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1968. 1040 pages. Out of print.
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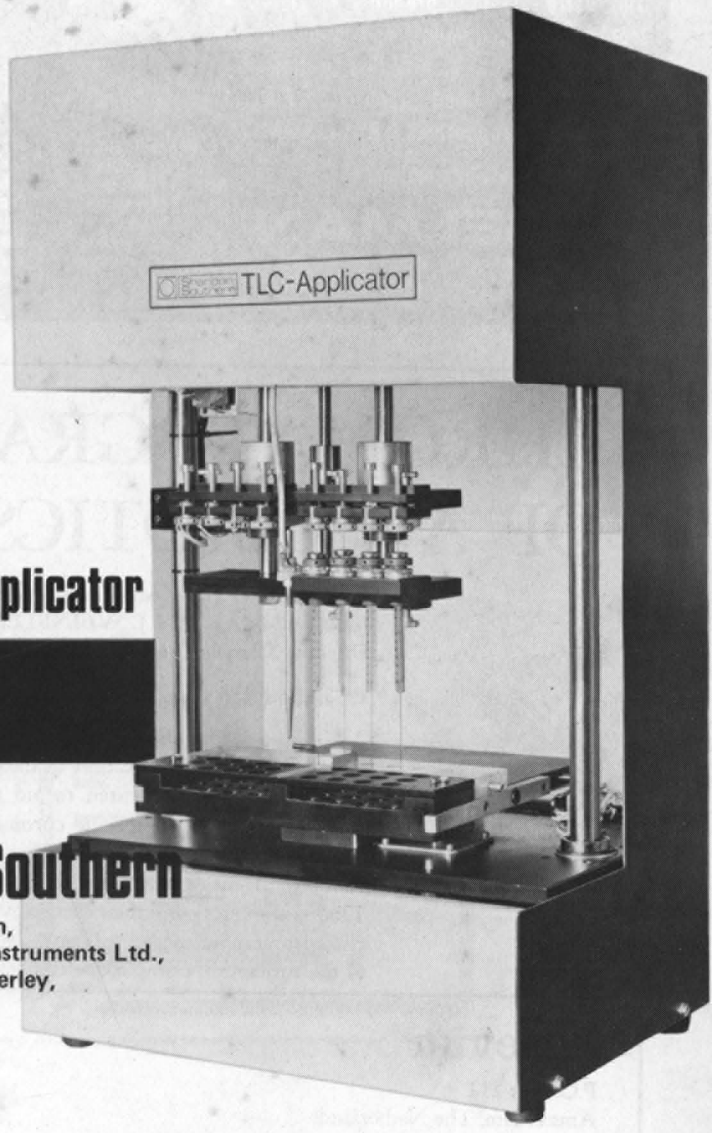

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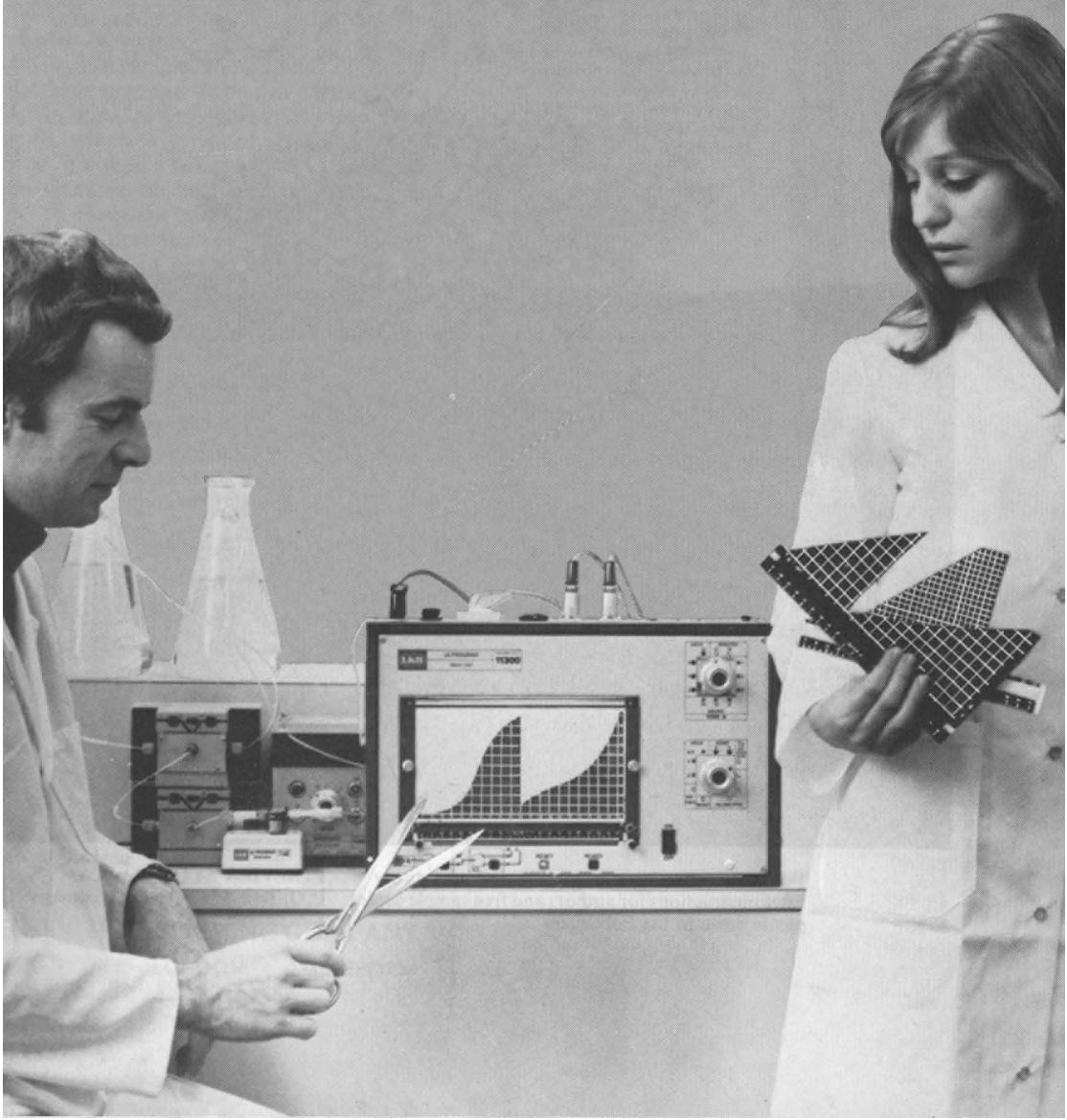

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VOL. 97 1974

Obituary

Prof. Domenico Marotta

On March 20th, 1974, the death occurred in Rome of Prof. Domenico Marotta, founder and first Director of the Istituto Superiore di Sanità, Rome, Italy.

Prof. Marotta was born in 1886 in Palermo and, after completing his studies there, he became assistant to the great Italian chemist Emanuele Paternò.

In 1934, he was offered the Chair of Analytical Chemistry, which he declined in order to become the Director of the newly created Istituto Superiore di Sanità. There he achieved outstanding results in creating in only a few years a world-famous Institute that attracted personalities such as Professor E. B. Chain and Prof. D. Bovet to head research groups.

Of special interest is that one of the early important papers in paper chromatography came from this Institute [D. Cavallini, N. Frontali and G. Toschi, *Nature (London)*, 163 (1949) 568] and that the Institute carried out fundamental studies on the use of chromatography in metabolic studies [F. Pocchiari and C. Rossi, *Chromatogr. Rev.*, 4 (1962) 1] and the chromatography of natural products [G. B. Marini-Bettòlo and G. C. Casinovi, *Chromatogr. Rev.*, 1 (1959) 75].

Of the successors to Prof. Marotta, one is a member of the Editorial Board of this journal and another is the author of a review paper.

The Istituto Superiore di Sanità, in its function as the central institute for Italian government analysts, organised international symposia on thin-layer chromatography, on gel filtration and on the gas chromatography of alkaloids at a time when these techniques were in their development stages and when such symposia were invaluable for the discussion of further developments.

The guest lecturers at the Istituto Superiore di Sanità during recent years have included Prof. A. J. P. Martin, Prof. E. Lederer, Dr. J. Janák, Dr. K. Macek, Dr. L. Fishbein, Dr. K. Sakodinsky and Dr. Z. Deyl, to mention some chromatographers.

That such a centre of scientific interest was possible in Rome was due mainly to the unceasing interest and great organising ability and the kindhearted hospitality of Prof. Marotta. In his last years, after retiring from the Institute, Prof. Marotta dedicated himself to the publications of the Società Chimica Italiana and here again he achieved much that helped to put Italian chemistry on the map.

From 1912, when he became Docente at the University of Rome, until his death, *i.e.*, for 62 years, Prof. Marotta was continuously active in some aspect of Italian chemistry. With his death, Italy loses one of its outstanding men of science.

MICHAEL LEDERER

Journal of Chromatography, 97 (1974) 3–13

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RETENTION INDICES OF BIPHENYLS AND DIPHENYLALKANES

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(Received May 7th, 1974)

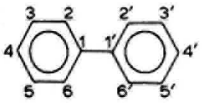
SUMMARY

The retention indices of biphenyl and all isomeric methyl-, ethyl-, isopropyl- and dimethylbiphenyls and those of several diisopropylbiphenyls, diphenylalkanes and stilbenes were measured on three capillary columns with Apiezon L, polyphenyl ether and SP 1000 at temperatures of 170, 185 and 200°.

The position of substitution has the greatest influence, while the influence of the size of the substituents is secondary. An equation that enables the retention indices (I) of polysubstituted homologues to be estimated is proposed. The relationship between the position of a substituent and the H'-factor, compound structure and ΔI value is discussed. A plot of the dependence of I on a non-polar phase showed the consistency of the chromatographic data and served for the estimation of the I values of several biphenyl homologues.

INTRODUCTION

Studies of the chromatographic behaviour of biphenyl-type hydrocarbons are concerned with theoretical, analytical and applicational aspects. Biphenyl can also be considered as a substituted benzene compound, and there are certain analogies in the behaviour of the two substances. On the other hand, only the biphenyl system involves the free rotation of two phenyl rings around a connecting bond, and the substitution of an alkyl group on the biphenyl molecule affects the mutual positions of the two rings. The sterically sensitive positions 2-, 6-, 2′- and 6′- are especially interesting in this respect. In gas–liquid chromatography (GLC), the substitution of a methyl group on these positions causes only a minor change in the retention time[1,2]. The increment per methyl group in a *meta*-position is comparable with the increment per carbon atom in the case of *n*-paraffins. For *para*-substitution, the figure is higher.



The analysis of biphenyls is of particular interest when they are identified in various hydrocarbon mixtures such as petroleum fractions, tars and petrochemical residues and intermediates[3–10]. In such materials they are usually accompanied by hydrocarbons of other types, which must be removed to some extent before GLC.

In this paper, the relationship between the structures and elution data of biphenyl, three isomeric methylbiphenyls, three ethylbiphenyls, three isopropylbiphenyls, all twelve dimethylbiphenyls, diphenylmethane, three methyldiphenylmethanes, two diphenylethanes and some stilbenes are considered.

EXPERIMENTAL

Chromatographic data were measured on three capillary columns with different polarities for aromatic hydrocarbons, expressed by means of McReynolds constants[11]. The stationary phase had to be resistant to operating temperatures of 170–200°, and possibly even higher. As the phase of lowest polarity, Apiezon L was chosen (McReynolds constant measured for benzene, $X' = 32$; maximum temperature, 250°), the phase of medium polarity was polyphenyl ether (5 rings) ($X' = 176$; maximum temperature, 200°) and the most polar phase was SP 1000 ($X' = 332$; maximum temperature, 275°)[12].

Apparatus

The standard Chrom 2 apparatus (Laboratorní Přístroje, Prague, Czechoslovakia) with flame-ionization detection was used, with nitrogen as the carrier gas. All analyses were carried out isothermally on stainless-steel capillary columns, length 50 m, I.D. 0.25 mm. A 1-μl Hamilton syringe was used for injection.

The operating conditions for the column with Apiezon L (Metropolitan-Vickers, Great Britain) were as follows: temperature, 170° and nitrogen flow-rate 0.42 ml/min; 185° and nitrogen flow-rate 0.33 ml/min; and 200° and nitrogen flow-rate 0.25 ml/min; the column efficiency was 55 000 theoretical plates. The operating conditions for the column with polyphenyl ether (5 ring) (Consol. Vac. Co.) were as follows: temperature 170° and nitrogen flow-rate 0.38 ml/min; 185° and nitrogen flow-rate 0.33 ml/min; and 200° and nitrogen flow-rate 0.25 ml/min; the column efficiency was 75 000 theoretical plates. The operating conditions for the column with SP 1000 (Supelco, Bellefonte, Pa., U.S.A.) were as follows: temperature 170° and nitrogen flow-rate 0.51 ml/min; and 185° and nitrogen flow-rate 0.34 ml/min; the column efficiency was 70 000 theoretical plates.

Most of the hydrocarbons of the biphenyl and diphenylmethane series were prepared in our laboratory[13,14]. 1,2-Diphenylethane was prepared by the hydrogenation of *trans*-stilbene. The stilbenes were of commercial origin, *viz.*, *trans*-stilbene (Lachema, Brno, Czechoslovakia), *cis*-stilbene (K & K Labs., Plainview, N.Y., U.S.A.) and α-methylstilbene (Koch-Light, Colnbrook, Great Britain).

Procedure

In order to measure the retention indices, seven mixtures of standards were prepared so as to have the individual components well separated chromatographically on all phases and at all temperatures. *n*-Paraffins with retention times corresponding to the given mixture and column used were added to the mixtures. The Kováts reten-

tion indices were calculated from the results of three measurements corrected for the column dead volume determined from the retention time of methane. The calculations were carried out using a Hewlett-Packard 9100 A electronic calculator. The reproducibility of the retention indices varied within a range of one unit for the Apiezon L and polyphenyl ether columns and within a range of three units for the SP 1000 column.

RESULTS AND DISCUSSION

The retention indices measured are shown in Table I. It is clear that the retention indices of biphenyls are predominantly influenced by the position of substitution, while the influence of the size of the substituents is secondary. The first compounds

TABLE I

RETENTION INDICES

Compound	*Symbol*	*Apiezon L*			*Polyphenyl ether*			*SP 1000*	
		200°	*185°*	*170°*	*200°*	*185°*	*170°*	*185°*	*170°*
Biphenyl	BP	1475	1459	1446	1737	1719	1705	2078	2029
2-Methylbiphenyl	2-MBP	1464	1449	1438	1709	1694	1683	2000	1960
3-Methylbiphenyl	3-MBP	1571	1556	1545	1833	1812	1801	2167	2120
4-Methylbiphenyl	4-MBP	1587	1570	1558	1845	1824	1811	2182	2134
2,3-Dimethylbiphenyl	2,3-DMBP	1588	1574	1562	1842	1827	1811	2137	2100
2,4-Dimethylbiphenyl	2,4-DMBP	1569	1556	1545	1813	1800	1784	2100	2063
2,5-Dimethylbiphenyl	2,5-DMBP	1557	1544	1535	1800	1788	1774	2086	2050
2,6-Dimethylbiphenyl	2,6-DMBP	1489	1477	1465	1721	1705	1693	1983	1948
3,4-Dimethylbiphenyl	3,4-DMBP	1713	1697	1683	1981	1962	1946	2324	2279
3,5-Dimethylbiphenyl	3,5-DMBP	1667	1652	1641	1928	1912	1898	2252	2211
2,2′-Dimethylbiphenyl	2,2′-DMBP	1471	1458	1446	1700	1685	1671	1950	1916
2,3′-Dimethylbiphenyl	2,3′-DMBP	1548	1536	1526	1797	1780	1768	2074	2037
2,4′-Dimethylbiphenyl	2,4′-DMBP	1570	1556	1546	1818	1800	1787	2100	2061
3,3′-Dimethylbiphenyl	3,3′-DMBP	1669	1655	1642	1928	1910	1895	2254	2212
3,4′-Dimethylbiphenyl	3,4′-DMBP	1683	1668	1656	1941	1922	1906	2267	2224
4,4′-Dimethylbiphenyl	4,4′-DMBP	1697	1681	1669	1952	1933	1916	2281	2236
2-Ethylbiphenyl	2-EBP	1513	1500	1488	1757	1743	1729	2033	1994
3-Ethylbiphenyl	3-EBP	1647	1633	1621	1909	1892	1877	2234	2190
4-Ethylbiphenyl	4-EBP	1678	1661	1647	1938	1920	1903	2268	2220
2-Isopropylbiphenyl	2-IPBP	1524	1512	1500	1763	1750	1738	2015	1980
3-Isopropylbiphenyl	3-IPBP	1687	1671	1660	1939	1923	1909	2244	2204
4-Isopropylbiphenyl	4-IPBP	1730	1713	1700	1986	1968	1953	2300	2255
3,5-Diisopropylbiphenyl	3,5-DIPBP				2077	2066			
3,3′-Diisopropylbiphenyl	3,3′-DIPBP				2132	2119			
3,4′-Diisopropylbiphenyl	3,4′-DIPBP				2191	2175			
4,4′-Diisopropylbiphenyl	4,4′-DIPBP				2239	2223			
Diphenylmethane	DPM	1503	1488	1477	1774	1756	1745	2092	2047
2-Methyldiphenylmethane	2-MDPM	1601	1583	1570	1868	1850	1835	2179	2135
3-Methyldiphenylmethane	3-MDPM	1591	1576	1565	1862	1847	1834	2168	2128
4-Methyldiphenylmethane	4-MDPM	1611	1594	1582	1876	1859	1844	2185	2144
1,1-Diphenylethane	1,1-DPE	1548	1532	1522	1818	1800	1790	2116	2074
1,2-Diphenylethane	1,2-DPE	1593	1576	1563	1862	1841	1827	2171	2126
1,4-Diphenylbutane	1,4-DPBu	1810	1793	1780	2085	2066	2052	2398	2355
trans-Stilbene	*t*-ST	1803	1784		2109	2088	2071	2547	
cis-Stilbene	*c*-ST	1585	1570		1866	1847	1832	2205	
α-Methylstilbene	α-MST	1776	1760		2071	2053	2038	2446	

to be eluted are the *ortho*-substituted biphenyls, followed by the *meta*-substituted and finally the *para*-substituted derivatives. As with benzenes substituted in adjacent positions[15], the elution time of analogous biphenyls is prolonged: in the dimethylbiphenyl series in which one substituent is in the *ortho*-position, the longest elution time is that of 2,3-dimethylbiphenyl. Of the *meta*- and *para*-substituted derivatives, 3,4-dimethylbiphenyl is eluted last. The number of biphenyl homologues eluted before biphenyl itself increases with increase in phase selectivity. On the Apiezon L column, only 2-methylbiphenyl has a shorter elution time than biphenyl on polyphenyl ether, 2,2′-dimethylbiphenyl and 2,6-dimethylbiphenyl are also eluted before biphenyl, and on SP 1000, 2-ethylbiphenyl, 2-isopropylbiphenyl and 2,3′-dimethylbiphenyl are further eluted before biphenyl.

