric acid = 1) being



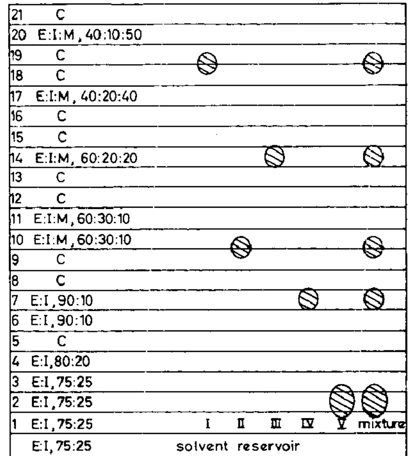


Fig. 1. Chromatogram of a mixture of compounds separated by conventional TLC. Development over 15 cm; solvent system: ethanol–isopropanol (75:25). I = 1-Benzoyl-5,5-diethylbarbituric acid; II = N-benzoyl diethylacetylurea; III = diethylmalonic acid diamide; IV = diethylmalonic acid monoamide; V = diethylmalonic acid.

Fig. 2. Chromatogram of a mixture of compounds separated by vapour programmed TLC. C = Chloroform; E = ethanol; I = isopropanol; M = methanol. I = 1-Benzoyl-5,5-diethylbarbituric acid; II = N-benzoyl diethylacetylurea; III = diethylmalonic acid diamide; IV = diethylmalonic acid monoamide; V = diethylmalonic acid.

(II) 0.50, (III) 0.77, (IV) 0.35, and (V) 0.07. Moreover, the spots were round and compact without tailing and this was important having regard to the need for their quantitative determination by means of densitometry[5].

Experimental

Layer: MN Silica Gel G (Macherey, Nagel & Co.).

Plate size: 20 × 20 cm.

Layer thickness: 250 mμ.

Activation: Air dried for 15 min, heated at 110° for 1 h, stored on a desiccator over silica gel and reheated at 110° for 15 min prior to use.

Solvents: ethanol, isopropanol, methanol, chloroform (all redistilled).

Running distance: 20 cm (time, approx. 60 min).

Temperature: 20–22°; temperature of evaporating unit 27–28°.

Load: 4 μl 0.2% solution in methanol.

Spray reagent: 0.05% dithizone in carbon tetrachloride.

The troughs of the vapour phase TLC chamber were partially filled (5 ml) with mixtures of the solvents as indicated in Fig. 2. The running solvent was ethanol–isopropanol (75:25) and this was introduced into the solvent reservoir after equilibration had been allowed to proceed for 10 min.

We would like to thank Mr. R. Brown for constructing the vapour programme TLC chamber (from the description given by De Zeeuw[3,4]). One of us (M.M.) would also like to thank the Chelsea College for awarding her a post doctoral research scholarship enabling her to work in the pharmacognosy research laboratories of the College.

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CHROM. 4698

Thin-layer chromatography of substituted methyl β-maltosides

During the synthesis[1] of substituted methyl β-maltosides, extensive recourse was made to chromatography. This note records the chromatographic behavior of these maltoside derivatives; both thin-layer (TLC) and dry-column[2] chromatography are reported.

Experimental

Thin-layer plates (0.25 mm thickness) were prepared with a Quickfit Instrument* apparatus from a slurry of Silica Gel G (25 g) and water (50 ml). Before use, the plates were air dried, horizontally, for 16 h. Solvents were purified by distillation. Plates were developed until the solvent front had ascended 13 cm above the spotting site. Benzene–absolute ethanol (2:1) was used to develop the unacylated maltosides, and toluene–methanol (50:1) for the acylated maltosides as well as for multiple ascents. Spots were detected by spraying with a solution of ethanol–water–concentrated sulfuric acid (10:5:1) and heating until charred. All spots turned black upon charring except the deoxy-maltosides, which charred dark brown after changing from various shades of yellow. Iodine vapor also located the *p*-tolylsulfonyl (tosyl) maltosides when a nondestructive method was needed.

Dry-column chromatography was used to isolate the tosylated maltosides (compounds 3, 7, and 11, Table I). A 3-g sample of the partially tosylated reaction mixture of methyl β-maltoside was introduced onto the dry column by first dissolving the mixture in a slurry consisting of 15 g of Davison Grade 12 silica gel and 50 ml 95% ethanol. After the ethanol had evaporated, the residue was introduced on top of a dry Silica Gel G column (200 g, 4×40 cm) and the column was developed with

* The mention of firm names or trade products does not imply that they are endorsed or recommended by the Department of Agriculture over firms or similar products not mentioned.

TABLE I

R_F VALUES OF SUBSTITUTED METHYL β-MALTOSIDES

Compound No.	R	X	Y	R_F values × 100 Solvent 1[a]	Solvent 2
1	H	OH	OH		12
2	Ac	OAc	OAc	20	
3	H	OTs	OTs		79
4	Ac	OTs	OTs	25	
5	Ac	I	I	45	
6	Ac	H	H	37	
7	H	OTs	OH		42
8	Ac	OTs	OAc	25	
9	Ac	I	OAc	33	
10	Ac	H	OAc	30	
11	H	OH	OTs		35
12	Ac	OAc	OTs	25	
13	Ac	OAc	I	34	
14	Ac	OAc	H	30	
15	Ac	OTs	I	34	
16	Ac	OTs	H	31	
17	Ac	OBz	H	38	

[a] Solvent 1 = toluene–methanol (50:1). Solvent 2 = benzene–absolute ethanol (2:1). Ac = acetyl, Bz = benzoyl, Ts = p-tolylsulfonyl.

benzene–absolute ethanol (2:1). Fractions (1–2 ml) were collected, monitored, and appropriately combined. Their R_F values are given in Table I.

Discussion

Synthesis of these substituted maltosides began with the partial tosylation of methyl β-maltoside (compound 1) to give a mixture of mono- and disubstituted derivatives, along with a large amount of starting material. Small amounts of tri, tetra, and higher orders of substitution were also present. To separate this mixture a modification of the dry-column chromatographic technique of LOEV AND GOODMAN[2] proved useful. Where LOEV AND GOODMAN halted elution when the dry column was fully developed, we simply modified the technique by continuing the elution and collecting fractions, as is usually done in wet-column chromatography. Excellent separation between compound 3 and higher order of substitution was obtained. Compound 3 overlapped only slightly with compounds 7 and 11, which eluted as a mixture. Sufficient separation between compounds 7 and 11 was realized when pure compound 11 crystallized in the last few fractions collected. Compound 1 remained on the column and was removed by eluting with 95% ethanol. The corresponding iodides (compounds 5, 9, and 13) were prepared quantitatively by heating under reflux with sodium iodide the respective acetylated tosyl products (compounds 4, 8, and 12), which were prepared by reaction of acetic anhydride–pyridine with compounds 3, 7, and 11. Toluene–methanol (50:1) separated well every reaction sequence in the acetylated series.

With the substituted maltosides the order of migration varied, *viz.* deoxyiodo > deoxy > tosyl > acetate and the sequence was found to be general; for example, compound 5 > 6 > 4 > 2. This sequence occurred also with 6-substituted methyl α- and β-glucopyranosides[3]. EVANS *et al.*[4] report for 6-chloro-6-deoxy and 6-deoxy methyl D-glucopyranosides a sequence deoxy > chlorodeoxy > hydroxy. With both the acetylated and unacetylated chlorodeoxy and deoxy glucosides in several solvent systems we observed the sequence chlorodeoxy > deoxy > acetate (or hydroxyl). No explanation can be offered for this difference.

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CHROM. 4701

Thin-layer gel filtration of modified proteins on 4% fine pearl-condensed agar

The possibility of preparing fine pearl agar suitable for thin-layer gel filtration has been reported in some preliminary results[1]. The major advantages of this thin-layer technique are reduced running time and very small sample size; the latter is especially important if we are working with high molecular weight substances which have been prepared by complicated and difficult isolation methods.

The fractionation experiments to test the feasibility of thin-layer gel filtration on agar gel were performed with nonprotein material–Blue Dextran 2000.

In the present study we tried to use the same thin-layer method for simple orientation estimation of aggregate and molecular sizes of modified and denatured proteins of an experimental blood expander "Modified Bovine Serum" (MBS)[2–4]. This protein material is a heterogeneous and complex mixture of modified serum protein and gelatin, together with their aggregates and degradation products.

Gel filtration on agar, resp. agarose gel, which has been introduced by POLSON[5], was shown to be more suitable for fractionation of MBS than gel filtration on Sephadex G-200. Dextran gel has too rigid a structure and its network detains only a smaller part of the comparatively large molecules and aggregates of MBS.

Materials and methods

"Modified Bovine Serum" was a standard preparation, batch No. "DG 472", prepared by heat denaturation and formolation of bovine serum in the presence of

partially degraded gelatin and by oxidation of this product with hydrogen peroxide in our laboratory. Proteins and Blue Dextran 2000 used for calibration were commercial preparations, bovine ribonuclease (Reanal, Hungaria), human serum albumin and human gamma globulin (Institute of Sera and Vaccines, Prague), Blue Dextran 2000 (Pharmacia, Uppsala). α_2-Macroglobulin was a gift from Mr. ŠTEFEK of the Research Institute for Immunology, Prague.

A standard buffer, 0.1 M Tris + 0.1 M NaCl adjusted to pH 7 by HCl, was used in the experiments for both gel filtration on columns and thin-layer techniques. The preparation of fine agar pearls by a modified method according to HJERTÉN[6] has been described previously[1]. The pearls having a 40–100 mesh size were used for gel filtration on the column and those of 100–400 mesh for the thin-layer technique.

Gel filtration was performed on a column of 4% agar pearls, equilibrated with the solution of standard Tris buffer. The column was 1.4 cm in diameter and 90 cm high. A 2 ml portion of the 4% MBS was applied to the top of the column and the flow rate was adjusted to 10 ml/h. Fractions of 4 ml were collected and measured after a reaction with trichloroacetic acid. The fractions of MBS selected for thin-layer gel filtration were concentrated by dialysis against 20% dextran solution.