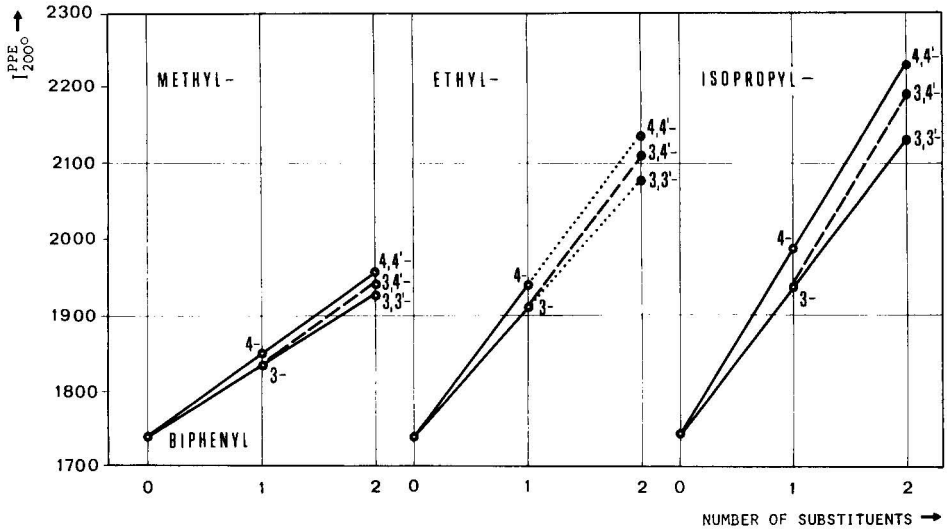


Fig. 1. Plot of retention indices *versus* number of substituents.

An interesting relationship follows from the plot of retention indices as a function of the number of carbon atoms in the substituent alkyl groups, in the modified form of a plot of the dependence of retention indices on the number and size of the substituents (Fig. 1). This plot shows a linear dependence of retention times on the number of substituents for *meta*- and *para*-substituted biphenyls, which can be expressed in general terms by the following equation:

$$I_{X_i} = k \cdot p + I_{BP} \tag{1}$$

where

I_{X_i} = retention index of mono-substituted biphenyls in position 3 or 4 and of biphenyls substituted on both rings in positions 3,3′ or 4,4′;
I_{BP} = retention index of biphenyl;
k = slope of the straight line;
p = number of substituents.

The equation can be used, for example, to calculate the retention indices of *meta*- and *para*-substituted biphenyls if the retention index of biphenyl and of its mono-substituted derivative is known. In this case the equation is transformed into:

$$I_{X_1} = 2\,(I_{X_2} - I_{BP}) + I_{BP} = 2\,I_{X_1} - I_{BP} \qquad (2)$$

where I_{X_1} and I_{X_2} are the retention indices of mono- or disubstituted biphenyls, respectively. The mean deviation of the retention index measured from the value calculated is 2 for dimethylbiphenyls and 6 for diisopropylbiphenyls, for all columns and temperatures.

3,4′-Disubstituted biphenyls are eluted approximately mid-way between the homologues substituted in positions 3,3′- and 4,4′- and for a given column, this position is a function of the temperature. When a column with polyphenyl ether was operated at 200°, the difference between the retention indices for the pairs 4-methylbiphenyl–3-methylbiphenyl and 4,4′-dimethylbiphenyl–3,4′-dimethylbiphenyl was the same, and the straight line passing through points 3-MBP and 3,4′-DMBP is thus parallel to the line connecting 4-MNP and 4,4′-DMBP (Fig. 1). The retention index of the 3,4′-disubstituted derivative can then be expressed analytically by the equation

$$I_{3,4'} = I_{4,4'} - (I_4 - I_3) \qquad (3)$$

and applying eqn. 2 adjusted for calculating $I_{4,4'}$ disubstituted:

$$I_{4,4'} = 2\,I_4 - I_{BP} \qquad (2a)$$

eqn. 3 is transformed into

$$I_{3,4'} = I_4 + I_3 - I_{BP} \qquad (4)$$

Eqn. 4 also contains eqn. 2 and can be used to estimate the retention indices of 3,4′-disubstituted biphenyls. The average deviation of the actually measured retention index from the calculated value over all columns and temperatures for 3,4′-dimethylbiphenyl is 2. The average deviation of the calculation of the retention index of 3,4′-diisopropylbiphenyl at two temperatures on a column of polyphenyl ether is 3.5. Eqn. 4 can also be used for the estimation of the retention indices of multi-substituted biphenyls or of biphenyls with different substituents according to the expression

$$I_{RS} = I_R + I_S - I_{BP}$$

where I_R, I_S and I_{RS} are retention indices of biphenyls with a substituent R in one ring only, a substituent S in one ring only and a substituent R in one ring and S in the other ring, respectively.

Variation of the retention index with temperature

The changes in retention indices with temperature are shown in Table II for the hydrocarbons studied on all three stationary phases. The values denoted by $\delta I/10°$ characterize the increase in the retention index with a 10° increase in temperature. The $\delta I/10°$ values increase with increasing selectivity of the stationary phase: the increment I for biphenyl on Apiezon L is 9.7 and on polyphenyl ether 10.8; the

TABLE II

VARIATION OF RETENTION INDEX WITH TEMPERATURE

Compound	$\delta I^{API}/10°$	$\delta I^{PPE}/10°$	$\delta I^{SP\ 1000}/10°$
Biphenyl	9.7	10.7	32.7
2-Methylbiphenyl	8.7	8.7	26.7
3-Methylbiphenyl	8.7	10.7	31.3
4-Methylbiphenyl	9.7	11.3	32.0
2,3-Dimethylbiphenyl	8.7	10.3	24.7
2,4-Dimethylbiphenyl	8.0	9.7	24.7
2,5-Dimethylbiphenyl	7.3	8.7	24.0
2,6-Dimethylbiphenyl	8.0	9.3	23.3
3,4-Dimethylbiphenyl	10.0	11.7	30.0
3,5-Dimethylbiphenyl	8.7	10.0	27.3
2,2′-Dimethylbiphenyl	8.3	9.7	22.7
2,3′-Dimethylbiphenyl	7.3	9.7	24.7
2,4′-Dimethylbiphenyl	8.0	10.3	26.0
3,3′-Dimethylbiphenyl	9.0	11.0	28.0
3,4′-Dimethylbiphenyl	9.0	11.7	28.7
4,4′-Dimethylbiphenyl	9.3	12.0	30.0
2-Ethylbiphenyl	8.3	9.3	26.0
3-Ethylbiphenyl	8.7	10.7	29.3
4-Ethylbiphenyl	10.3	11.7	32.0
2-Isopropylbiphenyl	8.0	8.3	23.3
3-Isopropylbiphenyl	9.0	10.0	26.7
4-Isopropylbiphenyl	10.0	11.0	30.0
3,5-Diisopropylbiphenyl	—	7.3	—
3,3′-Diisopropylbiphenyl	—	8.7	—
3,4′-Diisopropylbiphenyl	—	10.7	—
4,4′-Diisopropylbiphenyl	—	10.7	—
Diphenylmethane	8.7	9.7	30.0
2-Methyldiphenylmethane	10.3	11.0	29.3
3-Methyldiphenylmethane	8.7	9.3	26.7
4-Methyldiphenylmethane	9.7	10.7	27.3
1,1-Diphenylethane	8.7	9.3	28.0
1,2-Diphenylethane	10.0	11.7	30.0
1,4-Diphenylbutane	10.0	11.0	28.7
trans-Stilbene	12.7	12.7	—
cis-Stilbene	10.0	11.3	—
α-Methylstilbene	10.7	11.0	—

highest increment of 32.7 occurs on the SP 1000 column. The variation in I with temperature is influenced primarily by the type and position of substitution, the influence of the number of substituents being secondary. For sterically hindered biphenyls substituted on positions 2 and 6, the increment is lower although the order of minimum increments on each of the three phases is not the same. This is valid for the columns of Apiezon L and SP 1000. The largest increment in I on SP 1000 is shown by biphenyl, on polyphenyl ether by 4,4′-dimethylbiphenyl and on Apiezon L by 2-methyldiphenylmethane. The highest increments are in general shown by *para*-substituted biphenyls and stilbenes. In individual cases, operation at different temperatures can be used in order to separate certain pairs in small groups. For example, two pairs eluted together at 185°, *i.e.* 3,4′-dimethylbiphenyl–4-ethylbiphenyl and 3-methylbiphenyl–3-methyldiphenylmethane, on a column of SP 1000, show a differ-

ence of 4 and 8 I units, respectively, at 170°. The three methyldiphenylmethanes are well separated on a polyphenyl ether column at 200°. By reducing the temperature, the difference in I between 2-methyldiphenylmethane and 3-methyldiphenylmethane is decreased to such an extent that at 170° the two compounds are eluted together. In general, however, the variations in the retention index increments with temperature are not sufficiently great for the individual hydrocarbons of the biphenyl and diphenylmethane series to be of help in their resolution. For hydrocarbons with condensed rings, higher $\delta I/10°$ variations were found than in the case of aromatic compounds with isolated rings, *i.e.*, biphenyls and diphenylalkanes. This result, which becomes more marked on a column with higher polarity, could be of more substantial use in the analysis of both types of compounds.

Relationship between the substituent position and the H'-factor

The H-factor is defined as the difference between the retention index of the biphenyl and the I value of an n-paraffin with the same number of carbon atoms. If we relate the H-factor to biphenyl, we can write $H'_{BP} = 0$ and obtain the expression

$$\begin{aligned} H' = H - H_{BP} &= I_{BP(z)} - 100\,z - H_{BP} \\ &= I_{BP(z)} - I_{BP} - 100\,(z - 12) \end{aligned} \tag{5}$$

where z is the number of carbon atoms in the molecule. The H'-factor expresses the change in the retention index of the investigated hydrocarbons brought about by the introduction of an alkyl group into the biphenyl configuration.

The values calculated from eqn. 5 range from +43 to −270 and are listed in Table III in descending order. The H'-factor decreases to such an extent that the biphenyl–stationary phase interaction is weakened in the case of sterically hindered *ortho*-substituted biphenyls. Homologues with most limited rotation of the rings around their connecting bond, resulting from the introduction of either two small alkyl groups or one large alkyl group, have the lowest values. Relatively smaller changes in the H'-factor in the negative sense are caused by the transition from a purely aromatic system to an aliphatic–aromatic system by an increase in the number of alkyl groups or their size. We can assume that the alkyl groups located in *meta*-positions in particular help to reduce the H'-factor in this manner. Only methylbiphenyls and dimethylbiphenyls substituted in *para*-position have positive values. The highest H'-factor occurs with the *ortho*-substituted 3,4-derivative. An increase in the size of the alkyl group has a negative influence on the H'-factor in the case of *para*-substituted derivatives also. In the series 4-methyl-, 4-ethyl- and 4-isopropyl-, its values move from positive through zero to negative. For the hydrocarbons studied, a linear dependence of the H'-factor on the number of substituents occurs, as shown in Fig. 2. The H'-values for diethylbiphenyls were calculated on the basis of retention indices assessed with the help of eqn. 2.

Dependence of ΔI on compound structure

The quantity ΔI is defined as the difference between the retention indices of a substance on a polar and a non-polar phase, or on phases with different selectivities with respect to the eluted substance.

When three columns are used, three combinations of the difference in retention

TABLE III

DEPENDENCE OF H'-FACTOR ON SUBSTITUTION

Results obtained on polyphenyl ether at 185°.

Compound	H'	*Number of aliphatic carbon atoms*	*Position*
3,4-Dimethylbiphenyl	43	2	*m,p**
4,4′-Dimethylbiphenyl	14	2	*p,p′*
4-Methylbiphenyl	5	1	*p*
3,4′-Dimethylbiphenyl	3	2	*m,p′*
4-Ethylbiphenyl	0	2	*p*
Biphenyl	0	0	—
3-Methylbiphenyl	−7	1	*m*
3,5-Dimethylbiphenyl	−7	2	*m,m*
3,3′-Dimethylbiphenyl	−9	2	*m,m′*
3-Ethylbiphenyl	−27	2	*m*
4-Isopropylbiphenyl	−51	3	*p*
2,3-Dimethylbiphenyl	−92	2	*o,m**
4,4′-Diisopropylbiphenyl	−96	6	*p,p′*
3-Isopropylbiphenyl	−96	3	*m*
2,4′-Dimethylbiphenyl	−119	2	*o,p′*
2,4-Dimethylbiphenyl	−119	2	*o,p*
2-Methylbiphenyl	−125	1	*o*
2,5-Dimethylbiphenyl	−131	2	*o,m*
2,3′-Dimethylbiphenyl	−139	2	*o,m′*
2-Ethylbiphenyl	−176	2	*o*
2,3′-Diisopropylbiphenyl	−200	6	*m,m′*
2,6-Dimethylbiphenyl	−214	2	*o,o*
2,2′-Dimethylbiphenyl	−234	2	*o o′*
2-Isopropylbiphenyl	−269	3	*o*

* *Vicinal*-substitution.

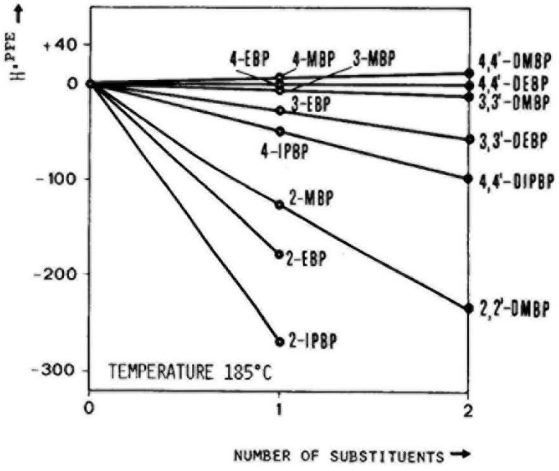


Fig. 2. Dependence of H'-factor on number of substituents for polyphenyl ether columns.

indices are possible at each temperature. At the same time, the values obtained by measuring ΔI for biphenyl at 185° are approximately double the difference in the McReynolds constants (X') for benzene on the individual phases[11]. Thus, for the pair SP 1000–polyphenyl ether, the values are $X' = 156$ and $\Delta I_{\text{biphenyl}} = 359$. For the pair polyphenyl ether–Apiezon L, $X' = 144$ and $\Delta I_{\text{biphenyl}} = 260$. The largest difference in retention indices is encountered on columns with the largest difference in polarity, *i.e.*, SP 1000–Apiezon, where $X' = 300$ and $\Delta I_{\text{biphenyl}} = 619$.

Table IV lists the ΔI values calculated for all three cases at 185°. The order of the individual components according to the decreasing values $\Delta I^{\text{SP 1000–API}}$ and $\Delta I^{\text{SP 1000–PPE}}$ is similar to the order for H'-factors. The *ortho*-substituted derivatives are the lowest, followed by the *meta*-substituted and finally the *para*-substituted derivatives. The ΔI value is also reduced with increasing size of the alkyl group. In the case of the $\Delta I^{\text{PPE–API}}$ values, a relative increase in the ΔI values for *meta*-substituted hydrocarbons was found. The usual order *ortho* < *meta* < *para* alters for methyl derivatives into *ortho* < *para* < *meta*, which is also illustrated in reverse order for

TABLE IV

ΔI VALUES

SP 1000–API L		*SP 1000–PPE*		*PPE–API L*	
Compound	ΔI	*Compound*	ΔI	*Compound*	ΔI
2,2′-DMBP	492	2,2′-DMBP	265	2,2′-DMBP	227
2-IPBP	503	2-IPBP	265	2-IPBP	228
2,6-DMBP	506	2,6-DMBP	278	2,6-DMBP	238
2-EBP	533	2-EBP	290	2-EBP	243
2,3′-DMBP	538	2,3-DMBP	294	2,5-DMBP	244
2,5-DMBP	542	2,5-DMBP	298	2,4-DMBP	244
2,4-DMBP	544	2,4-DMBP	300	2,3′-DMBP	244
2,4′-DMBP	544	2,4′-DMBP	300	2,4′-DMBP	244
2-MBP	551	2-MBP	306	2-MBP	245
2,3-DMBP	563	2,3-DMBP	310	3-IPBP	252
3-IPBP	573	1,1-DPE	316	4,4′-DMBP	252
1,1-DPE	584	3-IPBP	321	2,3-DMBP	253
4-IPBP	587	3-MDPM	321	3,4′-DMBP	254
4-MDPM	591	4-MDPM	326	4-MBP	254
3-MDPM	592	2-MDPM	329	3,3′-DMBP	255
1,2-DPE	595	1,2-DPE	330	4-IPBP	255
2-MDPM	596	4-IPBP	332	3-MBP	256
3,4′-DMBP	599	DPM	336	3-EBP	259
3,3′-DMBP	599	3,5-DMBP	340	4-EBP	259
4,4′-DMBP	600	3-EBP	342	BP	260
3,5-DMBP	600	3,3′-DMBP	344	3,5-DMBP	260
3-EBP	601	3,4′-DMBP	345	4-MDPM	265
DPM	604	4,4′-DMBP	348	1,2-DPE	265
4-EBP	607	4-EBP	348	3,4-DMBP	265
3-MBP	611	3-MBP	355	2-MDPM	267
4-MBP	612	4-MBP	358	DPM	268
BP	619	*c*-ST	358	1,1-DPE	268
3,4-DMBP	627	BP	359	3-MDPM	271
c-ST	635	3,4′-DMBP	362	*c*-ST	277
α-MST	686	α-MST	393	α-MST	293
t-ST	763	*t*-ST	459	*t*-ST	304

the dimethyl-substituted compounds 4,4′-DMBP < 3,4′-DMBP < 3,3′-DMBP. In this series, diphenylalkanes have the highest $\Delta I^{\mathrm{PPE-API}}$ values, whereas in the remaining two cases they ranged approximately in the centre of the alkylbiphenyl series. Of the whole series of investigated substances, stilbenes have the highest difference in retention indices on two phases.

Plot of retention indices on two phases

By plotting the retention indices on two different phases, a diagram is obtained that enables, in some instances, minute details in the configuration of the investigated substances to be established[16]. In another case it may serve as an estimate of the retention indices of further members of homologous series[17] or to characterize individual positions of substitution[18].

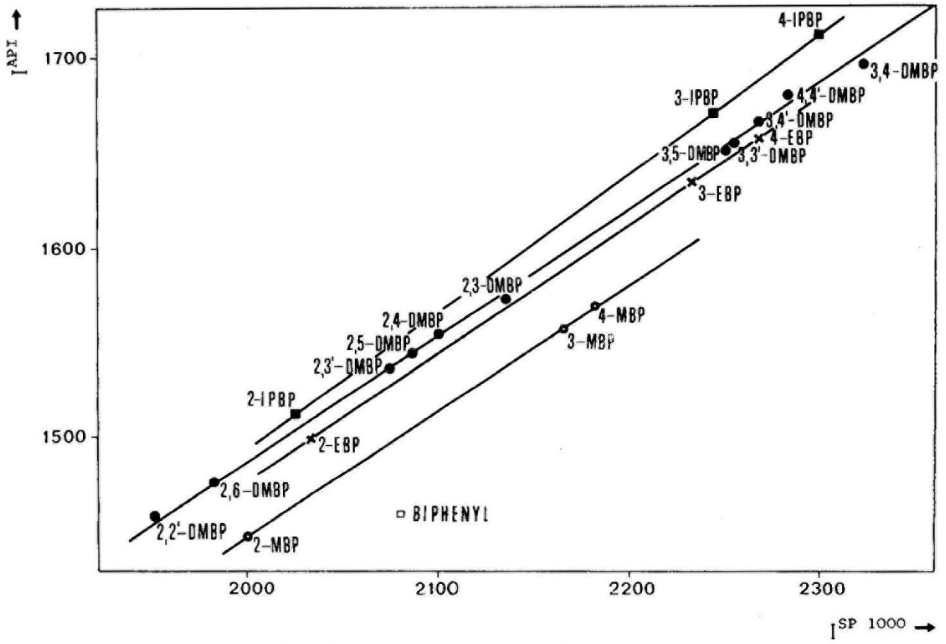


Fig. 3. Plot of retention indices for substituted biphenyls on Apiezon L column *versus* SP 1000 column at 185°. ○, Methylbiphenyls; ●, dimethylbiphenyls; ×, ethylbiphenyls; ■, isopropylbiphenyls.

A most interesting relationship appears when plotting the retention indices for the Apiezon L column against the *I* values on the SP 1000 column (Fig. 3). The plot shows a straight-line dependence for biphenyls substituted with the same alkyl group, *i.e.* methyl, ethyl and isopropyl, and of biphenyls substituted with two of the same alkyl group, as in the case of dimethylbiphenyls. Biphenyl, located below the group of straight lines, has a unique position.

The straight lines can be expressed analytically by the following general equation:

$$I^{\mathrm{API}} = k \cdot I^{\mathrm{SP\ 1000}} + q \qquad (6)$$

where k is the slope of the straight line and q is the intercept on the I^{API} axis.

Both constants were calculated for all straight lines with the help of the method of least squares together with the correlation factor (r), indicating the extent to which the points measured comply with the equation calculated. The values are given in Table V.

TABLE V

PARAMETERS OF RELATIONSHIP I^{API} *versus* $I^{SP\,1000}$ (ACCORDING TO EQN. 6)

Biphenyls	*Slope of the straight line*	*Intercept on the* I^{API} *axis*	*Correlation factor*
Methyl-	0.6544	140.0	0.99961
Dimethyl-	0.6572	174.5	0.99892
Ethyl-	0.6761	125.2	0.99965
Isopropyl-	0.7002	101.0	0.99991

The calculated values listed in Table V show that the angles formed by the straight lines and the axis do not differ by more than 2°. Their distance from the point indicating biphenyl increases with increasing substitution. The plot clearly indicates that for better separation of the individual components, it is advantageous to use a polar column or a column with a high Rohrschneider or McReynolds constant for benzene.

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CHROM. 7563

APIEZON L AS A STATIONARY PHASE FOR THE DETERMINATION OF ACTIVITY COEFFICIENTS BY LIQUID–LIQUID CHROMATOGRAPHY

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SUMMARY

The use of a stationary phase of high molecular weight in the determination of activity coefficients at infinite dilution by liquid–liquid chromatography (LLC) is described.

With Apiezon L as the stationary phase, the problem of mutual solubility with the mobile phase is overcome. Activity coefficients of hydrocarbons in acetonitrile and aniline determined by LLC are in good agreement with those reported in the literature.

INTRODUCTION

Liquid–liquid chromatography (LLC) is useful in the determination of thermodynamic quantities, for example activity coefficients, in those cases where gas–liquid chromatography (GLC) fails, that is when volatile solvents are used as the stationary phase. However, the activity coefficients obtained by LLC are higher than those found by other techniques.

In earlier work[1,2], we ascribed these discrepancies mainly to the mutual, even if slight, solubilities of the two solvents used as stationary and mobile phases. There are two means of lowering these mutual solubilities: decreasing the temperature (as discussed in previous work[2]), or changing the stationary phase. As the solubilities of the compounds of a homologous series in a pure solvent generally decrease with increasing molecular weight, in this work we have studied the variation of activity coefficients determined by LLC with changes in the molecular weight of the stationary phase.

EXPERIMENTAL

The experiments were carried out with an LLC apparatus of our own construction. The whole system was thermostatically controlled at 25 $\pm$ 0.01°. The separation columns were made of stainless steel with a length of 2 m and an I.D. of 4 mm, while the pre-columns were 1 m long. The packings for both columns were 25% (w/w) of stationary phase (*n*-hexadecane, squalane, Apiezon L) supported on 60–80

mesh Chromosorb P. The mobile phases (aniline, acetonitrile) were deaerated before use. The analyses of the fractions were carried out by GLC. As the main column pressure was close to 1 atm, the pressure term (A) in eqn. 1 (see later) was not considered because its value was negligible.

The GLC apparatus was a thermal conductivity chromatograph (Fractovap Model B, Carlo Erba, Milan, Italy). The eluent used was high-purity hydrogen. The stainless-steel pre-columns and columns were 2 m long and 4 mm I.D., packed with 25% (w/w) of stationary phase supported on 60–80 mesh Chromosorb P. The stationary phases were Apiezon L, squalane saturated with aniline and *n*-hexadecane saturated with aniline, saturation being achieved by equilibrating the two solvents at 25°. The various quantities appearing in eqn. 2, which gives the activity coefficients, were evaluated according to refs. 3 and 4. The solvents and solutes used in the LLC and GLC tests were reagent-grade products (Fluka, Buchs, Switzerland and BDH, Poole, Great Britain) with a minimum purity of 99.5 mole-%.

RESULTS AND DISCUSSION

By combining LLC and GLC, it is possible to determine the activity coefficients of solutes in polar solvents (mobile phase in LLC) using as the stationary phase in both GLC and LLC an apolar solvent of high molecular weight, for example, a paraffin (*n*-hexadecane, squalane, etc.). However, even for these solvents, the influence of the mutual solubilities is considerable, as confirmed by the results obtained using acetonitrile[5] and aniline as mobile phases (Tables I and II). In fact, the values obtained by LLC in these solvents should be considered as the activity coefficients of the solutes not in the pure solvents but rather in the saturated solvents, because the solvents as used in LLC are saturated, as shown in previous work[2].

TABLE I

COMPARISON BETWEEN ACTIVITY COEFFICIENTS IN ANILINE AT 25° DETERMINED BY LLC USING SQUALANE ($\ln \gamma_{i_{Sq}}^{\infty,A}$), *n*-HEXADECANE ($\ln \gamma_{i_{Hex}}^{\infty,A}$) AND APIEZON L ($\ln \gamma_{i_{AL}}^{\infty,A}$) AS STATIONARY PHASES AND THOSE IN THE LITERATURE[3] ($\ln \gamma_i^{\infty,A}$)

Compound	$\ln \gamma_i^{\infty,A}$	$\ln \gamma_{i_{Sq}}^{\infty,A}$	$\ln \gamma_{i_{Hex}}^{\infty,A}$	$\ln \gamma_{i_{AL}}^{\infty,A}$
n-Hexane	3.17	3.19	3.26	3.17
n-Heptane	3.40	3.44	3.46	3.41
n-Octane	3.67	3.68	3.70	3.67
1-Hexene	2.50	2.51	2.53	2.50
1-Heptene	2.72	3.74	2.76	2.72
1-Octene	2.94	2.99	3.01	2.93
Cyclohexane	2.54	2.56	2.59	2.55
Methylcyclohexane	2.83	2.86	2.87	2.83
Ethylcyclohexane	3.10	3.10	3.13	3.10
Benzene	0.79	0.82	1.04	0.80

In particular, Table I reports the activity coefficients of solutes in aniline as obtained by LLC in combination with the GLC determination of the activity coefficients of the same solutes in the stationary phases saturated with aniline (see eqn. 1). From this table, it can be seen that the increase in molecular weight of the stationary

TABLE II

COMPARISON BETWEEN ACTIVITY COEFFICIENTS IN ACETONITRILE AT 25° DETERMINED BY LLC USING SQUALANE[5] ($\ln \gamma_{i_{Sq}}^{\infty,ACN}$) AND APIEZON L ($\ln \gamma_{i_{AL}}^{\infty,ACN}$) AS STATIONARY PHASES AND THOSE IN THE LITERATURE[6,7] OBTAINED BY OTHER METHODS ($\ln \gamma_i^{\infty,ACN}$)

Compound	$\ln \gamma_i^{\infty,ACN}$	$\ln \gamma_{i_{Sq}}^{\infty,ACN}$	$\ln \gamma_{i_{AL}}^{\infty,ACN}$
n-Pentane	3.01 (ref. 6)	3.06	3.00
n-Hexane	3.24 (ref. 6)	3.42	3.24
n-Heptane	3.51 (ref. 7)	3.74	3.52
n-Octane		4.11	3.96
1-Pentene	2.24 (ref. 6)	2.24	2.24
1-Hexene		2.66	2.52
1-Heptene	2.70 (ref. 7)	3.00	2.73
1-Octene		3.37	3.04
Cyclohexane		3.20	3.05
Methylcyclohexane		3.46	3.34
Ethylcyclohexane		3.78	3.62
Benzene	0.99 (ref. 6)	1.12	1.02
Toluene		1.51	1.40
Ethylbenzene		1.83	1.75

phase has a positive influence: the discrepancy between the experimental results and those reported in the literature decreases on passing from *n*-hexadecane to squalane.

Therefore, a stationary phase with a higher molecular weight must be chosen, such as paraffin waxes, greases and polymers. When polymers are employed, the adsorption phenomena and the risk of swelling by the action of the mobile phase give rise to some difficulties.

For all the above substances, however, there is the problem of the lack of a well defined molecular weight (M_s), which interferes in the determination both of the ratios of activity coefficients by LLC[5] and of the activity coefficients of the stationary phase by GLC[3]:

$$\ln \left(V_{g_i}^0\right)_{\text{LLC}} = \ln \left(\frac{\gamma_i^{m,\infty}}{\gamma_i^{s,\infty}}\right) + \ln \left(\frac{M_m}{M_s \varrho_m}\right) + A \tag{1}$$

$$\ln \gamma_i^{s,\infty} + \ln M_s = \ln \omega_s = \ln \left(\frac{273\ R}{(V_{g_i}^0)_{\text{GLC}}\ P_i^0}\right) + B \tag{2}$$

where $V_{g_i}^0$ is the retention volume, M_m and M_s are the molecular weights of the mobile and stationary phase, ϱ_m is the density of the mobile phase, P_i^0 is the vapour pressure of pure solute, A is the pressure correction term and B is the correction for nonideality in gas phase.

This difficulty can be overcome by determining, by GLC, the quantities $\gamma_i^{s,\infty} M_s = \omega_s$ instead of the activity coefficients ($\gamma_i^{s,\infty}$). Considering eqns. 1 and 2 and taking into account $\gamma_i^{s,\infty} M_s = \omega_s$, we obtain

$$\ln \gamma_i^{m,\infty} = \ln (V_{g_i}^0)_{\text{LLC}} - A + \ln \left(\frac{\varrho_m}{M_m}\right) + \ln \omega_s \tag{3}$$

Consequently, as the retention quantities are known from LLC and the term $\ln \omega_s$ from GLC, it is possible to calculate the activity coefficients of the solutes in the mobile phase.

According to eqn. 3, the activity coefficients of different hydrocarbons in aniline were determined by LLC using Apiezon L as stationary phase in GLC and LLC (Table I). The results obtained by LLC agree exactly with those found by GLC or by other methods[3], as confirmed by the complete insolubility between Apiezon L and aniline.

Apiezon L was then used for the determination of activity coefficients in acetonitrile, for which only a few values have been reported in the literature. The values so determined were compared with the "apparent activity coefficients" obtained by Locke[5] using squalane as the stationary phase and, where possible, with the values in the literature that had been obtained by other methods[6,7]. The agreement between the literature values and the values obtained by LLC using Apiezon L as stationary phase was very good.

It follows that an increase in the molecular weight of the solvent used as the stationary phase has the same effect as a decrease in the operating temperature: in both instances the mutual solubility decreases so that the activity coefficients in pure solvents can be determined directly.

It can therefore be stated that Apiezon L can be used successfully as a stationary phase in LLC with all polar solvents that are insoluble in it; this opens a new and interesting method, complementary to GLC, for the determination of activity coefficients.

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GAS–LIQUID CHROMATOGRAPHY OF N-HEPTAFLUOROBUTYRYL ISOBUTYL ESTERS OF AMINO ACIDS*

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SUMMARY

The N-heptafluorobutyryl isobutyl esters of the protein amino acids have been separated by gas–liquid chromatography using a single column of 3% SE-30 on Gas-Chrom Q. The separation is superior to that of previous single-column methods and is particularly suitable for the analysis of plant seed proteins. The method is also applicable to glycoproteins.