The thin-layers of agar pearls were prepared on 8 × 15 cm glass plates, thickness of the layers being 0.5 mm. The samples (20 μl of 0.5–2% protein solution) were applied with a micropipette as round spots 5 mm in diameter. The flow rate through the gel of 1.5 cm/h was regulated by the angle of the plate (approx. 20°) to horizontal. After completion of a run the substances were transferred from the gel layer by adsorption into a sheet of a slightly wet filter paper. After 1 min the sheet was removed, dried for 10 min and stained for 30 min by 0.1% Nigrosine. The migration distances of the spots of proteins were measured from their start to their centre and expressed as D_r values[7] (ratio of the migration distance of the retarded species to that of the excluded species).

Results and discussion

Fig. 1 shows a typical elution curve of MBS without degraded gelatin on 4%

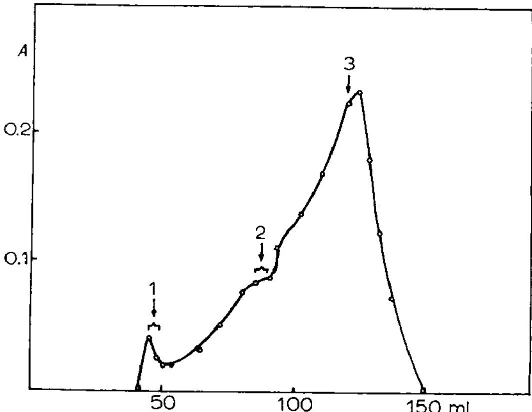

Fig. 1. Column gel filtration of MBS on pearl-condensed agar, particle size 40–100 mesh. Column 90 × 1.4 cm; buffer 0.1 M Tris + 0.1 M NaCl + HCl, pH 7. The fractions selected for thin-layer gel filtration are indicated by arrows.

pearl-condensed agar[8]. On the agar column, only a small part of the MBS is in the region of the exclusion limit, which is different from gel filtration on Sephadex G-200 (ref. 9), where MBS is almost totally excluded. The main part of the MBS is considerably slowed down on agar. Thus the structure of 4% agar gel is more convenient for the fractionation of the large molecules of the modified proteins of bovine serum than the structure of Sephadex G-200. The elution volume of the first small peak, which consists of relatively large particles corresponding to a mol. wt. of the order of 10^6, is close to the void volume of the agar column. The main peak according to the calibration curve was found to have a mol. wt. of about 300,000.

Since modified proteins of MBS possess such a wide range of sizes they seemed to be suitable for testing thin-layer gel filtration on 4% pearl agar, when we take into consideration that for example the commercial product Sepharose 4 B (Pharmacia, Uppsala)[10] with approx. 4% agarose has a fractionation range of mol. wt. of about $300{,}000\text{–}3 \times 10^6$ (booklet 1967) and the exclusion limit for protein mol. wt. is about 20×10^6 (booklet 1969).

Thin-layer gel filtration of MBS and its column fractions on 4% agar gel can be seen on Fig. 2. Only the first fraction from column gel filtration had an almost equal length of run to Indian ink, which was used for the determination of the exclusion limit of the plate. The other fractions of MBS and unfractionated MBS were distinctly slowed down compared with Indian ink. A short table of D_r values of MBS and its fractions on 4% agar gel and Sephadex G-200 is given below (Table I).

If we compare the D_r values in Table I we can see that thin-layer gel filtration

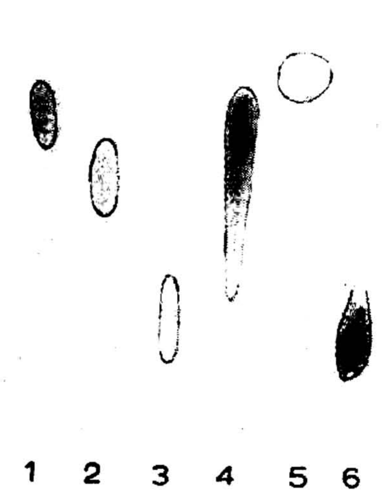

Fig. 2. Thin-layer gel filtration on agar of the MBS fractions from column gel filtration. Pearl-condensed agar, 4%, particle size 100–400 mesh. Plates 8 × 14 cm; thickness of the layers 0.4 mm; buffer 0.1 *M* Tris + 0.1 *M* NaCl + HCl, pH 7; flow rate 1.5 cm/h; 20 μl of 0.5–2% protein solution applied. 1, 2, 3 = fractions from Fig. 1, 4 = unfractionated MBS; 5 = Bromphenol Blue; 6 = dilution of Indian ink.

TABLE I

D_r VALUES OF MBS AND ITS FRACTIONS

Sample	*4% agar*	*Sephadex G-200*
MBS	0.62	1.00
Fraction 1	0.98	1.00
Fraction 2	0.58	1.00
Fraction 3	0.42	0.80

on agar pearl has advantages over the same technique on a dextran gel of the type Sephadex G-200 for fractionation of the large size molecules of MBS. All the fractions of MBS are separated on agar gel which may be used to separate molecules and particles up to a molecular weight of several millions. Only the third fraction is different from the exclusion limit and can be identified on Sephadex G-200.

We were interested in the possibility of a rough estimation of molecular (particle) sizes directly from the data of the thin-layer technique. For this purpose we calibrated the agar gel plate with various proteins of known molecular weight and calculated the D_r value for each protein (Fig. 3). A linear relationship is seen to exist when the D_r values are plotted against the log of the molecular weight of the retarded protein, a relationship similar to that described by ANDREWS[11]. The calibration curve was considerably steeper than that for the same proteins on Sephadex G-200. D_r values of standard proteins and Blue Dextran 2000 on 4% agar pearl and Sephadex G-200 are shown in Table II.

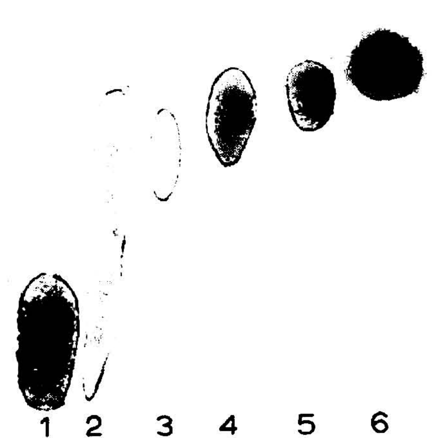

Fig. 3. Thin-layer gel filtration on agar of: 1, Indian ink (the second spot is probably the protein of the glue that is in commercial Indian ink); 2, Blue Dextran 2000; 3, α_2-macroglobulin; 4, human γ-globulin; 5, human serum albumin; 6, bovine ribonuclease. Experimental conditions see Fig. 2.

TABLE II

Dr VALUES OF STANDARD SUBSTANCES

	Mol. wt.	D_r *values*	
		4% agar	*Sephadex G-200*
Ribonuclease	13,000	0.36	0.35
Serum albumin	69,000	0.43	0.60
γ-Globulin	156,000	0.48	0.81
α_2-Macroglobulin	820,000	0.57	1.00
Blue Dextran 2000	2,000,000	0.76	1.00

The first fraction of MBS according to the calibration curve was found to have a molecular weight of the order 10^6, the second fraction 700,000–800,000 and the third fraction 50,000–60,000.

Our results presented in this communication show that under suitable conditions thin-layer gel filtration on pearl-condensed agar can serve as a simple and convenient method for the orientation estimation of relatively large molecular sizes of modified proteins.

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CHROM. 4725

The use of PVC membrane filters as specific chromatographic carriers for rapid ultra-micro-analysis of proteins

Membrane chromatography and electrophoresis on cellulose nitrate[1-3] and acetate[4] membranes have proved to be very convenient techniques, especially for the simple and rapid analysis of very small amounts of biological material. In the course of further investigations on other types of membrane filters for similar purposes, we have recently studied polyvinylchloride (PVC) filters (Sartorius) which do not seem to have been used yet in this field. The results we achieved with these membranes indicated that the behaviour of the PVC carriers towards proteins is more like that of cellulose nitrate than of cellulose acetate. However, important differences between the behaviour of proteins on PVC and nitrocellulose membranes were observed, in particular the firmer binding of the proteins, which opens many new and promising experimental possibilities. Some special details of the pretreatment necessary when working with PVC membranes are briefly given below.

Materials and methods

PVC membrane filters (Sartorius, Göttingen, G.F.R.) SM 12801, 12804, 12806 and 12807, in strips 3–10 mm wide and 10–30 mm long, were used. They were first soaked in 40–50% aqueous ethanol, and then washed thoroughly with the developing solution, *e.g.*, water; 0.9% and 10% NaCl; 0.1 *M* acetate buffer, pH 3.7 and pH 6; 0.1 *M* phosphate buffer, pH 7.5; 0.1 *M* veronal acetate buffer, pH 8.6; 0.1 *M* borate buffer, pH 9.06. To prevent desiccation of the "slower" membranes (12804, 12806, 12807) during manipulation, 10% glycerine or ethyleneglycol was added to those solutions. 0.1–1 μl of human serum albumin, whole serum, dog haemoglobin, horse myoglobin, ovalbumin, trypsin, as 0.01–2% solutions, were placed on the wet membrane by means of a thin calibrated capillary[1] or a wick[5] of filter paper or cellulose acetate membrane (Millipore). In quantitative estimations it was convenient to let the drop of sample soak into the starting edge of the strip from the surface of the plexi-glass. Ascending one-dimensional development was used during chromatography. The starting edge of the strip was placed in contact, perpendicularly, with a filter paper wick wetted with buffer, while the other end was pressed gently between a dry filter paper wick and a glass slide in order to maintain a regular flow of buffer. In short runs (1–5 min) and especially on the "faster" SM 12801 strips no closed humid chamber was necessary. Indelible pen or crystal violet were suitable for marking the start, 5–10% solutions of either potassium bichromate or copper sulphate or chloride were used to visualise the rate of flow of the buffer. Proteins were stained on the wet membranes with 0.5% Amido Black 10 B or 0.01% nigrosine in 5% trichloroacetic acid and the background destained with water[1-5].