INTRODUCTION

The analysis of amino acids by gas–liquid chromatography (GLC) can be performed only after the conversion of the amino acids to suitably volatile and stable derivatives. The most extensively investigated derivatives have been the esters and acyl derivatives of the polar groups. Due largely to the work of Gehrke and his associaties[1–4], a procedure was developed for the separation and quantitation of the protein amino acids as the N-trifluoroacetyl butyl esters using two columns and two different liquid phases[5]. Subsequently, the same amino acid derivatives were separated in about 45 min using a single column[6]. An excellent single-column separation of the protein amino acids has also been achieved by Moss *et al.*[7], using the N-heptafluorobutyryl (HFB) *n*-propyl esters. More recently, Zanetta and Vincendon demonstrated the separation of the N-HFB isoamyl esters using a single-column procedure and showed that the method could be applied to acid-hydrolysed proteins or glycoproteins[8].

In our studies of seed proteins we have used both of the above methods but have found that each method presents certain disadvantages. Seed proteins commonly contain substantial amounts of aspartic and glutamic acids but relatively little methionine[9]. However, the amount of methionine in a seed protein is frequently of considerable interest. In the method of Moss *et al.*[7] the accurate quantitation of a small methionine peak close to a large aspartic acid peak is difficult. Similarly, using the method of Zanetta and Vincendon[8], the quantitation of lysine and tyrosine is made difficult by the proximity of a large glutamic acid peak. We have found that these problems are avoided by using the N-HFB isobutyl esters, the single-column GLC separation of which we now report.

* NRCC No. 14189.

EXPERIMENTAL

Reagents

Standard amino acid mixtures were obtained from Hamilton (Whittier, Calif., U.S.A.) and heptafluorobutyric anhydride was obtained from Eastman-Kodak (Rochester, N.Y., U.S.A.).

Methanol, isobutyl alcohol and ethyl acetate were refluxed for 3–4 h over magnesium turnings, decanted, refluxed for 3–4 h over calcium hydride and redistilled from an all-glass apparatus. Dry HCl gas was added to methanol and isobutyl alcohol, in the appropriate amounts, after passing the gas through a sulfuric acid tower. The normality was verified by titration. The reagents were stored at −20°.

Preparation of derivatives

All derivatization steps were performed in a 1-ml Reactivial (Pierce, Rockford, Ill., U.S.A.). All evaporations were performed using a stream of dry nitrogen at room temperature. All residues were dispersed into solution by placing in an ultrasonic bath for 30 sec.

Inter-esterification

Samples containing 0.05–0.25 μM of each amino acid were dried either by freeze drying or under a stream of dry nitrogen. The last traces of water were removed azeotropically using methylene chloride. The residue was dissolved in 200 μl of methanol–1.25 N HCl and placed in an ultrasonic bath for 30 sec. After 30 min at room temperature the reagent was evaporated, 200 μl methylene chloride was added and again evaporated. The residue was dissolved in 200 μl of isobutyl alcohol–1.25 N HCl and the vial heated at 110° for 150 min in an oven. After cooling the reagent was evaporated. The residue was dissolved in 50 μl of ethyl acetate, 20 μl of heptafluorobutyric anhydride was added and the vial was heated for 10 min at 150° in an oil bath. The vial was then thoroughly cooled, the reagent evaporated just to dryness and the residue was dissolved in an appropriate volume of ethyl acetate. An appropriate volume of the sample was injected along with acetic anhydride in the sample to anhydride ratio of 2:1.

Direct esterification

Direct esterification, using the procedure of Roach *et al.*[10], was also performed. Samples containing 0.05–0.25 μM of each amino acid were dried. The residue was dissolved in 200 μl of isobutyl alcohol–3 N HCl. After heating at 110° for 1 h and evaporating the excess reagent, the sample was acylated as described above.

Chromatography

All analyses were performed using a Hewlett-Packard, Model 7611 gas chromatograph equipped with dual-flame ionisation detectors. The column packing (3% SE-30 on Gas-Chrom Q, 100–200 mesh) was obtained from Applied Science Labs., State College, Pa., U.S.A.). Pyrex columns (11–12 ft. × 2.5 mm I.D., thin-walled) were filled with the stationary phase by gentle tapping under suction and conditioned overnight with a carrier gas (nitrogen) flow-rate of 30 ml/min. The chromatographic conditions were as follows: temperature program, 90°–240° at 4°/min; injector tem-

perature, 250°; detector temperature, 280°; air flow-rate, 300 ml/min; hydrogen flow-rate, 30 ml/min; nitrogen flow-rate, 30 ml/min. Quantitation was performed using an Infotronics Model CRS 208 automatic digital integrator.

RESULTS AND DISCUSSION

The separation of the N-HFB isobutyl esters of the protein amino acids shown in Fig. 1 and the retention temperatures shown in Table I represent the results obtained by using a temperature program of 4°/min. However, the separation was only marginally decreased by programming at 6°/min and a complete analysis could thus be obtained in less than 30 min. The order of elution of the amino acids was identical to that observed for the N-HFB propyl esters by Moss *et al.*[7]. However, methionine was completely separated from aspartic acid and phenylalanine was completely separated from glutamic acid. The order of elution of the N-HFB isobutyl esters is somewhat different from that of the N-HFB isoamyl esters[8]. The main difference is in the separation of lysine, tyrosine and glutamic acid. The removal of glutamic acid from close proximity to lysine and tyrosine is important for the accurate quantitation of

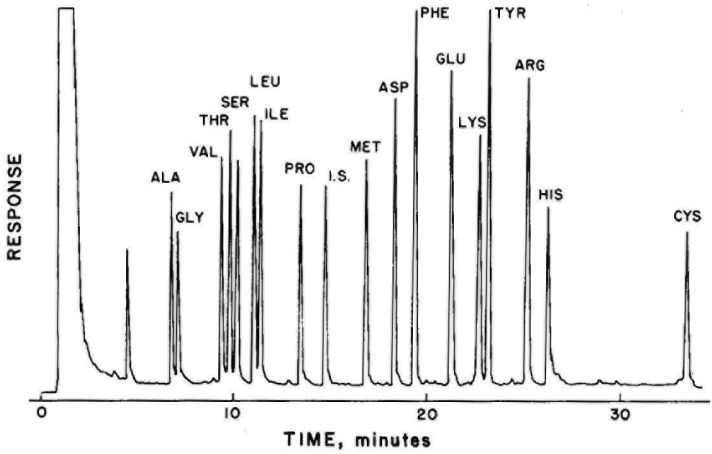

Fig. 1. Gas chromatogram of the N-HFB isobutyl esters of the protein amino acids. I.S. = internal standard, pipecolic acid used in an amount of 0.8 mole per mole of each of the other amino acids. The other peaks each represent 0.0075 μM of amino acid. GLC conditions are given in the text.

TABLE I

RETENTION TEMPERATURES OF THE N-HEPTAFLUOROBUTYRYL ISOBUTYL ESTERS OF AMINO ACIDS

Amino acid	R_T (°C)	*Amino acid*	R_T (°C)	*Amino acid*	R_T (°C)
Alanine	119.0	Histidine	204.5	Pipecolic acid	153.5
Arginine	200.0	Hydroxyproline	158.5	Proline	148.0
Aspartic acid	170.0	Isoleucine	138.5	Serine	133.5
Cysteine	149.0	Leucine	137.0	Threonine	132.0
Cystine	236.5	Lysine	189.5	Tyrosine	191.5
Glutamic acid	183.0	Methionine	163.5	Valine	130.5
Glycine	120.5	Phenylalanine	175.0		

these amino acids. This is particularly important when analysing proteins such as plant proteins which contain substantial amounts of glutamic acid. The flow-rate was not critical for the separation of any of the amino acids. These points are illustrated by the chromatographic separation of the amino acids from a typical seed source shown in Fig. 2.

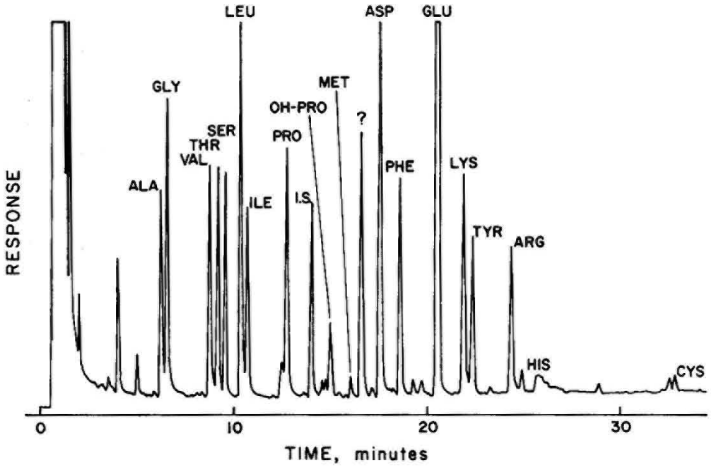

Fig. 2. Gas chromatogram of the N-HFB isobutyl esters of the amino acids in an acid hydrolysate of defatted mustard seed (*Brassica hirta* Moench). I.S. = internal standard. Temperature program, 95°–240° after a 1-min hold. Other GLC conditions are given in the text. No acetic anhydride was injected.

The relative molar responses of the N-HFB isobutyl esters of the protein amino acids relative to that of the corresponding derivative of the internal standard, pipecolic acid, are shown in Table II. The responses obtained by the direct esterification procedure compared favourably with those obtained by the more tedious procedure of inter-esterification. Since, for most of the amino acids maximum or near maximum relative responses were obtained after 1 h of direct esterification, this time was used for routine amino acid analysis. This procedure appeared to be the best compromise between the most rapid derivatisation and optimum response. The variability of the relative molar responses was less than $\pm$ 2%.

Zanetta and Vincendon[8] experienced loss of the more volatile components during evaporation when concentrating the *n*-butyl trifluoroacetyl and the *n*-propyl heptafluorobutyryl derivatives. We have not experienced this problem but we have routinely performed all evaporations in the production of these derivatives at room temperature. However, when using the isoamyl-HFB derivatives we have experienced losses of alanine and glycine when evaporations were performed at 50° and 80° as directed[8]. We therefore evaporated at room temperature after methylation and at 40° after inter-esterification. For the N-HFB isobutyl ester method, no loss of the more volatile derivatives was observed when all evaporations were performed at room temperature. Unlike the N-HFB isoamyl esters of the amino acids, the N-HFB isobutyl esters were not more soluble in acetonitrile than in ethyl acetate. The latter

TABLE II

RELATIVE MOLAR RESPONSE OF AMINO ACID DERIVATIVES AS A FUNCTION OF ESTERIFICATION TIME AND DERIVATISATION PROCEDURE*

Amino acid	*Direct esterification*				*Inter-esterification*	
	15 min	*30 min*	*60 min*	*120 min*	*60 min*	*150 min*
Ala	0.63	0.63	0.59	0.59	0.50	0.67
Gly	0.55	0.54	0.49	0.49	0.43	0.50
Val	0.83	0.85	0.85	0.85	0.75	0.85
Thr	0.89	0.88	0.87	0.87	0.84	0.87
Ser	0.73	0.73	0.74	0.74	0.70	0.75
Leu	0.99	0.98	0.98	0.98	0.93	0.99
Ile	0.90	1.00	1.00	1.01	0.96	1.04
Pro	0.84	0.84	0.83	0.83	0.82	0.79
Met	0.93	0.94	0.94	0.94	0.90	0.92
Asp	1.15	1.18	1.18	1.18	1.17	1.17
Phe	1.43	1.45	1.45	1.45	1.43	1.38
Glu	1.30	1.32	1.34	1.37	1.30	1.30
Lys	1.22	1.23	1.24	1.24	1.20	1.22
Tyr	1.48	1.50	1.50	1.50	1.49	1.50
Arg	1.28	1.31	1.41	1.42	1.46	1.40
His	0.82	0.84	0.87	0.89	0.88	0.75
Cys	0.60	0.60	0.60	0.61	0.84	0.57

* Molar response relative to the internal standard pipecolic acid. Each value represents an average of at least six determinations.

solvent was therefore used as the acylation solvent to minimise the preparation of anhydrous solvents.

The chromatography of the N-HFB isobutyl ester of histidine was similar to that of the N-HFB propyl ester[7]. A sharp peak was observed only when the sample was co-injected with acetic anhydride and thus, presumably, on-column acylation took place[11]. The difficulties in derivatising histidine were confined to the acylation step as the isobutyl ester was readily prepared in a large-scale derivatisation which was monitored by methods such as infrared spectroscopy. We have not been able to chromatograph histidine as the isoamyl ester, unlike the observations of Zanetta and Vincendon[8], without co-injecting with acetic anhydride. The derivatisation of histidine was observed to be particularly sensitive to the presence of water in any of the solvents or reagents.

The acyl derivatives of glucosamine and galactosamine had retention temperatures of respectively 146.5° and 149° and the method would thus appear to be applicable, without modification, to the analysis of the protein moieties of glycoproteins.

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HIGH-PRESSURE LIQUID CHROMATOGRAPHY OF CARBOHYDRATES

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SUMMARY

A procedure is described for the separation of water-soluble wood polysaccharides on Bio-Glas (granular porous glass) and Bio-Gel P (polyacrylamide) packed columns using high-pressure liquid chromatography (HPLC). An example of HPLC employing non-aqueous solvent systems and EM Gel OR-PVA (vinyl acetate copolymer) type packing materials is also discussed.

INTRODUCTION

A technique is described for the separation and isolation of some water-soluble wood polysaccharides and low-molecular-weight carbohydrates using high-pressure liquid chromatography (HPLC). The polysaccharides surveyed include arobinogalactans from western larch (*Larix occidentalis*), water-soluble polysaccharides from loblolly pine (*Pinus taeda*) and a synthetic polymer formed by thermal polymerization of methyl-α-D-glucopyranoside.

EXPERIMENTAL

Apparatus and materials

A Waters Ass. (Framingham, Mass., U.S.A.) liquid chromatograph (Model 202) equipped with a 1000 p.s.i. pumping system and both ultraviolet and differential refractometer detectors was employed. All chromatograms were obtained at room temperature.

Bio-Gel P-2 and P-60 (polyacrylamide gel) and Bio-Glas 500 (granular porous glass) packing materials were obtained from Bio-Rad Labs., Rockville Center, N.Y., U.S.A. EM Gel OR-PVA 500 (vinyl acetate copolymer) packing material was obtained from EM Labs., Elmsford, N.Y., U.S.A.

Molecular weight standards (dextran polymers) were purchased from Pharmacia, Uppsala, Sweden.

Column preparation

Stainless-steel columns, 4 ft. × 3/8 in., were used for both Bio-Gel and Bio-Glas materials while a column 2 ft. × 1/8 in. was employed for the EM Gel packing.

Granular Bio-Gel was suspended in water and allowed to swell overnight. The water was then decanted off in order to remove any fine particles. Sufficient fresh water was added to form a thick slurry, which was then poured slowly into the column. Subsequently, the column was attached to a solvent output line on the liquid chromatograph and allowed to purge with the solvent for ½ h. The process of adding packing material and purging was repeated until the column was completely packed.

Porous Bio-Glas was suspended in water in a stoppered Büchner funnel, and a vacuum was applied. When all bubbling of the slurry had ceased, the column was packed in an analogous manner to the Bio-Gel column.

As EM Gel OR-PVA materials can be used only with non-aqueous solvents, this packing was allowed to swell in methanol overnight. After clamping the 2 ft. × 1/8 in. column in a vertical position, a vacuum was applied at the lower end while a slurry of the packing material was slowly introduced at the top. Final pressure packing of the column was accomplished by using a high-pressure pump. Furthermore, to prevent foreign matter from being introduced into the UV or refractometer cells, all newly packed columns were allowed to purge with appropriate solvents for 3 h before being connected to the inlet lines of the detectors.

Analysis of the carbohydrate materials

The polysaccharides were hydrolyzed and the hydrolyzate was silylated using the procedure of Sweeley *et al.*[1]. The silylated monosaccharides were separated and identified by gas–liquid chromatography (GLC) and quantitatively determined using an electronic integrator as previously described by Laver *et al.*[2]. The value of each sugar was corrected for any loss due to decomposition and for addition of a water molecule during hydrolysis.

Preparation of samples

Water-soluble polysaccharides from loblolly pine[3]. Boards, cut from the sapwood of a green loblolly pine (*Pinus taeda*) log, were ground into small chips and passed through a Wiley mill equipped with a 60-mesh screen. The sapwood meal (*ca.* 1500 g) was then placed in two large containers and extracted with distilled water (4 l) for 24 h at room temperature. The two solutions were filtered and the aqueous filtrates combined. A rotary evaporator was employed to concentrate the aqueous filtrate to a thick, dark brown syrup. This material was labeled total, cold-water-soluble fraction. Fehling's reagent was then added to an aqueous solution of this syrup, causing a light green material to precipitate. The precipitate was collected and washed with a large volume of distilled water and then allowed to air dry. Decomposition of this polysaccharide–copper complex was performed by adding it to ice-cold (0°) ethanol containing hydrochloric acid (5%, v/v). The precipitate was collected by filtration and washed successively with ethanol, acetone and diethyl ether. This procedure was repeated three times to yield a purified galactoglucomannan.