Results

It was found that all the proteins tested were firmly adsorbed to the PVC membranes even in alkaline developing buffers and during chromatography formed a very homogeneous layer with very sharp contours. The area covered by the adsorbed protein under defined conditions was directly proportional to the absolute amount

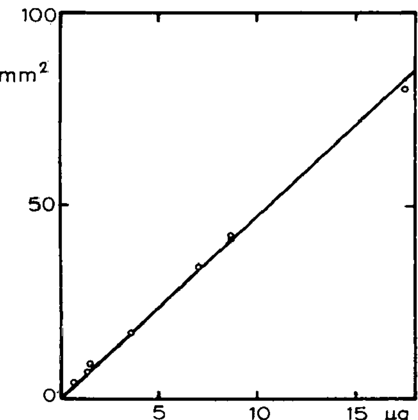

Fig. 1. Calibration curve of dog haemoglobin (fresh stroma-free haemolysate). PVC membranes Sartorius SM 12801 were used, the given amounts of haemoglobin applied in volumes 1 μl–3 μl, and chromatographed in 0.9% NaCl for 1–2 min.

of the given protein (Fig. 1). This fact extends remarkably the range for the rapid quantitative estimation of proteins by membrane chromatography[1] to the region of neutral and alkaline pH values and to low molecular weight proteins (on nitrocellulose there was some restriction to acid pH values and higher molecular weights[3]).

The adsorption of the proteins could be prevented by previous impregnation of the membranes with either the same protein, or polyethyleneglycol (Polywachs 20,000, Hüls, G.F.R.), or neutral detergents such as 2% aqueous Tween 20 (Atlas, U.S.A.). The latter is able to substitute and thus elute adsorbed proteins from PVC strips similarly to what was observed on nitrocellulose[2,3]. Impregnated PVC membranes are then suitable as carriers for the microelectrophoresis of proteins under the usual conditions[2,3,5]. Certain changes in the adsorption of the proteins tested were observed in 0.1 *M* NaOH and 8 *M* urea; however, no marked changes were found in 0.1 *M* HCl and 10% NaCl.

Further details and results concerning the use of intact or impregnated PVC membranes in biochemical analysis and the binding mechanism of substances to PVC will be reported on later.

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CHROM. 4692

A method for the one-dimensional thin-layer chromatographic separation of serum phospholipids*

The requirement for a rapid analytical procedure for the resolution and quantitation of the principal serum phospholipid classes, utilizing TLC separations, led to the development of this method. To meet the requirements of the desired analytical procedure, the method had to exhibit the following characteristics: one-dimensional separation to accommodate multiple samples per plate, resolution of the principal phospholipid classes with minimal overlapping, stable and reproducible operation on a day-to-day basis, and adaptability to densitometric scanning for quantitation. Attempts to use reported systems did not permit precise resolution of individual lipid classes in one dimension[1–4], and therefore the following method was developed.

Materials and methods

Glass plates and spreader were obtained from Quickfit Reeve Angel, Clifton, N.J., U.S.A. Adsorbosil-5 silica gel was obtained from Applied Science Laboratories, State College, Pa., U.S.A. This silica gel was selected after testing of numerous other commercially available silica gels. Spectroquality chloroform and methanol, and chromatoquality tetrahydrofuran were obtained from Matheson, Coleman and Bell, East Rutherford, N.J., U.S.A. The individual lipid standards and mixture were obtained from Supelco, Inc., Bellefonte, Pa., U.S.A. Spotting syringes and dispenser were obtained from Hamilton Company, Whittier, Calif., U.S.A.

The 20×20 cm glass plates were coated with a 250 μ thick slurry of silica gel and allowed to air-dry at ambient temperature for about 2 h. The air-dried plates were scribed into 1.0 cm wide lanes and activated in a convection oven at 110° for 20 min. After activation, the plates were cooled in a nitrogen-flushed desiccator containing silica gel desiccant. Routinely a sample was spotted under nitrogen in a narrow band in the center third of a lane. Each sample contained 100 μg of lipid in 10 μl of chloroform–methanol (95:5). After evaporation of the spotting solvent, the plate was developed in a paper-lined TLC tank for 9.0 min in tetrahydrofuran–methanol (3:1). The plate was allowed to dry for 20 min under dry nitrogen and then transferred to a second paper-lined TLC tank and developed in the same dimension to a height of 17 cm in chloroform–methanol–4 *M* ammonium hydroxide (75:37:7). The plate was then removed from the tank, dried at ambient temperature, sprayed with a 20% aqueous solution of ammonium bisulfate[5] and charred 90 min at 170° for detection.

Discussion

Fig. 1 illustrates a typical chromatogram obtained by the above method with phospholipid standards. Fig. 2 illustrates a typical chromatogram showing the separation of phospholipid classes obtained by this method with a phospholipid fraction isolated from human serum. Changes in room temperature, relative humidity (10–60%)

* The following abbreviations will be used in this manuscript: LPC = lysophosphatidylcholine; SPH = sphingomyelin; PC = phosphatidylcholine; PS = phosphatidylserine; PI = phosphatidylinositol; PE = phosphatidylethanolamine; PG = phosphatidylglycerol; CAR = cardiolipin; PA = phosphatidic acid; MIX = mixture of all nine phospholipids listed above.

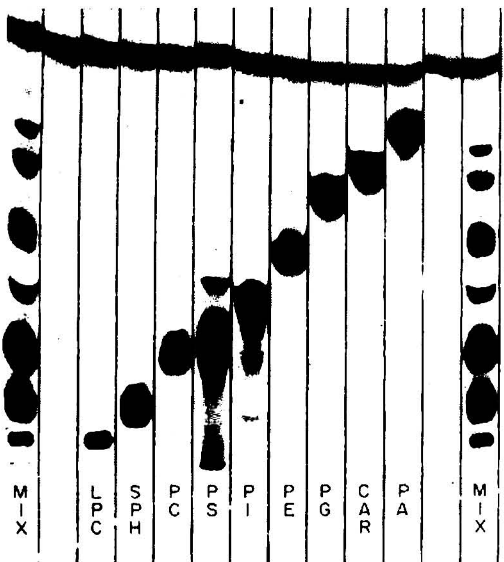

Fig. 1. Thin-layer chromatogram of standard phospholipid classes.

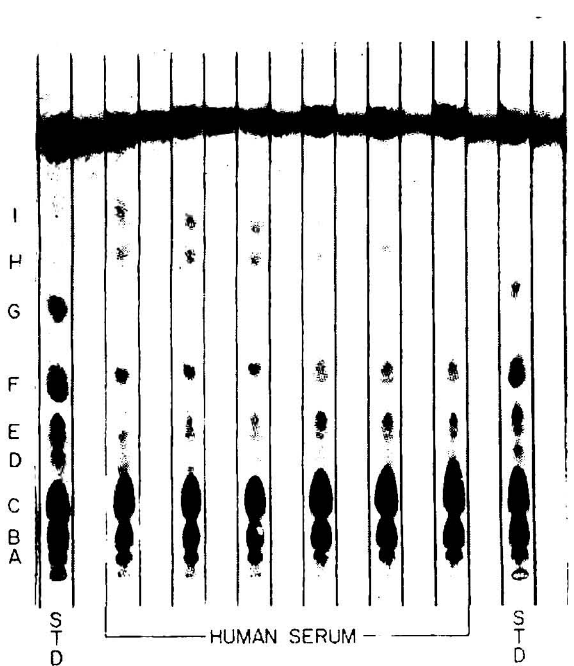

Fig. 2. Thin-layer chromatogram of human serum phospholipids (six inside lanes) and a standard phospholipid mixture (two outside lanes). A = Lysophosphatidylcholine; B = sphingomyelin; C = phosphatidylcholine; D = phosphatidylserine; E = phosphatidylinositol; F = phosphatidylethanolamine; G = phosphatidylglycerol; H = cardiolipin and I = phosphatidic acid.

and lot variations in silica gel result in changes in the observed separations. These changes can be corrected by varying the volumes of methanol and ammonium hydroxide solution used in the second developing solvent. Separations between PG and CAR vary slightly with the activation time. Under some conditions, this pair will not separate on plates activated for longer or shorter times. The separation of PS is somewhat variable, and the conditions controlling this separation are not completely understood at this time. In our experience tetrahydrofuran has proven to be unstable during storage. Placing an iron nail or paper clip into the bottle when first opened, flushing with nitrogen, and storage at 4° enable the compound to be used for periods up to two weeks.

The large amounts of PC and SPH in serum present the principal difficulty in utilizing one-dimensional TLC methods. At this concentration, these two lipid classes tend to trap the minor lipid classes. The use of the first solvent eliminated this problem and allows the observed separations. This method has also been successfully applied to phospholipid class separations of lipoprotein fractions, tissue samples, and bacterial samples in our laboratory.

The excellent technical assistance of PHILIP J. FRAPPAOLO is gratefully acknowledged.

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The opinions in this paper are those of the authors and do not necessarily reflect the views of the Navy Department or the naval service at large.

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CHROM. 4723

Sterol metabolism

XIII. Chromatographic resolution of the epimeric 24-hydroxycholesterols

Although chromatographic resolution of epimeric hydroxycholesterol derivatives bearing hydroxyl groups in the 20- (ref. 1), 22- (refs. 2–7), and 23- (ref. 8) positions has been reported, chromatographic resolution of the epimeric 24-hydroxycholesterols essential to their study in human tissues has not been achieved. Only one epimer of 24-hydroxycholesterol, named cerebrosterol, has been isolated from human brain[9–11]. Whereas this sterol may be detected readily in extracts of human brain by paper[12] and thin-layer[13] chromatography (TLC) and in human feces and meconium by gas chromatography (GC) (refs. 5, 6), the stereochemical purity of the sterol from tissues has not heretofore been demonstrated chromatographically.

We have attempted to no avail to resolve the epimers of 24-hydroxycholesterol, as the free sterols and as their diacetates and dibenzoates, by a variety of column chromatographic means, including Sephadex LH-20 (ref. 14), by direct TLC methods and by GC (ref. 1). We have now devised a TLC resolution of the epimeric 24-hydroxycholesterols as their dibenzoate esters by extended ascending irrigation for 15 h. This method, though time consuming, permits analysis for the first time of the stereochemical purity of 24-hydroxycholesterol samples isolated from human tissues.

Experimental

Both epimers of 24-hydroxycholesterol[10,11] were obtained by sodium borohydride reduction of 24-ketocholesterol. The epimer identified with the naturally occurring 24-hydroxycholesterol cerebrosterol and designated as the $24\xi^1$-hydroxycholesterol initially[10,11] has been assigned absolute configuration as the $24\beta_F$(24S)-hydroxycholesterol[15]. However, we have suggested that the assignment be reversed[8], and for the present we retain the original $24\xi^1$- and $24\xi^2$-nomenclature for cerebrosterol and its epimer, respectively. Cerebrosterol was isolated from human brain by modifications of previously described methods of extraction and recovery[9–13].

Crude sterol preparations and purified reference sterols were benzoylated by dissolving 1 mg of sample in 2 ml of dry pyridine and adding 1 ml of benzoyl chloride. The solution was held at room temperature overnight, whereafter 1 ml of water was added. After 4 h the mixture was extracted with 100 ml of diethyl ether. The ether extract was washed with dilute sodium hydroxide solution, with water three times, and with brine once, after which the extract was dried over anhydrous sodium sulfate and evaporated under vacuum. TLC examination of the benzoylated samples using benzene–ethyl acetate (3:2) showed that none of the free sterol remained unesterified and that only one esterified component was formed.

A 20 × 40 cm chromatoplate prepared with Silica Gel HF_{254} (E. Merck GmbH., Darmstadt, G.F.R.), 0.25 mm thick for analysis, 1 or 2 mm thick for preparative work, is washed chromatographically along the 40 cm dimension with methanol–chloroform (2:1) and redried (1 h at 110°). Samples (10–50 μg) of the benzoylated samples are spotted along the 20 cm dimension in the usual manner, with reference samples of both $24\xi^1$-hydroxycholesterol (cerebrosterol) and its epimer $24\xi^2$-hydroxy-

cholesterol as the 3β,24-dibenzoates. The chromatoplate is irrigated in ascending fashion with benzene–hexane (1:1) in a chromatographic chamber closed at the top so that the long dimension of the chromatoplate protrudes from the chamber into the laboratory atmosphere (about 10–12 cm is exposed). Ascending solvent evaporates from the exposed portion of the chromatoplate, and after 15 h irrigation is terminated. The chromatoplate is dried and visualized under 254 nm UV light. Visualization may also be accomplished by spraying the chromatoplate with 50% aqueous sulfuric acid in the usual fashion, with warming to full display of coloration.

For quantitation the zones detected under UV light are marked, the silica gel excised from the chromatoplate and packed into short glass columns for elution of the sterol dibenzoates with 5 ml of redistilled methanol. The methanol solution so obtained was scanned between 210–250 nm against a blank similarly prepared by methanol extraction of an equivalent area of the irrigated chromatoplate not bearing any steroid. The absorption spectrum of both dibenzoates was a symmetric peak, λ_{max} 228 nm, from which the absorption intensities were directly determined. Two known mixtures of the epimeric 24-hydroxycholesterols, carried throughout the benzoylation, chromatographic, and spectrophotometric procedure, gave very good results. A mixture containing 12.0% of the naturally occurring epimer (cerebrosterol) and 88.8% of the unnatural epimer was analyzed to contain 12.5% of cerebrosterol and 87.5% of the epimer. A mixture containing 44.2% of cerebrosterol was analyzed to contain 43.6%.

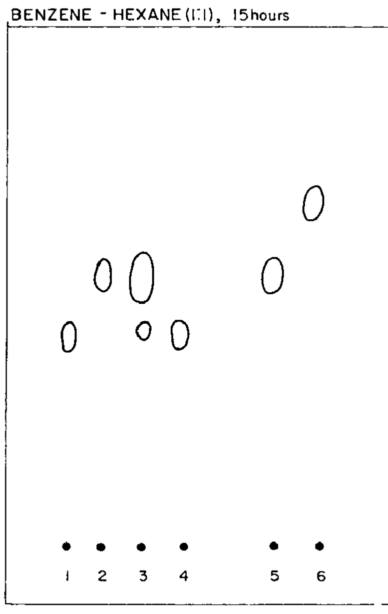

Fig. 1. Resolution of 24-hydroxycholesterol dibenzoates on Silica Gel HF_{254}, 15 h ascending irrigation with benzene–hexane (1:1). Dibenzoate esters of: (1) 24ξ^1-hydroxycholesterol from borohydride reduction of 24-ketocholesterol; (2) 24ξ^2-hydroxycholesterol by borohydride reduction; (3) a 1:2 mixture of the 24ξ^1- and 24ξ^2-hydroxycholesterol epimers; (4) 24ξ^1-hydroxycholesterol (cerebrosterol) from human brain; (5) 23R-hydroxycholesterol; (6) 23S-hydroxycholesterol.

Fig. 1 shows the resolution obtained using this system with the epimeric 24-hydroxysterols. Complete resolution is achieved. A mixture of 24-hydroxycholesterol epimers in which the unnatural $24\xi^2$-hydroxycholesterol epimer predominated is also given to show the ready recognition of minor amounts of one epimer in the presence of larger amounts of the other. For comparison we also present in Fig. 1 the resolution of the epimeric 23-hydroxycholesterols as the 3β,23-dibenzoates.

We confirm with these studies that the human brain 24-hydroxycholesterol previously isolated as a single epimer is indeed stereochemically pure and that human brain preparations do not appear to have any of the other epimer present.

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Thin-layer chromatography of the chromogens from the Allen reaction

As part of an investigation of the mechanism of the ALLEN[1] reaction, separation of the components of the reaction mixtures has been effected by thin-layer chromatography. The chromogens were prepared by a modification[2] of the ALLEN reaction. The steroids used were 3β-hydroxyandrost-5-en-17-one (dehydroepiandrosterone, DHEA), 17β-hydroxyandrost-4-en-3-one (testosterone, TT) and androst-4-ene-3,17-dione (androstenedione, AD). The steroids were dissolved (100 mg per ml) in 66% H_2SO_4 (Analar grade, B.D.H.), and from this solution two sets of derivatives were prepared.

Firstly, the acidic solutions were poured into a tenfold excess of water, and the resultant precipitate was filtered off under reduced pressure provided by a water-pump, washed with water and acetone, and dried. In the case of TT and AD, the derivatives were crystallised from methanol. In the second experiment, the acidic solutions were heated in a boiling water-bath for 4 min (DHEA) or 30 min (TT and AD), the resulting blue-green solutions were poured into a tenfold excess of water and the precipitates recovered as previously. The two derivatives each from DHEA, TT and AD were dissolved in 1,2-dichloroethane (B.D.H.), spotted onto pre-coated aluminium-backed alumina plates (Merck) with fluorescence indicator and developed using the Eastman chromatographic apparatus with ethyl acetate–benzene (Analar grade, B.D.H.) as solvent. Optimal concentrations were ethyl acetate–benzene in a ratio of 1:10 for DHEA chromogens and 1:2 for TT and AD chromogens.

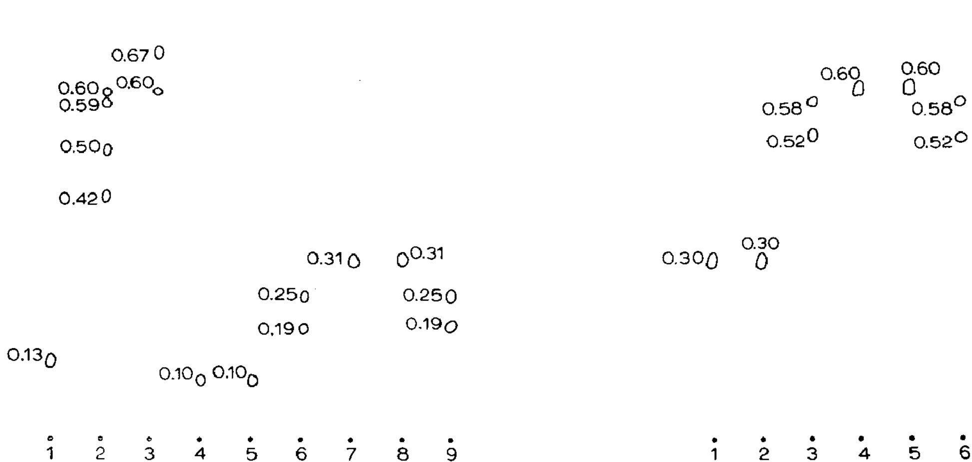

Fig. 1. Thin-layer chromatogram of chromogens from dehydroepiandrosterone (1–3), testosterone (4–6), and androstenedione (7–9), developed with ethyl acetate–benzene (1:10). 1, 4, 7 = parent steroid; 2, 5, 8 = derivative after solution in sulphuric acid without heating; 3, 6, 9 = derivative after solution in sulphuric acid and heating for 4 or 30 min.

Fig. 2. Thin-layer chromatogram of chromogens from testosterone (1–3) and androstenedione (4–6), developed with ethyl acetate–benzene (1:2). 1, 4 = parent steroid; 2, 5 = derivative after solution in sulphuric acid without heating; 3, 6 = derivative after solution in sulphuric acid and heating for 4 or 30 min.

The results are shown in Figs. 1 and 2; the R_F values for each component are indicated in these Figs. The chromogens were visualised by spraying with acetic anhydride–concentrated sulphuric acid (4:1)[3-5]. No further spots could be detected using phosphomolybdic acid[6,7], phosphotungstic acid or the ZIMMERMANN reagent[8] or by irradiation with UV light[9].