The Fehling's-soluble fraction from above was deionized with MB-3 ion exchanger and filtered. The filtrate was then concentrated to a small volume and added to an excess of ethanol. The white precipitate which formed was washed successively with ethanol, acetone and diethyl ether. This material was labeled Fehling's-soluble fraction.

Water-soluble polysaccharides from western larch[4]. Small blocks were cut from

a dry sample board of larch. These blocks were then planed into chips and passed through a Wiley mill. The resultant meal was placed in a Soxhlet unit and exhaustively extracted successively with benzene, benzene–ethanol (1:1) and ethanol. After the sample had been air dried, it was likewise extracted with distilled water (24 h, 20°). The aqueous extract was filtered, concentrated to a small volume and added dropwise to a four-fold excess of denatured ethanol. The fluffy white precipitate which formed was collected, washed sequentially with ethanol, acetone and diethyl ether and dried in a vacuum oven.

Stachyose hydrate (lupeose; α-D-galactosyl-α-D-glucosyl-β-D-fructose), D(+)-cellobiose and D(−)-ribose samples. These materials were obtained in a highly pure grade from Sigma, St. Louis, Mo., U.S.A.

Products from thermal polymerization of methyl-α-D-glucopyranoside. A small sample of methyl-α-D-glucopyranoside was introduced into the heating chamber (initial temperature 310°) of a Chromalytics MP-3 thermal analyzer while the chamber was being purged with nitrogen. The reaction was allowed to proceed isothermally at 310° for 10 min, at which time the sample was removed and dissolved in a small amount of water.

The above temperature and reaction time were chosen because earlier studies had shown that methyl-α-D-glucopyranoside would polymerize under these conditions[5].

RESULTS AND DISCUSSION

Fig. 1 shows the separation of the two water-soluble arabinogalactans from larch on a Bio-Glas 500 packed column. This packing material withstood high pressures well; hence larger flow-rates were employed. Sharp, well separated peaks were obtained for the polymers on Bio-Glas, which illustrated that a random molecular weight distribution did not exist. Subsequently, each component was isolated from the liquid chromatograph and characterized by GLC. The results, which are shown in Table I, agree well with previously reported values for these compounds[6–8].

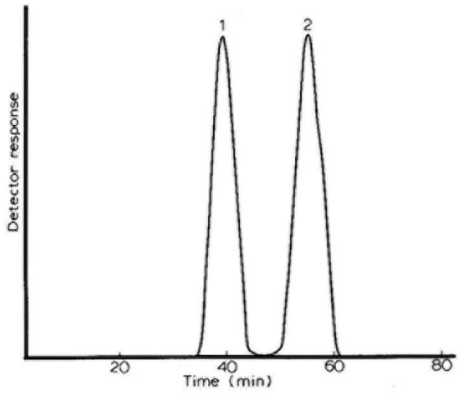

Fig. 1. HPLC refractive index chromatogram of larch arabinogalactans. Column: Bio-Glas 500 (500 Å pore diameter), 4 ft. × 3/8 in., stainless steel. Solvent: water. Flow-rate: 0.9 ml/min. Pressure: 275 p.s.i. (1) Arabinogalactan, $M_w > 100{,}000$; (2) arabinogalactan, $M_w = 18{,}195$.

TABLE I

ANALYSIS OF LARCH ARABINOGALACTANS

Polymer	*Molar ratio (Arab/Galac)*		*Molecular weight*	
	Reported[7,8]	*Found*	*Reported*[6]	*Found*
Component 1	4.3	4.14	100,000	⩾100,000
Component 2	3.8	3.38	16,000	18,195

Molecular weights ($\bar{M}_w$ and $\bar{M}_n$ values) for the two water-soluble arabinogalactans were obtained by making standard runs of dextran polymers on the Bio-Glas 500 column. Apparently, owing to the relative inertness of the porous glass beads, little if any affinity interaction took place between the column and dextran standard. Hence, molecular weights were determined accurately on this column except in the region near the void volume (molecular weight operating range of column = 10,000–100,000). The high-molecular-weight component eluted very close to the void volume of the column; therefore, little fundamental information about its polydispersity or molecular weight could be obtained. However, the other component had a much lower molecular weight, so characterization was easy. This species possessed a polydispersity of 2.16 and $\bar{M}_n$ and $\bar{M}_w$ values of 8405 and 18,195, respectively.

Fig. 2 shows the separation of the total, cold-water-soluble polysaccharides on a Bio-Gel P-60 packed column. The Bio-Gel materials were found to compress very easily under high pressure so a low flow-rate had to be employed. The total elution time was slightly over 4 h.

After introducing large samples of total, cold-water-soluble material via the 2-ml loop injector, fractions were isolated from the total run (see Fig. 2) and analyzed by GLC. The results indicated the presence of an arabinose-, mannose- and galactose-containing polymer, a galactoglucomannan and large amounts of glycerol. One frac-

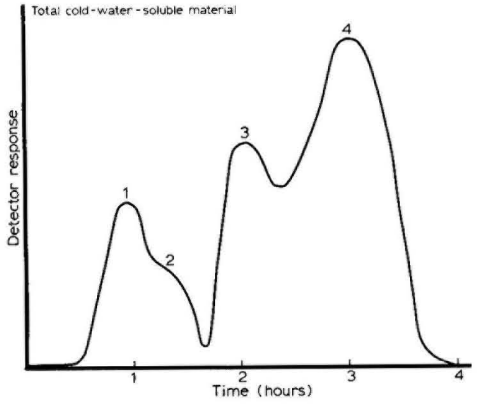

Fig. 2. HPLC refractive index chromatogram of total, cold-water-soluble material. Column: 4 ft. × 3/8 in., stainless steel. Bio-Gel P-60 packing material. Solvent: water. Flow-rate: 0.3 ml/min. (1) Unknown; (2) galactoglucomannan; (3) arabinose-, mannose- and galactose-containing polymer; (4) glycerol.

tion (peak 1) appeared to be a mixture and, because its elution volume was so near the void volume of the column, it could not be resolved any further.

As stated previously, the total, cold-water-soluble material was chemically fractionated using Fehling's solution. The Fehling's-insoluble fraction (copper complex precipitate) yielded a galactoglucomannan similar to that reported by Jones and Painter[9] and by Timell[10] ($[\alpha]_D^{25°} = -19.6°$). Acid hydrolysis and GLC analysis confirmed the presence of galactose, glucose and mannose in the ratio 1.1:1.0:3.8. The periodate consumption was determined spectrophotometrically[11] and found to be 0.98 mole/anhydro unit, while the formic acid liberation was 0.19 mole/anhydro unit.

The Fehling's-soluble fraction afforded a white material containing arabinose, mannose and galactose in the ratio 1.0:1.0:2.7. This material was homogeneous by HPLC criteria and further chemical purification with Fehling's solution failed to alter its mannose content. This polysaccharide appeared completely different from arabinogalactan material reported from larch[4] and Monterey pine[12] and even lobolly pine[3] total wood. A complete structural analysis, which is beyond the scope of this paper, would be necessary in order to confirm this material as being a true arabinogalactan species.

Fig. 3 shows the Fehling's products as they appeared on a Bio-Gel P-60 column. Scan A and B of the Fehling's-insoluble fraction were run in order to illustrate the utility of liquid chromatography in monitoring a chemical purification. Chromatogram A shows the cold-water-soluble galactoglucomannan contaminated with an unknown component (peak 1). After repeated precipitation with Fehling's solution, the galactoglucomannan was resolved into one peak (peak 2), exclusive of any contaminating material (scan B). Peaks 1 and 2 are analogous to those on the chromatogram of the total, cold-water-soluble material. The Fehling's-soluble fraction contained three compounds corresponding to peaks 1, 3 and 4 on the chromatogram of the total, cold-water-soluble material. Components generating peaks 3 and 4 were easily characterized, but peak 1, which was caused by a high-molecular-weight species,

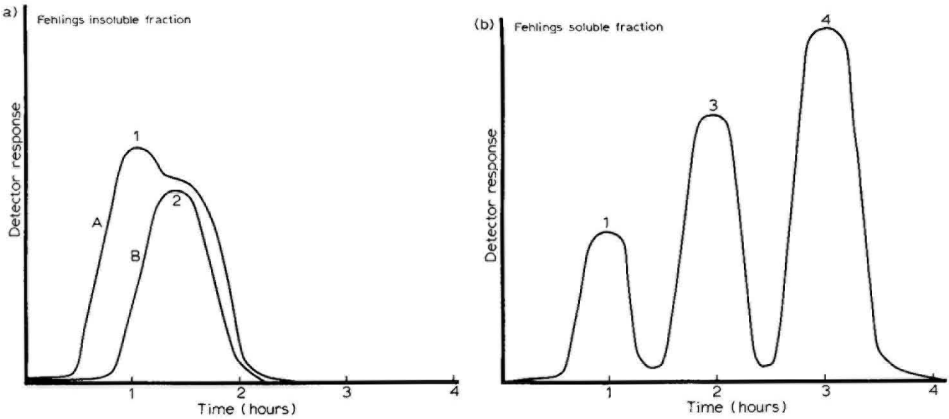

Fig. 3. HPLC refractive index chromatograms of Fehling's-insoluble (a) and Fehling's-soluble (b) materials. Column: Bio-Gel P-60, 4 ft. × 3/8 in., stainless steel. Solvent: water. Flow-rate: 0.3 ml/min. A, unpurified Fehling's-insoluble fraction; B, purified Fehling's-insoluble fraction. (1) Unknown; (2) galactoglucomannan; (3) arabinose-, mannose- and galactose-containing polymer, (4) glycerol.

could not be further purified. As stated earlier, it eluted at the void volume of the column. A Bio-Gel P series column possessing a higher molecular weight operating range would have resolved this material, however.

Fig. 4 illustrates the separation of some low-molecular-weight carbohydrate materials when a non-aqueous solvent system was employed. Again, the packing material was easily compressed at high pressures (high flow-rates), so a low flow-rate had to be used.

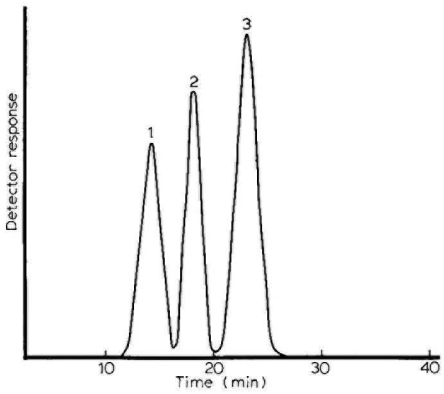

Fig. 4. HPLC refractive index chromatogram of some low-molecular-weight carbohydrates. Column: EM Gel OR-PVA 500, 2 ft. × 1/8 in., stainless steel. Solvent: methanol. Flow-rate: 0.3 ml/min. (1) Stachyose hydrate; (2) D(+)-cellobiose; (3) D(−)-ribose.

Resolution of peaks on the EM Gel column was excellent. And, although a number of compounds were surveyed, the separation of tetrasaccharides from disaccharides and monosaccharides seemed to be one of the greatest assets of the column.

In order to study further the utility of the Bio-Gel packing materials, polymerization products from a thermal decomposition reaction of methyl-α-D-glucopyranoside were investigated. Fig. 5 shows the initial and final products as well as the intermediate compounds formed in the polymerization process.

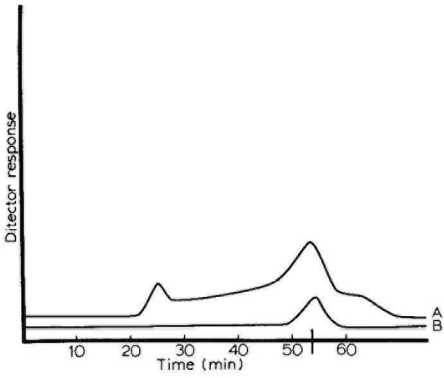

Fig. 5. HPLC refractive index chromatogram of products from thermal polymerization of methyl-α-D-glucopyranoside. Column: Bio-Gel P-2, 4 ft. × 3/8 in. Solvent: water. Flow-rate: 0.9 ml/min. A, Total reaction mixture; B, methyl-α-D-glucopyranoside.

The first peak eluted from the Bio-Gel P-2 column was the highest-molecular-weight compound or "compounds" in the reaction mixture. For this particular reaction, the first peak of scan A corresponded to mol. wt. $\geqslant$ 1300 while the last peak corresponded to mol. wt. $\leqslant$192. The intermediate area between the first and last peak of scan A can be associated with materials in the reaction mixture possessing random molecular weights from 192 to 1300. Scan B of pure methyl-α-D-glucopyranoside was run for comparison. Perhaps the most important fact to be ascertained from the above illustration is that through the use of HPLC, polymerization processes, and many other types of reactions, can be monitored from initiation to termination. Also, the total reaction mixture can be analyzed directly, eliminating the formation of any derivatives. For example, neutral sugars can be studied in their natural states when using HPLC, while analysis by GLC would require common derivative formation (*e.g.*, trimethylsilyl, acetyl and trifluoroacetyl derivatives).

CONCLUSION

Through the use of HPLC, water-soluble polysaccharides, together with low-molecular-weight carbohydrates and polymerization products, were separated, collected and identified. Bio-Glas packed columns gave the most rapid separation of high-molecular-weight, water-soluble polysaccharides, while Bio-Gel was found most useful for separating complex mixtures of polymers. Lower molecular weight carbohydrates were separated on both Bio-Gel and EM Gel OR-PVA columns, with EM Gel giving the best resolution. The only drawback found with the EM Gel packings was that non-aqueous solvents had to be employed. Consequently, analysis of those carbohydrate oligomers which possess more than four units was somewhat limited owing to solubility problems.

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CHROM. 7515

PURIFICATION OF RIBONUCLEASE T_1 ON POROUS GLASS AFFINITY ADSORBENTS

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SUMMARY

Purification of ribonuclease T_1 has been achieved using a porous glass–2′,(3′)-GMP affinity adsorbent. This adsorbent exhibits specific affinity for ribonuclease T_1 and is almost devoid of non-specific ion-exchange properties.

INTRODUCTION

Ribonuclease (RNase) A[1] and tobacco RNase[2,3] have been purified on Sepharose-based affinity adsorbents. The tendency of agarose gel beads to distort limits the use of agarose-based adsorbents for large-scale work. In addition, agarose is susceptible to microbial attack and cannot be regenerated from adsorbents for re-use. Porous glass beads have been used as an alternative insoluble matrix for affinity chromatography adsorbents[4,5]. They are resistant to distortion and microbial attack and can easily be regenerated for re-use by treating adsorbents with boiling nitric acid.

This paper reports the preparation of several porous glass derivatives and describes their use in the purification of RNase T_1.

MATERIALS AND METHODS

Sources of materials were as follows: Taka diastase (Parke & Davis, London, Great Britain); highly polymerized yeast RNA, calf thymus DNA, Corning CPG 10 (2000 Å pore diameter) 200-mesh glass beads (BDH, Poole, Great Britain); 3-aminopropyltriethoxysilane (Ralph Emanuel, Wembley, Great Britain); guanosine 2′,(3′)-monophosphoric acid (2′,(3′)-GMP) (Sigma, St. Louis, Mo., U.S.A.).

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Preparation of 5'-(4-aminophenylphosphoryl)-guanosine 2',(3')-monophosphate (APP-2',(3')-GMP)

APP-2',(3')-GMP was synthesized from guanosine 2',3'-cyclic monophosphate and 4-nitrophenylphosphate as described elsewhere[3].

Preparation of 1,5-dihydroxynaphthalene–glass (DHN–glass)

Porous glass beads were cleaned and reacted with 3-aminopropyltriethoxysilane in dry acetone[4]. The resulting aminoalkyl glass beads were converted to aminoaryl glass, diazotized[6], and added to a solution of 1,5-dihydroxynaphthalene (5 mg/ml) in ice-cold ethanolic sodium acetate (1 g glass per 20 ml). The mixture was stirred gently for 1 h at 4° after which the DHN–glass (Fig. 1) was collected by filtration. Unreacted dihydroxynaphthalene was removed by washing the product with ethanol. Finally the DHN–glass was washed with ether and air dried. Preparations were found to contain 90 to 110 μmoles dihydroxynaphthalene per gram of glass.