From these chromatograms it is concluded that: (1) DHEA is quantitatively converted into chromogens by solution in 66% H_2SO_4 without heating. Comparable treatment of TT and AD produces changes which are easily reversible when the acidic solutions are poured into water. It is most likely that the TT and AD are converted into their conjugate acids by protonation of the oxygen functions; these steroids were recovered in 61 and 93% yield, respectively. The identity of these compounds indicated by thin-layer chromatography was confirmed by IR spectroscopy of KBr pellets of the steroids and by melting point determinations. As protonation of the C-17 ketone group in androstenedione is easily reversible, it is concluded that changes which are observed in DHEA involve the 5-en-3β-ol group. The three spots obtained from DHEA are coloured yellow, red and orange, respectively, by the spraying reagent in passing from the starting point towards the solvent point. Visible absorption maxima in the sulphuric acid solution are obtained at 408 (yellow), 551 (purplish pink) and 486 nm (orange), which probably corresponds to the three chromogens in the chromatogram. (2) The reaction product obtained on heating with 66% H_2SO_4 is identical from TT and AD and different from the DHEA chromogen mixture—a hypothesis which has been advanced previously[2]. Thin-layer chromatography of TT and AD chromogen mixtures obtained after various heating intervals between 1 and 30 min confirmed this relationship (the difference is not due to differences in heating time) and indicated that TT reacts much more readily than AD. The IR spectra of KBr pellets of the TT and AD chromogen mixtures have identical vibration bands, which are different from those given by the DHEA chromogen mixture.

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CHROM. 4693

Resolution of RNA hydrolysis products by two-dimensional anion-exchange thin-layer chromatography*

Separations of nucleic acid hydrolysates and complex nucleotide mixtures have been reported[1–4]. RAAEN AND KRAUS[5] recently presented a technique capable of group separation of the four major nucleotides of alkali-hydrolyzed RNA and the separation of the 2'- and 3'-isomers of adenylic acid monophosphate. LEECH *et al.*[6] have presented a method for the determination of base ratios of acid-hydrolyzed ribonucleic acid. CASHEL *et al.*[7] have demonstrated the effect of pH on nucleotide migration in phosphate solvents. The technique presented here is capable of resolving the four major nucleotides found in RNA and both the 2'- and 3'-isomers of each of them. Preliminary application of this technique to maize tRNA hydrolysates demonstrates an additional component, probably a substituted guanylic acid. The advantages of this system reside in (1) the high degree of resolution and (2) its adaptability to radioisotope studies involving quantitation in the $\mu\mu$mole range.

Materials and methods

Poly(ethyleneimine) (Chemicat P-145) was generously supplied by Chemirad Corporation, Baltimore, Md., U.S.A.; MN Cellulose 300, manufactured by Macherey, Nagel and Co., Duren, G.F.R., and a Desaga applicator, manufactured by Desaga, Heidelberg, G.F.R., were obtained from Brinkmann Instruments Inc., Westbury, N.Y., U.S.A. Bakelite ® Rigid Vinyl Sheets (21 × 50 in.) type VSA 3310 Clear 31 Matte 06, 0.010 in., manufactured by Union Carbide Corporation, Cincinnati, Ohio, U.S.A., were obtained from Commercial Plastics and Supply Corporation, New York, N.Y., U.S.A. RNA (Torula Yeast, Grade VI) was obtained from Sigma Chemical Company, Company, St. Louis, Mo., U.S.A. Nucleotide monophosphate standards, *i.e.*, adenylic acid, 2'(3') mixed isomers (2',3'-AMP); cytidylic acid, 2'(3') mixed isomers (2',3'-CMP); guanylic acid, 2'(3') mixed isomers (2',3'-GMP), and uridylic acid, 2'(3') mixed isomers (2',3'-UMP), were obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio, U.S.A.

Preparation of the PEI-cellulose thin layers has been reported elsewhere[2].

Effect of sample pH on migration and resolution. Three grams of commercially prepared RNA were dissolved in 250 ml of 0.3 *N* KOH and incubated in a water bath at 37° for 12–15 h. Any insoluble material was then centrifuged away. 10-ml aliquots were diluted with 20 ml of doubly distilled water and adjusted to the various pH values, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 5.0, 7.0, 9.0, 11.0, and 13.0 (the pH of the alkaline hydrolysate). The samples were applied 2 cm above the bottom of the plate, 2–3 cm apart. The sample contained approximately 3×10^{-6} g of the original 3-g sample. The plate was then run 10 cm from the sample origin in a closed rectangular chromatography chamber. Various solvents were used, including 1.0 *N* formic acid pH 2.0, 0.1 *N* formic acid pH 3.6, 1.0 *N* formic acid pH 3.6, 1.0 *M* LiCl pH 7.0, 0.5 *M* formic acid–sodium formate pH 3.6, and 2.0 *M* formic acid–sodium formate, pH 3.6.

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The standard nucleotide monophosphates were run in the various solvents for the purpose of identification of the hydrolyzed RNA components.

Two-dimensional chromatography. The solvent for the first dimension was either 1.0 N formic acid pH 3.6 or 0.1 N formic acid pH 3.6. The sample, with a pH of 3.0 ± 0.1 (3×10^{-6} g in 2 μl), was spotted 2 cm from the bottom of the plate and approximately 3 cm from either side. The solvent was allowed to run a distance of 10–15 cm from the base of the plate, after which the plate was dried in a current of air and the R_F values were determined. The plate was next trimmed below the front area to eliminate impurities that migrated with the front. The solvent for the second dimension (1 M LiCl, pH 7.0) was then run a distance of approximately 8 cm on the plate, dried in a current of air and the R_F values were determined.

The chromatographic plates were illuminated with a Mineralight UVS-11 short-wave UV light source and photographed with Agfa 500 color film. The 35-mm slide was then used to draw Figs. 1 and 2 by projection through a Beseler enlarger.

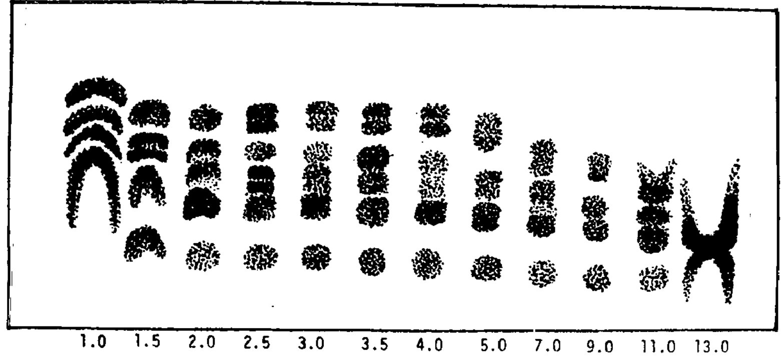

Fig. 1. One-dimensional chromatogram of alkaline hydrolysate of yeast RNA. The effect of sample pH on separation and resolution is shown; pH values are given for each sample from left to right. Solvent: 0.1 N formic acid, pH 3.6. The lower border represents the origin for all samples.

Results and discussion

Effect of sample pH. Sample pH was found to have a marked effect on component separation and resolution in the unbuffered system (Fig. 1). In the buffered system, the R_F values were similar regardless of sample pH. (R_F values not given.) Optimum

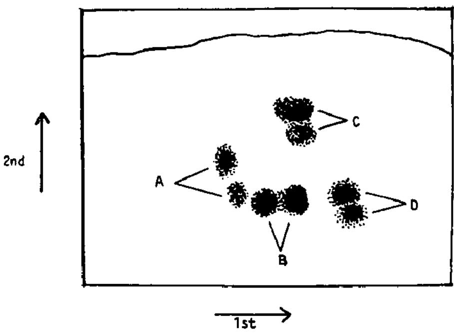

Fig. 2. Two-dimensional chromatogram of alkaline hydrolysate of yeast RNA. A = 2'- and 3'-GMP; B = 2'- and 3'-AMP; C = 2'- and 3'-UMP; D = 2'- and 3'-CMP. Pure 2' and 3' forms were not available for reference.

separation and resolution were found to occur at pH 3.5 ± 0.1; all samples subsequently tested were first brought to this pH.

Two-dimensional chromatography. It was of primary importance in a two-dimensional elution scheme to avoid any effect by the solvent system for the first dimension upon migration in the second dimension. RANDERATH *et al.*[1] thoroughly discussed the effects of the presence of salt on migration and separation of nucleotides. They proposed a methanol wash after the first dimension, but this involved some loss of sample. To avoid this we used a volatile solvent. The ideal solvent for the first dimension varied with the aims of the experimenter. 1.0 *N* formic acid and 3.0 *N* formic acid, pH 2.0, gave sharp group separation. In these systems, adenylic and cytidylic nucleotides migrated close to the solvent front and far ahead of the guanylic and uridylic nucleotides. Maximum resolution of nucleotides was obtained with 0.1 *N* formic acid, pH 3.6. This solvent separated the 2'- and 3'-isomers of adenylic and guanylic acid monophosphates in addition to the group separation of uridylic and cytidylic acid monophosphates (Fig. 2).

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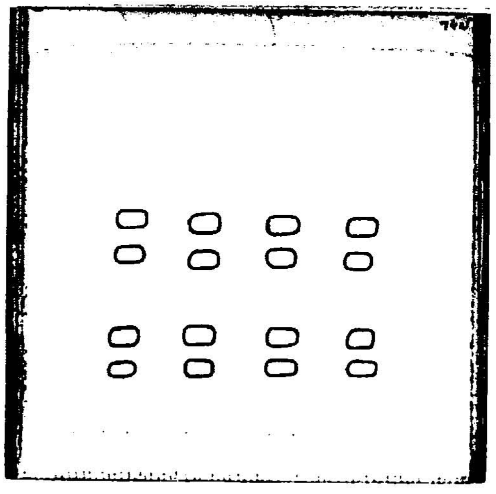
CHROM. 4699

Microméthode pour la détermination de la composition nucléotidique des ADN

Nous avons publié en 1967 (bibl. 1) une méthode de séparation des désoxyribomononucléotides sur couche mince de PEI-cellulose. Depuis, notre technique a été améliorée et appliquée au fractionnement des hydrolysats enzymatiques des ADN, en vue de la détermination de leur composition nucléotidique. Les dosages des nucléotides sont effectués par spectrophotométrie directe des plaques et les résultats sont précis et reproductibles.

Chromatographie

Nous utilisons des plaques de PEI-cellulose F Merck (réf. 5725/0025). Les dépôts sont effectués à 2 cm du bord inférieur de la plaque sur 1 cm de longueur et 3 mm de largeur. Quatre dépôts espacés de 2.5 cm peuvent être effectués sur une plaque. Le solvant chromatographique est une solution de formiate d'ammonium 0.5 *M* de pH 6.2–6.5 que l'on fait migrer jusqu'au sommet de la plaque.



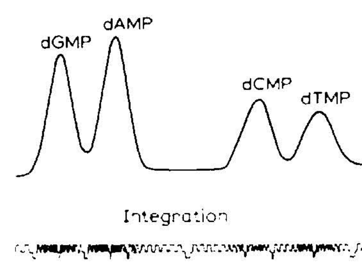


Fig. 1. Séparation des quatre désoxyribonucléotides sur couche mince de PEI-cellulose. De bas en haut: dGMP, dAMP, dCMP, dTMP.

Fig. 2. Diagramme obtenu après enregistrement d'une chromatoplaque au spectrophotomètre intégrateur Vernon.

Après séchage sous un courant d'air froid, l'emplacement des nucléotides est repéré en lumière UV à 254 mμ (lampe Camag) (Fig. 1). Les chromatoplaques sont lues au spectrophotomètre enregistreur intégrateur Vernon en transparence (source UV; largeur de fente maximum 5; hauteur de fente 2 cm; sensibilité maximum; vitesse d'avance R; densité 0.3; filtre jaune; absorption rapide).

Le diagramme obtenu (Fig. 2) permet de connaître les surfaces des pics des différents nucléotides, et de calculer les quantités de chacun de ces nucléotides.

Etalonnage du spectrophotomètre

Dans un premier temps nous avons vérifié que les surfaces des pics sont proportionnelles aux quantités des différents nucléotides.

Préparation de solutions titrées de nucléotides. 10 mg environ de chaque nucléotide* sont dissous séparément dans 5 ml d'eau distillée. La concentration exacte de ces solutions en µmoles/ml est déduite des dosages de phosphore effectués en triple exemplaire sur 1 ml de chaque solution. Les dosages de phosphore sont effectués selon une modification de la méthode d'ALLEN[2]:

minéralisation de 1 ml de la solution nucléotidique par 2 ml de mélange destructeur SO_4H_2-ClO_4H (3:2) pendant 2 h sur une rampe électrique;

hydrolyse des polyphosphates par 30 ml d'eau distillée à l'étuve à 80° pendant 2 h;

addition d'eau distillée qsp 50 ml;

dosage du phosphore sur 10 ml (en tube jaugé de 15 ml); addition de 1 ml de molybdate d'ammonium à 8.3% et 1 ml de solution d'amidol à 1% préparée extemporanément dans du sulfite de sodium à 20%. On ajoute de l'eau distillée pour compléter à 15 ml.

La coloration bleue se développe pendant 30 min à l'obscurité et les lectures sont faites à 650 mµ en cuve de 1 cm au spectrophotomètre (Jobin et Yvon). L'étalonnage préalable de l'appareil a été effectué avec des solutions de phosphate monopotassique.

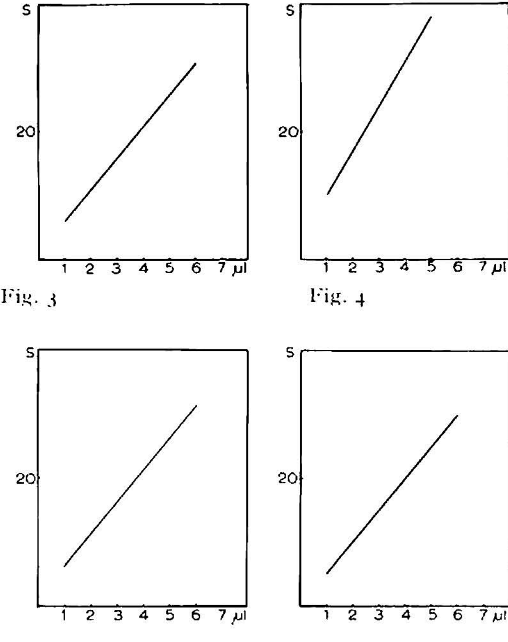


Fig. 3–6. Relation entre la surface des pics et le nombre de nanomoles déposées. Fig. 3: 1 µl dGMP — 3.966 nmoles; Fig. 4: 1 µl dAMP — 5.384 nmoles; Fig. 5: 1 µl dCMP — 5.904 nmoles; Fig. 6: 1 µl dTMP — 4.880 nmoles.

* Sels sodiques ou ammoniques de désoxyribonucléotides purs (Sigma).

TABLEAU I

DÉTERMINATION DU COEFFICIENT K DE L'ACIDE DÉSOXYGUANOSINE-5'-MONOPHOSPHATE

Dépôts (en µl)	*Nanomoles*[a]	*Surface des pics*[b]	K^c
1	3.966	6	
			1.260
2	7.932	11	
			1.260
3	11.898	16	
			1.260
4	15.864	21	
			1.260
5	19.830	26	
			1.260
6	23.796	31	

[a] Valeurs déduites des dosages de phosphore.

[b] Les nombres de la colonne correspondent aux indications fournies par l'intégrateur du spectrophotomètre Vernon.

[c] Ce coefficient (voir texte plus haut) est calculé pour chaque portion de courbe comprise entre deux points expérimentaux successifs.

$$\text{Exemple: } \frac{11-6}{3.966} = 1.260.$$

Préparation des plaques de chromatographie en couches minces. Nous avons effectué, sur couche mince de PEI-cellulose, à l'aide d'une micropipette (Glassfim) des dépôts de 1, 2, 3, 4, 5 et 6 µl de chaque solution de nucléotide pur.

Après la migration du formiate d'ammonium et séchage, l'enregistrement est effectué, comme nous l'avons décrit plus haut. Nous avons tracé les courbes des surfaces des pics en fonction du nombre de nanomoles déposées pour chacun des quatre nucléotides. Ceci nous a permis de déterminer la quantité minimale à déposer pour obtenir un résultat valable; ceci nous a permis aussi d'évaluer un coefficient (K) qui relie la surface des pics au nombre de nanomoles pour chaque nucléotide (voir Fig. 3–6 et Tableaux I–IV), coefficient qui dépend très étroitement des conditions expérimentales décrites dans ce texte. Le coefficient K de chaque nucléotide pour tous les intervalles expérimentaux étudiés (voir Tableaux) est toujours constant.

TABLEAU II

DÉTERMINATION DU COEFFICIENT K DE L'ACIDE DÉSOXYADÉNOSINE-5'-MONOPHOSPHATE

Dépôts (en µl)	*Nanomoles*	*Surface des pics*	K
1	5.384	10	
			1.300
2	10.768	17	
			1.300
3	16.152	24	
			1.300
4	21.536	31	
			1.300
5	26.920	38	

TABLEAU III

DÉTERMINATION DU COEFFICIENT K DE L'ACIDE DÉSOXYCYTIDINE-5'-MONOPHOSPHATE

Dépôts (en µl)	*Nanomoles*	*Surface des pics*	K
1	5.904	6	
			0.846
2	11.808	11	
			0.846
3	17.712	16	
			0.846
4	23.616	21	
			0.846
5	29.520	26	
			0.846
6	35.424	31	

Hydrolyse enzymatique des ADN

L'hydrolyse des ADN est effectuée par la désoxyribonucléase I (3-1-4-5) et la phosphodiestérase de venin de serpent (3-1-4-1) selon le protocole suivant: 2 mg d'ADN sont dissous dans 0.2 ml de tampon Tris/HCl 0.002 M, de pH 7.2 contenant du chlorure de magnésium 0.01 M, dans un tube à hémolyse, à la température du laboratoire. On ajoute 0.1 ml d'une solution de DNase I* (correspondant à 1200 unités) et on porte à 37° pendant une nuit. Le lendemain, le pH est ajusté à 9 avec de la NaOH 0.1 N et on ajoute 0.1 ml d'une solution de phosphodiestérase de venin de serpent** (5 mg/0.5 ml eau). Après incubation à 37° pendant 2 h, l'hydrolyse de l'ADN est totale et on peut procéder au fractionnement des désoxyribonucléosides 5'-phosphate sur couche mince de PEI-cellulose selon la technique décrite plus haut (dépôt de 5 µl sur 1 cm).

TABLEAU IV

DÉTERMINATION DU COEFFICIENT K DE L'ACIDE DÉSOXYTHYMIDINE-5'-MONOPHOSPHATE

Dépôts (en µl)	*Nanomoles*	*Surface des pics*	K
1	4.880	5	
			1.024
2	9.760	10	
			1.024
3	14.640	15	
			1.024
4	19.520	20	
			1.024
5	24.440	25	
			1.024
6	29.280	30	

* DNase I (DNEP) de Sigma Chemical Company, 3500 Dekalb Street, St.-Louis, Mo. 63118, U.S.A.

** Phosphodiestérase de venin de serpent (activité spécifique 0.3) Schwarz représenté en France par BD-Mérieux, Marcy-l'Étoile (69), France.

Dans un premier temps, il est préférable de faire migrer de l'eau distillée sur la plaque pour vérifier l'absence de nucléosides, qui ne sont pas fixés sur la PEI-cellulose et sont entraînés par l'eau et pour éliminer les cations qui peuvent gêner la chromatographie.

Cette technique a été mise au point sur l'ADN de thymus de veau; son application aux ADN des virus et des ADN des mitochondries de foie de cobaye est actuellement en cours d'étude.

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* Avec la collaboration technique de Mademoiselle A. HEMEZ.

CHROM. 4696

The use of acid dissociation constants in selecting buffers to effect the electrophoretic separation of bithionol, fenticlor, hexachlorophene, bromchlorophene, dichlorophene, tetrachlorophene and 4-hexylresorcinol

Halogenated *o,o'*-dihydroxydiphenyl sulphides and methanes are important compounds pharmaceutically, and hexachlorophene in particular has found widespread use in medicated soaps, shampoos and toothpastes. Other compounds, including bithionol and fenticlor, are available commercially for use as bacteriostats, fungicides, spermicides and anthelmintics.