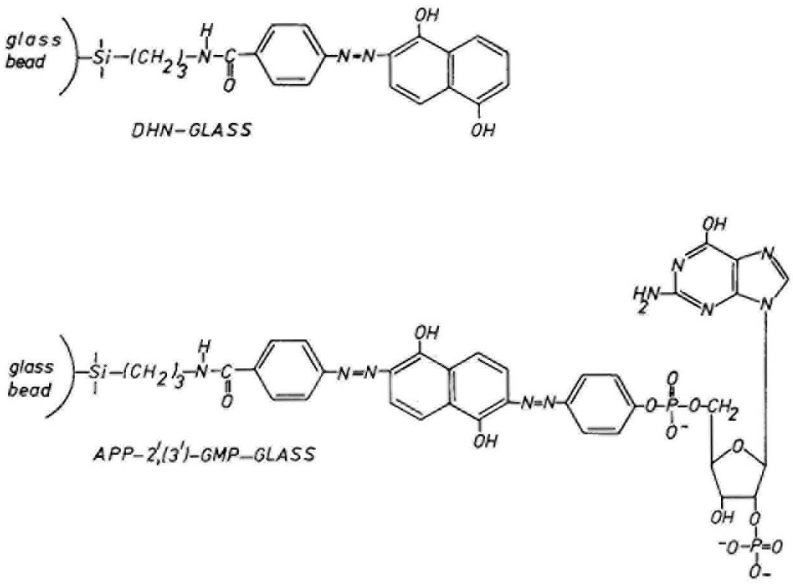

Fig. 1. Structures of porous glass derivatives.

Preparation of APP-2',(3')-GMP–glass

APP-2',(3')-GMP (200 μmoles) was dissolved in 5 ml of ice-cold 200 m*M* hydrochloric acid and diazotized by addition of 50 mg of sodium nitrite. After 10 min the solution was poured into a stirred suspension of DHN–glass (1 g) in 20 ml of ice-cold 500 m*M* pH 9.0 bicarbonate buffer. The reaction mixture was stirred for 1 h at 4°, after which the product was collected by filtration. The product was exhaustively washed, first with 200 m*M* ammonium acetate buffer pH 5.4 containing 500 m*M* KCl, and then with 200 m*M* Tris–HCl buffer pH 9.5 containing 500 m*M* KCl. This washing procedure was found to remove ionically bound material from the porous glass. Finally, the APP-2',(3')-GMP–glass (Fig. 1) was washed with distilled water

and stored at 4°. Preparations were found to contain 3–8 μmoles 2′,(3′)-GMP per gram of glass.

Preparation of RNA–glass and DNA–glass

Highly polymerized yeast RNA and heat-denatured calf thymus DNA were attached to aminoalkyl glass using glutaraldehyde as the coupling reagent[4]. Aminoalkyl glass was treated with 1% (v/v) ice-cold glutaraldehyde at 4° for 30 min. The treated glass was washed with cold distilled water and then packed into a small column (5 × 0.7 cm). Solutions of nucleic acid in 50 m*M* phosphate buffer pH 7.5 (1 mg/ml) were pumped over the treated glass for 24 h, at 4° using a peristaltic pump. The pumping ensures complete access of the nucleic acids to all surfaces of the porous beads during the coupling reaction. After coupling, derivatives were washed as described above to remove ionically bound material.

Preparations of RNA–glass contained 7.5 to 8.5 mg of RNA per gram of glass. DNA–glass preparations contained 10 to 12 mg of heat-denatured DNA per gram of glass. Control preparations made by omitting the glutaraldehyde treatment contained less than 0.5 mg of nucleic acid per gram of glass.

Determination of ligand content of derivatives

Derivatives were washed with ethanol and ether and air dried. Weighed samples of the dried derivatives were used to determine ligand content. Equal weights of aminoaryl glass were used as controls. Samples of derivatives were treated with orcinol–ferric chloride reagent[7] to determine GMP or RNA content, or with diphenylamine reagent[8] to determine DNA content.

The dihydroxynaphthalene content of DHN–glass was determined by treating a weighed sample of the derivative with a solution of sodium dithionite. The dithionite reduces the azo linkage binding the DHN to the glass and releases the DHN, which can then be measured spectrophotometrically.

Preparation of RNase T_1

RNase T_1 was partially purified from Taka diastase by extracting 2 g of the latter with 20 ml of 50 m*M* ammonium acetate buffer pH 5.4 containing 50 m*M* KCl (acetate–KCl buffer). Extracts were clarified by centrifugation and applied to a 4 × 100 cm column of Sephadex G-100, equilibrated and eluted with acetate–KCl buffer. Separation of RNase T_1 activity from RNase T_2 activity was achieved in this way. Fractions containing RNase T_1 activity were collected and used for affinity chromatography without further treatment.

RNase T_1 activity was assayed[9] using highly polymerized yeast RNA as substrate.

Column operation

Columns were made from small (0.7 cm I.D.) filter tubes containing sintered glass discs. These were packed to a height of 5 cm with glass derivatives suspended in acetate–KCl buffer. Solutions were pumped through the columns at a flow-rate of 30 ml/h using a peristaltic pump. Column effluents were monitored at 254 nm using an LKB Uvicord. Fractions were collected on an LKB Mini-rac fraction collector.

Elution of columns was carried out using the procedures described elsewhere for Sepharose–APP-2′,(3′)-GMP derivatives[3].

RESULTS AND DISCUSSION

APP-2′,(3′)-GMP–glass

RNase T_1 was completely adsorbed onto columns of APP-2′,(3′)-GMP–glass beads from solutions of Sephadex G-100-purified material. The contaminating proteins in the Sephadex preparations were not adsorbed by the glass derivative and passed through columns unretarded (Fig. 2). Elution of the bound RNase could be achieved either by changing the pH of the elution buffer (Fig. 2a), or by specific elution with substrate (Fig. 2b), or inhibitor (Fig. 2c). Elution of the bound RNase could not be achieved with an ionic strength gradient from 50 m*M* KCl up to 1 *M* KCl[3].

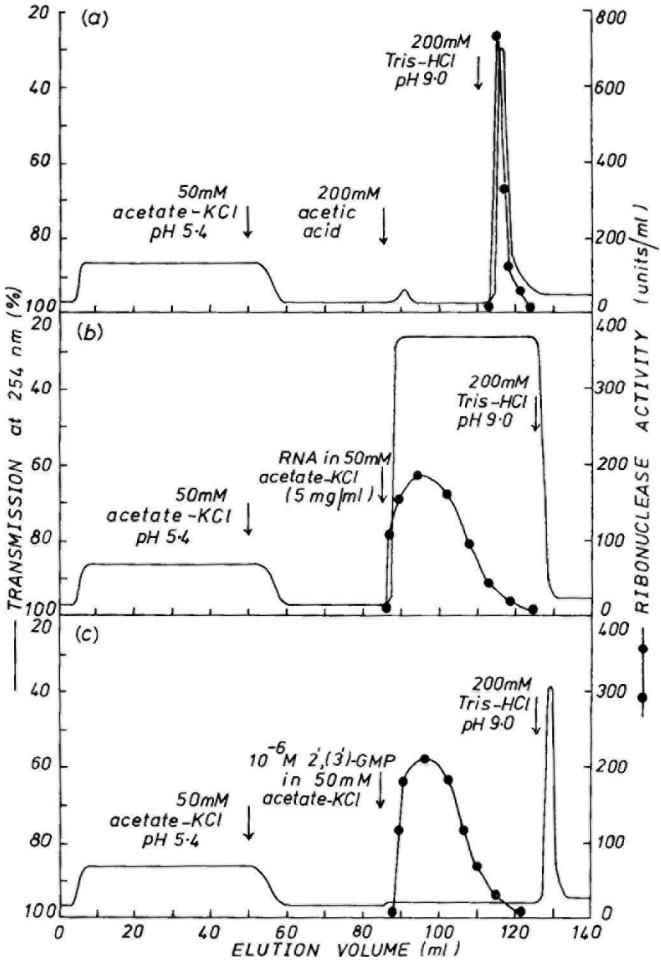

Fig. 2. Chromatography of Sephadex G-100-purified RNase T_1 on APP-2′,(3′)-GMP–glass. 50 ml of Sephadex effluent were applied to a 5-cm column of adsorbent and eluted as shown. (a) RNase was eluted by raising the pH of the eluting buffer to 9.0. (b) RNase was eluted by washing the column with a solution of RNA in 50 m*M* acetate–KCl buffer. (c) RNase was eluted by washing the column with 10^{-6} *M* 2′,(3′)-GMP in 50 m*M* acetate–KCl buffer.

Of the elution systems used, 10^{-6} *M* 2′,(3′)-GMP was the most satisfactory as this did not elute any of the non-specifically bound proteins.

DHN–glass, RNA–glass and DNA–glass

RNase T_1 was not adsorbed onto DHN–glass from Sephadex G-100-purified material. This result confirms that the APP-2′,(3′)-GMP–glass derivative functions as a specific affinity adsorbent for RNase T_1, rather than as a simple ion exchanger.

Neither RNA–glass nor DNA–glass bound RNase T_1. DNA–Sepharose has been used as an affinity adsorbent for *E. coli* RNase[10] and heat-denatured DNA does inhibit RNase T_1[11]. Evidently, the affinity of RNase T_1 for single stranded DNA is not sufficient to allow the latter to be used for affinity chromatography.

Purification of RNase T_1 on APP-2′,(3′)-GMP–glass

Purification of RNase T_1 on APP-2′,(3′)-GMP–glass beads yielded a product that appeared homogeneous on polyacrylamide disc gel electrophoresis[12,*]. The porous glass based adsorbent appeared to be at least as effective as similar Sepharose-based adsorbents. It also has the advantage that column flow-rate remained constant, even after prolonged use. The glass derivative was very stable under conditions used for RNase T_1 purification. However, very slight loss of ligand occurs at high pH, a problem also encountered with Sepharose-based adsorbents[13].

The porous glass adsorbents described here were primarily developed to aid the isolation of RNases from large volumes of waste liquor produced during plant protein manufacture[14]. In these liquors, RNase is present, and active, after most other proteins have been precipitated by heat treatment. However, the large volumes involved make isolation of the RNase by most conventional techniques very difficult. In preliminary experiments, columns of APP-2′,(3′)-GMP–glass were successfully used to remove the RNase from several litres of waste liquor. Under the conditions employed in these experiments, Sepharose-based adsorbents quickly lost their flow properties. Porous glass adsorbents do seem to have significant advantages over Sepharose derivatives for large-scale work. APP-2′,(3′)-GMP–glass adsorbents have been used continuously under non-alkaline conditions for several months without detectable loss of RNase T_1 binding capacity. Such adsorbents have been stored dry for up to two years and then successfully used for RNase T_1 purification.

ACKNOWLEDGEMENTS

The authors wish to thank N. W. Pirie F.R.S. for his helpful criticism of this work. One of us (N.M.P.) was on Industrial Training leave from Hatfield Polytechnic, Hatfield, Hertfordshire when this work was undertaken and wishes to thank Dr. B. Williams and Dr. I. Simpkins of the Biology Department, Hatfield Polytechnic for helpful discussions.

* Polyacrylamide disc gel electrophoresis was performed on a two-layer system[15]. The buffer system used was 0.1 *M* Tris–0.004 *M* EDTA–0.015 *M* boric acid, pH 8.9. After electrophoresis, protein bands were visualized by staining with Coomassie brilliant blue[16].

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DIRECT DETERMINATION OF MOLAR RATIOS OF VARIOUS CHEMICAL CONSTITUENTS IN ENDOTOXIC GLYCOLIPIDS IN SILICIC ACID SCRAPINGS FROM THIN-LAYER CHROMATOGRAPHIC PLATES

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SUMMARY

Analytical procedures are described for the determination of the ratios of nitrogen, phosphorus, hexosamine, carbohydrates, fatty acids and 2-keto-3-deoxy-octonate (KDO) in bacterial endotoxic glycolipids separated by thin-layer chromatography (TLC). The methods make it possible to carry out analyses in the presence of silicic acid to obtain the molar ratios of the chemical constituents of natural products. For some micro-determinations, such as nitrogen, phosphorus or KDO, scrapings from a single TLC plate are sufficient for carrying out the quantitative analyses.

INTRODUCTION

Thin-layer chromatography (TLC) at present can be considered to be one of the most effective and useful techniques for the separation of lipids. The advantages of TLC include simplicity, speed and high resolution. Preparative TLC usually effects better separations than silicic acid column chromatography. Some natural products can be separated to form a homogeneous single band and several milligrams of purified material can be recovered by collecting scrapings from five or more plates. The disadvantages of preparative TLC are two-fold. Firstly, the eluate is frequently contaminated with fine silicic acid particles[1]. Filtration of the eluate through ultrafine glass filters does not eliminate all the contamination. Centrifugation and partition in two-phase solvents have been designed to eliminate the fine particles[2], but the procedures are tedious and removal of the silicic acid is not complete. Secondly, recovery of the material from the scrapings is usually low, especially when the lipids must be developed in a rather polar solvent system[3].

Bacterial endotoxic glycolipids are heterogeneous. The purification and isolation of purified material is necessary before the structure–function relationship can be verified. The endotoxic glycolipids from *Salmonella minnesota* R595 have been separated by TLC[4]. In order to avoid the difficulties in the elution of glycolipids from silicic acid scrapings, quantitative analytical procedures have been developed in our laboratory for the determination of the molecular ratios in these glycolipids

in the presence of silicic acid. Scrapings from TLC plates were taken and subjected to quantitative chemical microanalyses, without attempting the elution of the components from the scrapings.

Chemical analyses included the measurement of total nitrogen, phosphorus, hexosamine, total carbohydrates, long-chain carboxylic acids and 2-keto-3-deoxy-octonate (KDO). The molecular ratios could be calculated from these data.

MATERIALS AND METHODS

Endotoxic glycolipid

Chloroform–methanol (4:1) soluble glycolipids were directly extracted from the lyophilized cells of a rough mutant, *Salmonella minnesota* R595, as reported elsewhere[4].

Thin-layer chromatography

All TLC was carried out with silicic acid Bio-Sil A (2–10 μm; Bio-Rad Labs., Richmond, Calif., U.S.A.) and with plates 20 × 20 cm in size and 0.25 cm in thickness. To 30 g of silicic acid, 56 ml of water containing 4 ml of concentrated ammonia solution were added. The plates were air-dried, activated at 120° for 1 h in a vacuum oven, then cooled in a vacuum desiccator. The solvent system used was chloroform–methanol–water–ammonia (100:50:8:4).

As these plates could not be used for the determination of nitrogen, other plates were prepared in which the slurry was made in 0.1 *M* phosphate buffer[5], pH 7.8, and the pH of the slurry was again adjusted to 7.8 with 10 *N* sodium hydroxide solution. In order to obtain reasonably good separations, the solvent systems used for these plates were either chloroform–methanol–water (3:3:1) or *n*-propanol–water (1:1).

The spray reagents used were either chromic acid–sulfuric acid (5% potassium dichromate in 40% sulfuric acid) or water. For preparative TLC, about 1 mg of glycolipid per plate was applied with a sample applicator (Applied Science Labs., State College, Pa., U.S.A.). The plates were developed, dried and sprayed with distilled water; those parts of the silicic acid which contained lipids remained white, while other zones became translucent. The outlines of the visible bands were marked and the plates were dried at room temperature. The bands were scraped off and the identical bands or zones from several TLC plates were pooled. The scraped silicic acid was dried at 70° in a vacuum oven over phosphorus pentoxide, then stored in vials in a vacuum desiccator over phosphorus pentoxide at room temperature. A blank zone was also scraped and treated in the same manner.

Determination of nitrogen

Before all quantitative chemical analyses, the pooled scrapings were thoroughly mixed. A 500-mg amount (the weighing should be carried out as rapidly as possible) of the scrapings was transferred into a 100-ml Kjeldahl digestion flask. Concentrated sulfuric acid (2 ml) saturated with anhydrous copper(II) sulphate was added and the digestion was carried out in the presence of alundum particles, as described elsewhere[6].

The distillation was performed with a Labconco micro-distillation apparatus (Labconco Corp., Kansas City, Mo., U.S.A.). A 5-ml volume of 10 *N* sodium hydroxide solution was added to the sample and the ammonia liberated was steam-distilled into a recipient 25-ml graduated cylinder containing 10 ml of 0.02 *N* sulfuric acid. After distillation, the cylinder was filled to 20 ml with water and its ammonia content was determined by Nesslerization. To a 5-ml aliquot, Nessler reagent (5 ml; Hartman–Leddon Co., Philadelphia, Pa., U.S.A.) was added and the color was measured at 440 nm. A calibration curve was prepared with ammonium sulphate.

Determination of phosphorus

The determination of phosphorus was carried out using a modified method of Chen *et al.*[7]. Liberation of organic phosphorus was achieved by heating 50-mg aliquots of silicic acid scrapings in 0.8 ml of 6 *N* sulfuric acid at 100° for 16 h in a glass-stoppered tube. A 4-ml volume of water was added and mixed well. After cooling, 0.8 ml of 2.5% ammonium molybdate mixed with 1.6 ml of water and 0.8 ml of 10% ascorbic acid was added. The tubes were thoroughly mixed and incubated at 37° for 1½ h. The mixture was centrifuged at 1000 *g* for 15 min, and the color intensity of the supernatant was determined at 820 nm. A calibration curve was prepared with potassium dihydrogen orthophosphate.