Dichlorophene	$X = CH_2$;	$R_1, R_2 = H$
Tetrachlorophene	$X = CH_2$;	$R_1 = Cl$; $R_2 = H$
Bromchlorophene	$X = CH_2$;	$R_1 = Br$; $R_2 = H$
Hexachlorophene	$X = CH_2$;	$R_1, R_2 = Cl$
Fenticlor	$X = S$;	$R_1, R_2 = H$
Bithionol	$X = S$;	$R_1 = Cl$; $R_2 = H$

The present authors have been investigating methods of determining these compounds and their use as analytical reagents for metals. Procedures for the determination of iron(III) with bithionol have been described[1,2]. As part of this investigation the proton–ligand and ligand–metal formation curves and stability constants of bithionol, fenticlor and hexachlorophene, and certain of their metal complexes, have been determined potentiometrically[3].

Many procedures for the determination of halogenated *o,o'*-dihydroxydiphenyl sulphides and methanes have been published. These include potentiometric pH titrations with sodium hydroxide[4], direct UV spectrophotometric determination[5–7] and colorimetric determination with 4-amino-antipyrine[8]. In each case, however, difficulty is experienced in distinguishing between individual compounds of this type and in avoiding interference from similar compounds.

Chromatographic procedures for the separation of certain of these compounds have been described. Hexachlorophene and dichlorophene have been separated by ascending paper chromatography and by thin-layer chromatography prior to their UV spectrophotometric determination[9]. R_F values for several compounds of this type have been given[10], and mixtures of 4-hexylresorcinol, bithionol, hexachlorophene and dichlorophene have been separated by two-dimensional thin-layer chromatography[11].

In the present communication, an investigation of the electrophoretic separation of several *o,o'*-dihydroxydiphenyl sulphides and methanes and 4-hexylresorcinol, which is found in certain similar pharmaceutical preparations, is described. As these compounds are dibasic acids, the movement achieved electrophoretically will increase with their degree of ionisation. A knowledge of their acid dissociation constants (K_a), therefore, is of value in selecting suitable buffers for effecting separations. In our earlier stability constant work[3] and in the electrophoretic work described here ethanol–water (3:1) solutions have been used, because of the water-insolubility of these compounds. The procedure for determining acid dissociation constants described previ ously[3] involved the use of a constant ionic background (1 M sodium perchlorate) and careful temperature control, titration technique and measurement of pH. Dissociation constants of sufficient accuracy for the present purpose may be obtained by the simple procedure described below.

The value of the dissociation constant is affected quite considerably by the ionic strength of the solution. In the present electrophoretic work buffer solutions of about 0.1 M concentration were used, and therefore the dissociation constants used were determined at an ionic background of 0.1 M sodium perchlorate.

Experimental

Reagents. The sample solution, 0.0065 M solution in ethanol, was prepared as follows: dissolve 6.5×10^{-4} mole of the halogenated *o,o'*-dihydroxydiphenyl sulphide or methane in ethanol (B.P. grade) and dilute the solution to 100 ml in a volumetric flask with ethanol. The standard sodium hydroxide solution, 10^{-1} M in ethanol–water (3:1), was prepared using a B.D.H. concentrated volumetric solution. Sodium perchlorate was used in the form of a 0.4 M solution in water.

Procedure. Add from a burette 37.5 ml of sample solution in ethanol to a 100-ml glass titration vessel or beaker, and add 12.5 ml of sodium perchlorate solution. Titrate the resulting solution with the standard sodium hydroxide solution (added from a

10-ml burette), following the titration potentiometrically using a pH responsive glass electrode and a calomel reference electrode.

All pH values quoted in this paper are strictly pH meter readings, and the values of dissociation constants refer to the ethanol–water (3:1) medium at the particular ionic strength indicated. The pH meter and electrode system was standardised using a 0.05 *M* aqueous solution of potassium hydrogen phthalate (pH 4.008 at 25°).

Estimation of acid dissociation constants. The two protons in the compounds studied react with hydroxyl ion in two quite separate pH regions, and it may be assumed that in the region where the first proton reacts only H_2A and HA^- are present, and in the region where the second proton reacts only HA^- and A^{2-} are present.

The first dissociation constant,

$$K_{a_1} = \frac{[HA^-][H^+]}{[H_2A]}$$

Thus at the half neutralised point $[HA^-] = [H_2A]$,

and

$$pK_{a_1} = (pH)_{[HA^-]=[H_2A]}$$

Similarly, at the half-neutralised point of the second proton,

$$pK_{a_2} = (pH)_{[A^{2-}]=[HA^-]}$$

Values of pK_{a_1} and pK_{a_2} for the *o,o'*-dihydroxydiphenyl sulphides and methanes studied here and for 4-hexylresorcinol are given in Table I. The effect of increasing ionic strength in lowering the values of pK_a is clearly illustrated.

Electrophoresis. Cellulose acetate membranes (Sartorius Membranfilter GmbH, 17 × 5 or 2.5 cm) were used in conjunction with a Gelman electrophoresis tank having 10 cm between supports. A Shandon Vokam stabilised power supply was operated in the constant current mode at 0.1–0.2 mA per cm width and 400 V. Approximately 0.1 *M* buffer solutions in ethanol–water (3:1) were prepared from molar aqueous solutions of sodium acetate and acetic acid, and ammonium acetate and ammonia.

TABLE I

ACID DISSOCIATION CONSTANTS IN ETHANOL–WATER (3:1)

	In 0.1 M sodium perchlorate[a]		*In 1 M sodium perchlorate*[a]		*In 1 M sodium perchlorate*[b]	
	pK_{a_1}	pK_{a_2}	pK_{a_1}	pK_{a_2}	pK_{a_1}	pK_{a_2}
Hexachlorophene	5.6	12.4	5.4	11.8	5.10 ± 0.03	≃ 11.5
Bithionol[c]	5.9	10.5_5	5.7	9.6	5.33 ± 0.02	9.05 ± 0.03
Bromchlorophene	6.4	12.3	6.1	11.6	5.75 ± 0.02	≃ 11.1
Tetrachlorophene	6.7_5	12.2	6.5	11.5	6.10 ± 0.03	≃ 11.0
Fenticlor	8.4	11.8	8.2	11.0	7.68 ± 0.02	10.38 ± 0.03
Dichlorophene	9.1	12.5	8.8	11.9	8.26 ± 0.03	≃ 12.1
4-Hexylresorcinol	11.6_5	12.6	11.2	12.0	10.53 ± 0.05	≃ 12.6

[a] Obtained by the method described in this paper; pK_a values based on pH meter readings.
[b] Obtained by the method described previously; pK_a values based on $-\log [H^+]$.
[c] The values of pK_{a_1} and pK_{a_2} obtained for bithionol without the addition of sodium perchlorate were 6.2 and 11.5.

TABLE II

RESULTS OF ELECTROPHORESIS

	Migration (cm) in 2.5 h	
	pH 6.4	*pH 8.9*
Hexachlorophene	5.3	6.3
Bithionol	4.4	6.4
Bromchlorophene	2.7	5.9
Tetrachlorophene	1.9	6.2
Fenticlor	0.7	4.6
Dichlorophene	0.7	2.5
4-Hexylresorcinol	0.7	0.5

The pH of each buffer was measured with a Pye 290 pH meter and a combined glass–calomel electrode. Minor adjustments to the pH were made where necessary by the addition of small amounts of strong acid or base.

The membranes did not stretch in these buffer solutions.

Aliquots of the compounds studied were applied to the membranes by the microscope cover slip technique 5 cm from the cathode end of the membrane. During the electrophoresis period—up to a maximum of 2.5 h—the voltage dropped slightly

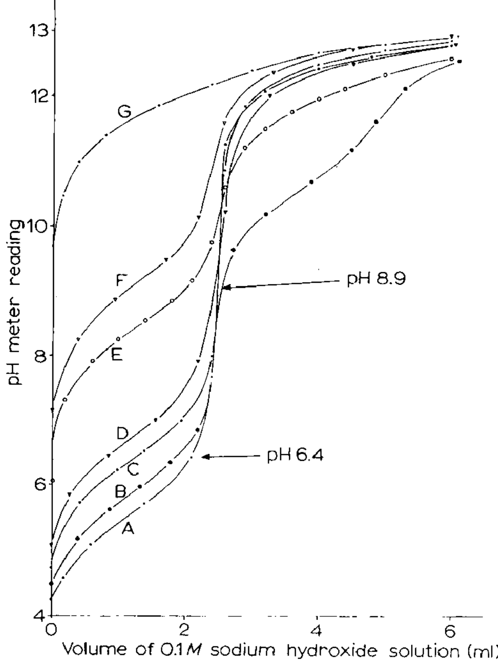

Fig. 1. pH titration curves. A = hexachlorophene; B = bithionol; C = bromchlorophene; D = tetrachlorophene; E = fenticlor; F = dichlorophene; and G = 4-hexylresorcinol.

to about 390 V. When the potential was limited to 400 V evaporation of solvent from the membranes did not exceed an acceptable level.

After electrophoresis the compounds were located by immersing the membrane in a freshly prepared aqueous solution of ferric nitrate and potassium ferricyanide[9]; the membrane was finally washed with water. After drying the membrane at room temperature the compounds appeared as various shades of blue.

Results and discussion

The use of dissociation constants in selecting a buffer suitable for effecting electrophoretic separation of particular compounds is clearly illustrated by reference to the pK_a values given in Table I and to the results of the electrophoretic runs given in Table II. When the titration data are available the selection of buffers can be made more conveniently directly from the titration curves (Fig. 1). Alternatively, the formation curves, $\bar{n}_A$ (= combined proton concentration/total reagent concentration) *versus* pH, can be calculated from the values of the dissociation constants determined at the correct ionic strength. As the two protons in each compound are ionised in quite separate pH regions, the following approximate formulae are valid.

$$\bar{n}_A = \frac{1 + 2\,\text{antilog}\,(pK_{a1} - \text{pH})}{1 + \text{antilog}\,(pK_{a1} - \text{pH})} \qquad \text{for pH} = pK_{a1} \pm 1$$

and

$$\bar{n}_A = \frac{\text{antilog}\,(pK_{a2} - \text{pH})}{1 + \text{antilog}\,(pK_{a2} - \text{pH})} \qquad \text{for pH} = pK_{a2} \pm 1$$

From examination of the titration curves shown in Fig. 1, it was considered that a buffer of pH 6.2 would be most suitable for the separation of hexachlorophene, bithionol, bromchlorophene and tetrachlorophene from each other and from the other compounds. Good electrophoretic separation was effected at this pH, but from an examination of the developed membrane and the titration curves, it seemed possible that the optimum separation could be effected at the slightly higher pH of 6.4. Optimum separation was in fact obtained at this pH.

Similarly, for the separation of fenticlor, dichlorophene and 4-hexylresorcinol, a buffer of pH 8.9 was selected. This proved to give optimum separation.

At all pH values studied in the present work the electrophoresis zones were well defined, with no evidence of adsorption.

In the above separations, the optimum pH in each case proved to be about 0.2 higher than the mean of the pK_{a1} values of the particular group of compounds. Thus, for hexachlorophene, bithionol, bromchlorophene and tetrachlorophene, the mean pK_{a1} value is 6.2 and the optimum pH for separation is 6.4. Similarly, the mean pK_{a1} value of fenticlor and dichlorophene is 8.75 and the optimum pH for separation is 8.9. Electrophoresis at these slightly higher pH values was necessary in order to obtain slightly greater movement of the less ionised compounds.

At pH values below 6.2 bromchlorophene and tetrachlorophene are unresolved, and at pH values just above 6.4 hexachlorophene and bithionol are unresolved. At pH 8.7 dichlorophene is not so well separated from 4-hexylresorcinol as at the optimum pH 8.9, but fenticlor is slightly better separated from the four compounds with pK_{a1} less than 7. At pH values above 9.1 there is less separation of fenticlor from the latter

compounds. All these results are closely similar to those expected from a consideration of the pH titration curves.

For routine use in electrophoretic work, the prior determination of dissociation constants (if these are not already known) can in many cases effect a considerable saving of time, and, because the pH data can be used in conjunction with the electrophoretic data, identification is made more certain. Indeed, the use of pH data makes the electrophoresis less empirical. By consulting a library of titration curves (or formation curves) not only can the optimum pH for electrophoresis be selected readily, but possible ambiguities of identity can be anticipated. It is assumed throughout that adsorption effects and strong ion association with the buffer ions are absent; this was clearly the case in the present work.

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CHROM. 4694

Ion-exchange paper chromatography of inorganic ions

XXVI. The adsorption of $Co(NH_3)_6^{3+}$ and $Co(en)_3^{3+}$ on various cation-exchange papers

In two previous papers[1,2] we have shown that complexes of the type $Co(NH_3)_6^{3+}$ are adsorbed by sulphonic resins with an apparent charge of about +5 (if a law of mass action equation is applied) and moreover that on sulphonic cellulose paper they have a charge of only about +2. Both can be explained if one assumes that the sulphonic groups of the resin (acting as if they were present in solution) form outer-sphere complexes with the cobalt complexes, and the degree of complexing varies with the "concentration" of the sulphonic groups.

This note gives additional data on the adsorption of $Co(NH_3)_6^{3+}$ and $Co(en)_3^{3+}$ on various cation-exchange papers. LiCl was used as eluent in this study, as numerous weak exchangers would not be ionised in HCl solutions. Chromatograms were obtained by ascending development using the Li^+ form of the various exchange papers in small jars at a temperature of 20 ± 1° with aqueous solutions of LiCl (A.R.) prepared by suitably diluting a 10 *N* stock solution. The developed chromatograms were sprayed with ammonium sulphide revealing dark spots for the cobalt complexes.

The "charge" of the complex was obtained graphically by plotting R_M values against log [LiCl], and the tangent of the line should then indicate the charge of the metal ion in a law of mass action equation (see ref. 3).

Results

On sulphonic resin paper, Amberlite SA-2, the R_M *vs.* log [LiCl] plots yield tangents of 3.9 and 3.3 for $Co(en)_3^{3+}$ and $Co(NH_3)_6^{3+}$ which are similar to those obtained with HCl on the same paper (namely 4.6 and 4.75, see ref. 1). This is again an example where the charge is above the theoretical value (which should be below 3).

On cellulose phosphate paper (Whatman No. P20) the tangents are 2.1 and 2.2. The usual hydrated trivalent ions such as Al^{3+} have such values on the Amberlite

TABLE I

THE CHARGE EXHIBITED BY SOME COBALT COMPLEXES ON CATION-EXCHANGE PAPERS

Cation-exchange paper	*Eluent*	*"Apparent charge" or tangent of the R_M vs. log[LiCl] plot for*	
		$Co(NH_3)_6^{3+}$	$Co(en)_3^{3+}$
Amberlite SA-2 paper	HCl	4.75	4.6 (from ref. 1)
Amberlite SA-2 paper	LiCl	3.3	3.9
Cellulose phosphate paper Whatman No. P20	LiCl	2.2	2.1
Cellulose citrate paper Whatman No. CT30	LiCl	1.5	1.5
Carboxymethyl cellulose paper Whatman No. CM50	LiCl	1.2	1.2
Zirconium phosphate paper	LiCl		2.4
Sulphonic acid cellulose paper (Macherey, Nagel and Co., Stark Sauer)	HCl	1.6	1.6 (from ref. 2)

sulphonic papers, and these can be considered to be closest to a "normal" behaviour of trivalent cations. Cellulose citrate paper (Whatman No. CT30) and carboxymethyl cellulose paper (Whatman No. CM50) give tangents of 1.5 and 1.2, respectively, and thus approach those obtained on the cellulose sulphonate paper (Macherey, Nagel and Co.; Stark Sauer)[2].

Zirconium phosphate paper gave rather erratic results perhaps due to a lack of uniformity of the paper employed. The only series of points which can be used to construct a line was obtained with $Co(en)_3^{3+}$ on a paper impregnated with 15% $ZrOCl_2$ (and precipitated with an excess of phosphoric acid). The tangent is 2.4.

The results are summarised in Table I and show that the "charge" exhibited in the law of mass action equation of the equilibrium of the complexes varies with the exchanger and can have (so far) any value from 5 to 1. If we assume that the adsorption of these complexes is due to outer-sphere complexing, these results appear reasonable as various degrees of outer-sphere complexing with various anions have been noted[4].

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News

Meetings

The Eighth International Gel Permeation and Liquid Chromatography Seminar will be held on July 1–3, 1970 at the Institute of Macromolecular Chemistry, Prague, Czechoslovakia. The co-sponsors of the seminar are Institute of Macromolecular Chemistry, Czechoslovakia Academy of Sciences and Water Associates Inc. Invitations for technical papers will be issued shortly by the Molecular Institute, however, due to the early date of the seminar, papers should now be sent to Dr. KOLINSKI, Institute of Macromolecular Chemistry, Prague, Czechoslovakia.

The Ninth International Gel Permeation Chromatography Seminar will be held on October 4–8, 1970 at the Eden Roc Hotel, Miami Beach, Fla., U.S.A. The sponsors of the Seminar will be Water Associates Inc., and you are invited to participate in this seminar by presenting a paper describing your current GPC activities. Such topics as new techniques and applications, data processing, and small molecule investigations will be of particular interest. A set of preprints will be distributed prior to the Seminar, and to meet this schedule, copies of completed manuscripts will be required by June 12th.

We gather that an American Chemical Society introductory course in Gel Permeation Chromatography at the Eden Roc Hotel is planned for October 3.

The Société Belge des Sciences Pharmaceutiques is organizing an International Symposium on Chromatography and Electrophoresis in Brussels on September 14th, 15th and 16th, 1970. Both the theoretical and practical aspects of the techniques will be dealt with.

Everyone interested is cordially invited to participate at this Symposium and to obtain a registration form from the Secretary of the Société Belge des Sciences Pharmaceutiques, Rue Archimede 11, 1040, Brussels, Belgium. The latest date for return of the registration form is August 1st, 1970. Members who intend to contribute a communication or a demonstration are invited to apply before July 1st. Each paper should be only 20 min long, and the official languages are English, French, Dutch and German. The conference fee is 1,500 BF, the fee includes the publication of lectures and communications.

The site of the lectures will be the Faculty of Medicine, The University of Brussels, Boulevard de Waterloo, 115, B-1000 Brussels. Plenary lectures will be given by Madam BEZANGER-BEAUQUESNE, Professor Doct. SCHULT, Professor LARS SVENNERHOLM and Dr. W. J. A. VANDENHEUVEL.

Apparatus

L.K.B. Instruments Ltd., have announced that their area of activity has now been extended to other areas of mass spectrometry, under an agreement signed with Electronic Associates Incorporated, Scientific Instrument Division, Palo Alto, Calif., U.S.A., so that L.K.B. can market in the U.K. and Eire the QUAD line of quadrupole mass spectrometers. The agreement completes the exclusive coverage of these instruments by L.K.B. companies throughout Europe.

For further information apply to the publisher under reference No. Chrom. N-273.

Manufacturers' Literature

A revised brochure "*Eastman Reagents for Protein and Polypeptide Synthesis and Structure Determination*" is available, especially to protein and peptide chemists. Eastman reagents listed in the brochure are volatile and nonvolatile, to enable chemists to determine amino acid sequences in both full peptide chains or peptide fragments.

For further information apply to the publisher under reference No. Chrom. N-271.

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Errata

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p. 274, eqn. 1 should read $F = \bar{t}_G/t_G$.
2nd line below eqn. 1, $\bar{t}_G$ should read t_G.
eqn. 3 should read $F = \bar{t}_S/(t_S + \Delta t)$.
eqn. 5 should read $R = \bar{t}_G/\bar{t}_S$.

J. Chromatog., 47 (1970) 400–407

p. 405, 2nd line up, 1% should be 0.1%.