Determination of hexosamine

A 300-mg amount of silicic acid scrapings was hydrolyzed with 2 ml of 4 *N* hydrochloric acid in a glass-stoppered test-tube at 100° for 18 h. The contents of the tubes were filtered into a 100-ml round-bottomed flask and washed about five times with 3 ml of water. The filtrate was dried thoroughly on a Büchi evaporator. The flask with the dried hydrolyzate was placed in a vacuum desiccator and dried overnight over potassium hydroxide pellets. A 3-ml volume of water was added to dissolve the residue and 0.5- and 1.5-ml aliquots were taken for the determination of hexosamine. The procedure for color development was essentially the same as that described by Rondle and Morgan[8]. A calibration curve was prepared with glucosamine.

Determination of carbohydrate

The carbohydrate content was determined according to the method of Dubois *et al.*[9]. To 200 mg of silicic acid scrapings and 0.5 ml of water in a test-tube, 1.0 ml of 5% phenol solution was added and mixed well. After the tubes had been chilled in an ice-bath, 5 ml of conc. sulfuric acid were added, mixed well and the tubes were returned to the ice-bath. The tubes were then immersed in a boiling water-bath for 15 min, after which they were cooled in an ice-bath. The sample was then centrifuged at 1000 *g* for 20 min and the supernatant was read at 490 nm. A calibration curve with standard dextran solution was used for the determination.

Determination of long-chain carboxylic acids (fatty acids)

A 2-ml amount of boron trifluoride reagent (Applied Science Labs.) was added to a test-tube with a standard-taper-joint which contained 500 mg of silicic acid scrapings and granules of alundum. The tubes were firmly attached to well cooled reflux condensers and the lower end (*ca.* ½ in.) was immersed in an 80–90° oil-bath for 5 h in order to transesterify the fatty acids[10]. The tubes attached to their condensers were cooled,

and 2 ml of redistilled methanol were added through the reflux condenser into the tubes. Water (2 ml) and *n*-hexane (2 ml) were added to the disconnected tubes, which were immediately closed with glass stoppers. The tubes were shaken vigorously for 30 sec and allowed to stand at room temperature for approximately 1 h until the silicic acid had settled and the two phases were clearly separated. The *n*-hexane phase was retained and 2 ml of fresh *n*-hexane were added for extraction. A total of four such extractions were repeated. Any contaminating water was removed with anhydrous sodium sulfate. The pooled *n*-hexane was then evaporated until completely dry with nitrogen gas at room temperature. The fatty acid methyl esters were quantitatively determined by the hydroxylamine method[11]. A calibration curve was made with glucose pentaacetate.

Gas–liquid chromatography (GLC)

The methyl esters used for quantitative fatty acid analysis were also examined by GLC using an F & M 609 instrument equipped with a hydrogen flame ionization detector. The components on the chromatograms were identified by co-chromatography with the authentic fatty acid methyl esters.

Determination of KDO

The hydrolysis of KDO is usually destructive and a hydrolysis curve is needed for each sample to obtain the value at zero time. When the sample is coated on silicic acid, however, KDO is protected against destruction by acid and a 20-min hydrolysis was found to be optimal.

A 100-mg amount of silicic acid scrapings was hydrolyzed with 1.0 ml of 0.025 *N* sulfuric acid in a boiling water-bath for 20 min. The cooled hydrolyzates were centrifuged at approximately 1000 *g* for 15 min. A 0.4-ml amount of the supernatant was used for the determination of KDO. The method was essentially the same as that described by Weissbach and Hurwitz[12] and modified by Osborn[13], except that the amounts of all the reagents were doubled. A calibration curve was prepared with ammonium KDO (a gift from Dr. O. Lüderitz, which the authors greatly appreciate) in the range 1–10 μg.

RESULTS AND DISCUSSION

The separation of endotoxic glycolipids from *S. minnesota* R595 by TLC with solvent system chloroform–methanol–water–ammonia (100:50:8:4) is shown in Fig. 1 Four major bands were obtained and all were found to be biologically active[4]. The KDO, hexosamine, phosphorus and fatty acid contents of each band were determined by the procedure described above. It was found that these four bands (a, b, c and d) had similar molar ratios, namely KDO:hexosamine:phosphorus:fatty acid = 2:2:3:6 (Table I). This ratio was in the range of reported data[14,15] for glycolipids from *S. minnesota* R595. In addition, the *n*-hexane extract of the transesterified methyl esters was also analyzed by GLC. Fig. 2 shows that the four biologically active bands revealed similar fatty acid compositions. Thus, by determining the amounts of the constituents of the silicic acid scrapings, it is possible to calculate the molecular ratios of various components in a natural product without knowing the exact organic material content of the silicic acid scrapings.

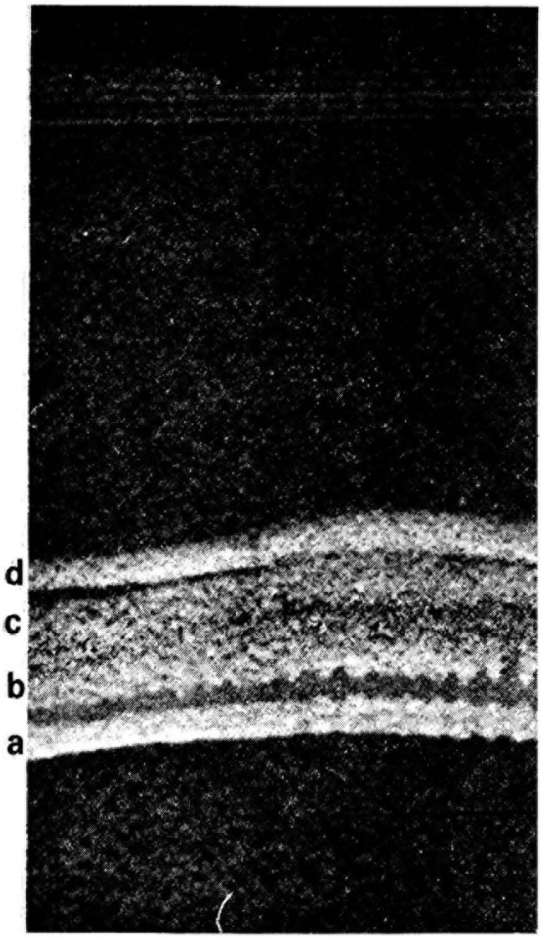

Fig. 1. Thin-layer chromatography of purified glycolipids with silicic acid Bio-Sil A (2–10 μm) and solvent system chloroform–methanol–water–ammonia (100:50:8:4). Silicic acid slurry was prepared with distilled water–ammonia. The plate was sprayed with water.

All the methods were tested with glycolipid or other known samples with blank silicic acid added. The results obtained from the known materials in the presence or absence of silicic acid were identical. The batches of silicic acid used in these experiments were free from contaminants which would interfere with the reac-

TABLE I

MOLAR RATIOS OF THE GLYCOLIPID FRACTIONS

Glycolipid fraction	*Hexosamine*	*Fatty acid ester*	*P*	*KDO*
a	1	2.90	1.69	1.07
b	1	2.75	1.60	0.93
c	1	3.10	1.36	0.83
d	1	2.67	1.34	1.08

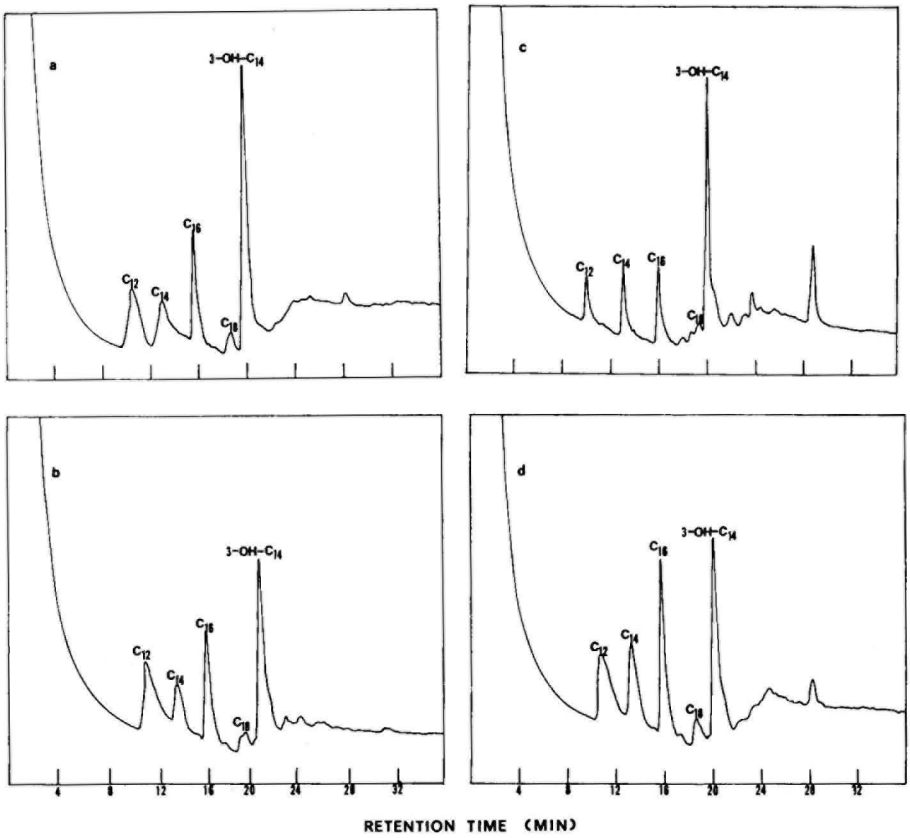

Fig. 2. Gas–liquid chromatogram of fatty acids present in glycolipid fractions. Column: 3% ECNSS-M on 100–120 mesh Gas-Chrom Q, 12 ft. Temperature programmed from 50° to 185° at 6.4°/min. a, b, c and d = fractions obtained from glycolipids by TLC. C_{12} = Lauric acid; C_{14} = myristic acid; C_{16} = palmitic acid; C_{18} = stearic acid; 3-OH-C_{14} = 3-hydroxymyristic acid.

tion. The silicic acid obtained from other sources may have a significant amount of contamination, in which case it should be pre-washed or a calibration curve with blank silicic acid plus known amounts of standard material should be used.

The amount of silicic acid scrapings needed for each analysis depends on the total organic material as well as the percentage of the particular component in the isolated band. The optimal ranges for the components to be determined are shown in Table II. Thus, if 1–3 mg of the total substance can be separated on one TLC plate, scrapings from a single band may be sufficient to determine nitrogen, phosphorus and KDO, and to calculate the molecular ratios if the compound in the band is relatively rich in these components.

The major source of error in these analyses is the variation in the water content of the silicic acid scrapings. The scrapings should be mixed and dried thoroughly and all samples should be kept constantly under vacuum (less than 0.1 mm Hg at room temperature over phosphorus pentoxide). The weighing procedure should be carried out as rapidly as possible.

TABLE II

RANGE OF THE OPTIMAL AMOUNTS OF THE COMPONENTS FOR DETERMINATION

Component	*Optimal range (μg)*
Nitrogen	5–25
Phosphorus	1–10
Hexosamine	20–80
Fatty acid	150–1250
Carbohydrate	20–100
KDO	2–10

While our procedures were developed for an endotoxic glycolipid, other natural products which can be separated by TLC may be subjected to similar analyses in order to obtain the chemical molar ratios without the difficulties of elution and contamination of silicic acid.

A similar TLC pattern to that of *S. minnesota* R595 glycolipids has been observed in our laboratories for glycolipids extracted from another rough mutant, *Salmonella typhimurium* SL1102. The molar ratios of KDO:hexosamine:phosphorus:fatty acid in pooled glycolipids was found[16] to be 2:2:4:7.

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THIN-LAYER CHROMATOGRAPHIC FLUORIMETRY OF INDOLE DERIVATIVES AFTER CONDENSATION BY A PARAFORMALDEHYDE SPRAY REAGENT

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SUMMARY

A sensitive paraformaldehyde spray reagent for the thin-layer chromatographic detection of indole derivatives at the nanogram level is described. Paraformaldehyde is dissolved in slightly alkaline ethanol and the ethanolic solution is neutralized by addition of acetic acid. The fluorescence is measured spectrophotofluorimetrically on thin-layer chromatograms and observations are made visually and on photographic films exposed through filters. Excitation and emission spectra are given for nine indole derivatives reacted with the paraformaldehyde spray reagent.

INTRODUCTION

Several methods have been described for the determination of tryptophan metabolites from biological tissues, based mainly on extraction and purification procedures[1–3] followed by measurements of the fluorescence obtained in the final solution when activated under UV light[4–6]. It is often desirable to start, or to make further investigations, with chromatographic procedures[1,7]. For the development of chromatographic spots of indole substances on thin-layer chromatograms, colour reactions have been described[1,7]. The most sensitive reagents, however, are those which give fluorescent products[8,10–13]. In histochemistry, gaseous formaldehyde is used for the detection of 5-hydroxytryptamine and catecholamines[14]. In paper and thin-layer chromatography (TLC)[9,12,13], it has also been used as a chromatographic reagent for indole substances and for catecholamines. It is believed that in the first step indoleamines form weakly or non-fluorescent tetrahydro-β-carbolines which will be fluorescent upon oxidation. In the histochemical method, the dehydrogenation reactions are catalyzed by protein. Fluorescent products are also obtained on silica gel thin-layer chromatograms, thus indicating that the dehydrogenation step will take place readily in the presence of silica gel[15]. Björklund *et al.*[15] found that the indoleamine fluorophores formed on dry protein and those formed on silica gel are identical.

In order to obtain gaseous formaldehyde, Björklund *et al.*[15] heated solid paraformaldehyde to 80–100° at 50% humidity, whereas Cowles *et al.*[13] heated paraformaldehyde to 150° to obtain drier conditions. In earlier experiments, Prochazka[7,16] introduced formaldehyde as a spray reagent for obtaining fluorescent products of

indoles. In the method described here, paraformaldehyde was dissolved in sligthly alkaline ethanol and the ethanolic solution was then evenly distributed over the thin-layer chromatogram by spraying. The fluorescence of various indole substances after gaseous formaldehyde condensation was studied in UV light and photographed through filters which increased the sensitivity. For nine fluorophores, the excitation and emission spectra were recorded *in situ* on the chromatogram.

EXPERIMENTAL

Reagents and equipment

The following reagents were used: Ethanol, absolute, spectrograde (AB Vin- & Spritcentralen, Stockholm, Sweden). Ethanol, 95% (AB Vin- & Spritcentralen). Hydrochloric acid, Titrisol, 0.1 *M* (Merck, Darmstadt, G.F.R.). Acetic acid, glacial, p.a. (Merck). Ammonia, 25% solution, p.a., Aristar (BDH, Poole, Great Britain). Methyl acetate, zur Synthese (Merck). Ethyl acetate, p.a. (Merck). Isopropanol, p.a. (Merck). *n*-Butanol, p.a. (Merck). Paraformaldehyde, purum (Kebo, Stockholm, Sweden). Sodium hydroxide, p.a. (Eka, Bohus, Sweden). Indole derivatives, listed in Table I.

Developing solvents used were: (A) methyl acetate–isopropanol–25% ammonia (9:7:4); (B) ethyl acetate–isopropanol–25% ammonia (9:7:4); (C) isopropanol –ethyl acetate–acetic acid–water (75:25:2:3); (D) *n*-butanol–acetic acid–water (4:1:5), upper layer. Solvents A, B and C were freshly prepared before each run.

The chromatography was performed on commercially pre-coated thin-layer plates of dimensions 20 × 20 or 5 × 20 cm with a 0.25-mm layer of silica gel (Merck). The plates were purified in a Shandon chromatography tank fitted with the equipment for descending chromatography. UV light was generated by a General Electric G8T5 tube mounted in a Chromatolux (Pleuger, Wijnegem, Belgium) and filtered through a UG 5 filter, thickness 2 mm (Carl Zeiss, Oberkochen/Württemberg, G.F.R.). The photographic recording was made on Agfa Scientia 50B65 or Kodak 2484 film with a Canon FT camera, lens 50 mm/1.8 or a Minolta SRT 101, lens 55 mm/1.7. A Kodak Wratten gelatin filter No. 8, yellow, and a sky-light filter (Hoya, Tokyo, Japan) were mounted on the lenses. The emission and excitation spectra were obtained from a Perkin-Elmer spectrophotofluorimeter to which a TLC scanning attachment was connected.

Procedure

Spray reagent. In 100 ml absolute ethanol, 60 mg of sodium hydroxide were dissolved. Paraformaldehyde (2 g) was added to the alkaline ethanol and, when it had completely dissolved, 0.1 ml of glacial acetic acid was added. The reagent can be stored refrigerated for at least 2 weeks before any loss in activity occurs. For comparison, paraformaldehyde was also dissolved in lower and higher concentrations and in 95% ethanol. When higher concentrations of paraformaldehyde were used, additional sodium hydroxide had to be dissolved in the alkaline ethanol.

Purification of chromatographic plates. The commercial pre-coated chromatographic plates were purified in a continuously descending chromatography system with a Whatman chromatography paper bridge between the solvent jar and the silica gel layer. Purification was performed with solvent A. The effluent was allowed to drip

from the lower part of the chromatogram. After a run of at least 24 h, the plates were dried in air for 15 min and then heated to 110° for 30 min. The plates were stored in a desiccator over silica gel. Omission of the purification step caused severe interactions between fluorescent products below the front.

Development of spots. The substances were dissolved according to the notes in Table I. Normally 1 μl of the solutions, with substance concentrations of 200, 100, 50, ..., 3.125 and 1.6, 0.8, 0.4 and 0.2 ng/μl of base, was spotted 1.5 cm from the edge of the plate. For the spectrophotofluorimetric measurements, 5–20 μg were spotted on the base line. After elution for 10 cm, the plates were dried *in vacuo* for 15–20 min, then sprayed with 10 ml of the paraformaldehyde reagent. The sprayed plates were heated at 150° for 20 min. When the silica gel layer was wetted with the spray reagent (which is not necessary), the plates were dried in air for 3–5 min before heating.

The plates were studied under UV light generated by the 254-nm lamp, the light from which was filtered through a UG 5 filter. The recording and further investigations were made on a panchromatic black and white film. The Wratten gelatin filter No. 8 cuts off the blue-violet background light from the silica gel and its use is necessary for the photographic recording. The UV lamp was placed 35 cm and the camera 65 cm above the chromatogram. The exposure time for the Agfa film was 2 min and for the Kodak 2484 15 sec with the diaphragm set at 4.0. The Agfa film was developed in Kodak D76 for 8 min, while the Kodak film was developed in Kodak D19 for 6 min.

RESULTS

Non-eluted system

In order to establish the optimal conditions for the action of the reagent, 5-hydroxytryptamine and other indoles were spotted on purified 5 × 20 cm silica gel plates. The plates were sprayed with paraformaldehyde in concentrations ranging from 0.05 to 10% (w/v) and each plate was dried separately in the oven at 150°. Optimal fluorescence was obtained when the reagent contained 2% (w/v) of paraformaldehyde in absolute ethanol.

At the optimal paraformaldehyde concentration, the temperature dependence for the formation of the fluorophores was studied at 10° intervals between 110 and 160°. The highest fluorescence intensity was obtained at 150 and 160°. When the heating time at 150° exceeded 20 min, maximal fluorescence occurred, while the fluorescence intensity decreased after 30 min.

In order to investigate the influence of water on the reagent, the paraformaldehyde was dissolved in a solution in which the absolute ethanol was replaced with 95% ethanol. The sensitivity of this reagent was greatly reduced, however: only 2 ng of 5-hydroxytryptamine could be detected, while the sensitivity was as high as 0.1 ng when absolute ethanol was used.

The fluorescence with the Prochazka reagent[7,16], a mixture of formaldehyde, ethanol and hydrochloric acid, is enhanced by exposure to *aqua regia*. In order to imitate the Prochazka reagent conditions, the plates were treated with paraformaldehyde in alkaline absolute ethanol without acetic acid. They were then sprayed either with 1–5 *M* hydrochloric acid in ethanol and heated at 150° for 20 min or first dried

TABLE I

THIN-LAYER CHROMATOGRAPHIC SEPARATION OF INDOLE DERIVATIVES AND SOME FLUORESCENCE DATA OBTAINED AFTER REACTION WITH GASEOUS FORMALDEHYDE

The sensitivities for the different derivatives are given after 10-cm elution with solvent A and development of the spots with 2% ethanolic paraformaldehyde solution. For the spectrophotofluorimetric determinations, 5–10 μg of indole substances were spotted on purified silica gel thin-layer plates and eluted with solvent A. Excitation and emission wavelength maxima for nine indole derivatives treated with the paraformaldehyde reagent are given. Wavelengths are uncorrected. When two wavelengths are given, the lower value gives the optimal fluorescence. The fluorescence of derivatives giving weak fluorescence or fluorescence not clearly distinguishable from the background (marked Weak) was measured from spots containing 20 μg/cm^2 of substance. Suppliers: A = Aldrich-Europe, Beerse, Belgium; C = Calbiochem, Los Angeles, Calif., U.S.A.; E = Eastman Organic Chemicals, Rochester, N.Y., U.S.A.; F = Fluka, Buchs, Switzerland; K = K & K Labs., Plainview, N.Y., U.S.A.; M = Mann Labs., New York, N.Y., U.S.A.; R = Professor L. Reio, Food Administration, Stockholm, Sweden; S = Sigma, St. Louis, Mo., U.S.A. Solutions (solvents for the derivatives): Ac = acetone; Et = ethanol; Et/HCl = ethanol 1 *M* with respect to HCl. Solvents A, B and C: see *Reagents and equipment*.

Indole derivative	*Supplier*	*Solution*	$R_F \times 100$			*Sensitivity (ng/spot)*	*Fluorescence maxima (nm)*		*Colour*
			A	*B*	*C*		*Excitation*	*Emission*	
N-Acetyl-5-hydroxytryptamine	S	Ac	77	76	68	25	410, 465	530	Reddish yellow
N-Acetyltryptophan	R	Ac	29	—	—	Not visible	—	—	—
DL-7-Azatryptophan	S	Ac	23	22	5	12.5	300	395	Blue
5-Benzyloxygramine	F	Et	78	81	4	25	Weak	Weak	Greyish blue
5-Benzyloxytryptamine	F	Et	68	69	4	1.6	Weak	Weak	Greenish yellow

Bufotenin	R	Ac	68	71	2	—	—	—	Brown
N,N-Dimethyltryptamine	F	Ac	78	—	—	1000	Weak	Weak	Blue
5-Fluorotryptamine hydrochloride	F	Ac	72	62	Decomp.	3.125	—	—	Blue
Gramine	M	Ac	79	—	1	500	—	—	Brown
5-Hydroxyindole	R	Ac	81	—	—	—	—	—	Brown
5-Hydroxyindole-3-acetic acid	S	Ac	21	18	67	6.25	395, 460	540	Yellow
6-Hydroxymelatonin	S	Ac	68	70	63	—	—	—	Grey
5-Hydroxytryptamine creatinin sulphate	S	Ac	58	51	4	1.6	410	530	Yellow
5-Hydroxy-L-tryptophan	S	Ac	18	18	11	3.125	395	525	Yellow
Indole	E	Ac	89	—	75	1000	—	—	Bluish
3-Indoleacetaldehyde sodium hydrogen sulphite	S	Ac	Decomp.			—	—	—	Bluish
Indole-3-acetic acid	S	Et	30	26	70	25	Weak	Weak	Bluish yellow
Indole-3-pyruvic acid	R	Ac	27	—	—	—	—	—	Brown
Melatonin	C	Et	82	76	60	—	355	450, 480	Bluish yellow
5-Methoxygramine	F	Et	71	76	2	25	Weak	Weak	Brown
5-Methoxyindole-3-acetic acid	S	Et	31	28	66	12.5	Weak	Weak	Bluish yellow
5-Methoxytryptamine	F	Et	71	61	8	1.6	310, 410	490	Bluish yellow
5-Methoxytryptophan	R	Ac	23	17	—	—	—	—	Yellow
5-Methylgramine	K	Et	79	—	2	250	Weak	Weak	Greyish blue
α-Methyltryptamine hydrochloride	A	Ac	68	71	4	1.6	—	—	Blue
5-Methyltryptamine hydrochloride	K	Et/HCl	77	63	38	1.6	310, 385	460	Blue
Tryptamine hydrochloride	S	Ac	68	67	Decomp.	1.6	310, 380	440	Blue
Tryptophan	S	Ac	25	18	6	1.6	310	440	Blue

at 150° for 20 min and then sprayed with the ethanolic hydrochloric acid. In the latter instance, the plates were heated again at 150° for 10 min. The first method resulted in a considerable reduction in the sensitivity, while the second treatment reduced the detectable amount of 5-hydroxytryptamine to 4 ng. The exposure to *aqua regia* of the original or the hydrochloric acid-treated plates did not affect the fluorescence intensity. The addition of 0.1 % (v/v) of glacial acetic acid to the reagent, however, increased the sensitivity above that of the alkaline paraformaldehyde reagent.

Eluted system

When the plates were chromatographed, the sensitivity of the reagent was found to depend on the solvent system. Thus, solvent D resulted in great losses in sensitivity. When an acidic solvent is desired, it is therefore recommended that solvent D be replaced with solvent C. The minimum sensitivities of the eluted substances are given in Table I.

The fluorescence spectra of a number of substances reacted with the paraformaldehyde reagent were investigated (Table I). No differences were observed for spectra recorded on non-eluted plates and on plates eluted with solvent A. Blank fluorescence was either measured between two adjacent spots or below the one measured. The fluorescence of each substance was measured on spots from three or more different experiments.

It is interesting to note that several of the substances did not give a defined peak (Table I), but very broad maxima ranging from 450 to 550 nm. The fluorescence intensity was also low and not clearly distinguishable from the background. The emission and excitation spectra of tryptamine, tryptophan, 5-hydroxytryptamine and other indole substances with blue or yellow fluorescence could easily be measured, however (Figs. 1–3).

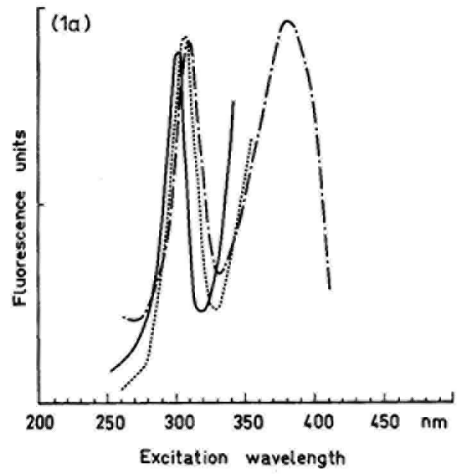
DISCUSSION

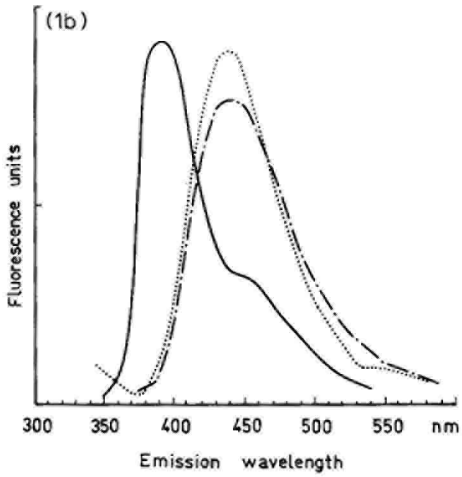
The use of paraformaldehyde as a spray reagent in the manner proposed here facilitates the rapid detection of indoles in TLC. The greatest sensitivity is obtained when spots are studied visually or by photography. The photographic recording makes it possible to measure the amounts densitometrically on the photographic

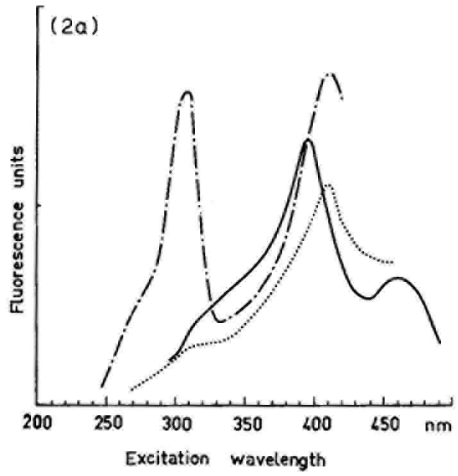
Fig. 1. (a) Excitation spectra and (b) fluorescence spectra for azatryptophan (———), tryptophan (· · · · · ·) and tryptamine (–·–·–) after reaction with the paraformaldehyde reagent. The measurements were made on spots obtained after elution with solvent A on silica gel TLC plates. Spectra are given after correction for blank fluorescence. Excitation and emission wavelengths, respectively: azatryptophan, 300 and 384 nm; tryptophan, 380 and 440 nm; and tryptamine, 310 and 440 nm. Wavelengths are uncorrected. Fluorescence is given in arbitrary units.

Fig. 2. (a) Excitation spectra and (b) fluorescence spectra for 5-methoxytryptamine (–·–·–), 5-hydroxyindoleacetic acid (———) and 5-hydroxytryptamine (· · · · · ·) after reaction with the paraformaldehyde reagent. Excitation and emission wavelengths, respectively: 5-methoxytryptamine, 310 and 480 nm; 5-hydroxyindoleacetic acid, 396 and 530 nm; 5-hydroxytryptamine, 410 and 530 nm. Other details as in Fig. 1.

Fig. 3. (a) Excitation spectra and (b) fluorescence spectra for 5-methyltryptamine (———), melatonin (· · · · · ·) and N-acetyl-5-hydroxytryptamine (–·–·–) after reaction with the paraformaldehyde reagent. Excitation and emission wavelengths, respectively: 5-methyltryptamine, 310 and 455 nm; melatonin, 348 and 450 nm; N-acetyl-5-hydroxytryptamine, 400 and 530 nm. Other details as in Fig. 1.


(1a)
Fluorescence units
200 250 300 350 400 450 nm
Excitation wavelength


(1b)
Fluorescence units
300 350 400 450 500 550 nm
Emission wavelength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Fluorescence units
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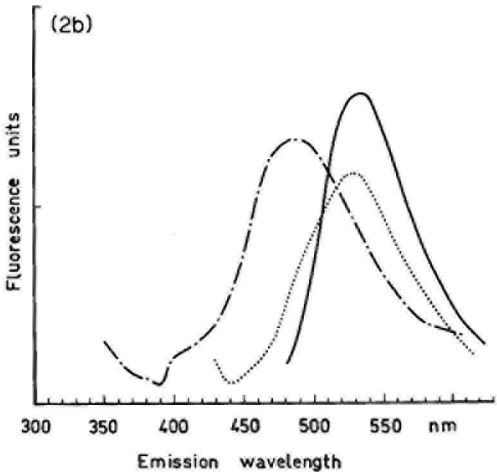
Excitation wavelength


(2b)
Fluorescence units
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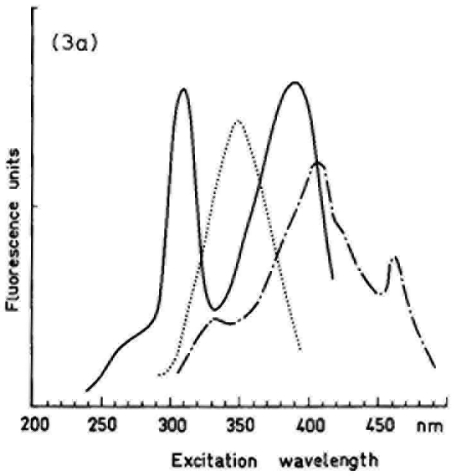
Emission wavelength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Fluorescence units
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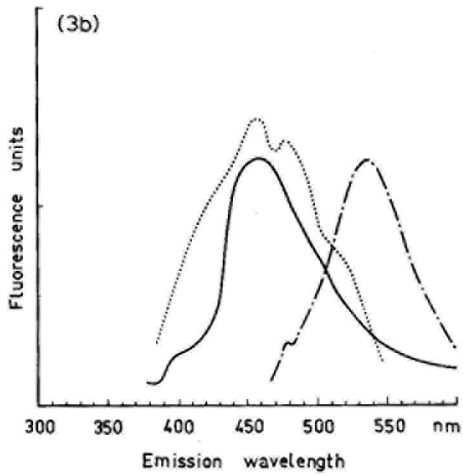
Excitation wavelength


(3b)
Fluorescence units
300 350 400 450 500 550 nm
Emission wavelength

negative. The use of easily obtainable gelatin filters increases the sensitivity of the method considerably.

Commercially pre-coated silica gel thin-layer plates must be purified thoroughly before use. This procedure is time consuming, however, but the use of a descending system is necessary only when two-dimensional chromatography is to be used. For one-dimensional chromatography, it is sufficient to purify the plates by running the solvent to the upper edge, preferably twice with a 30-min drying period at 110° in between.

The main choice of solvent is the alkaline solvent A and the acidic solvent C. The sensitivity of the reagent is higher when the acidic solvent is used, presumably owing to less degradation during elution. The use of solvent D is not recommended, as the loss in activity of the reagent is high. Solvent B is essentially identical with A, except that methyl acetate is replaced with ethyl acetate.

The heating times and temperatures for the development of the fluorophores are similar to those proposed by Cowles *et al.*[13]. In the method developed by Aures *et al.*[12], the temperature used is much lower (80°). As noticed by both groups of workers, the fluorescence of some of the fluorophores formed from indole derivatives is difficult to measure. This was also found in the present investigation (Table I). The spectra in Figs. 1–3 represent substances with mainly blue or yellow fluorescence.

The fluorescence of several of the derivatives listed in Table I is weak or not clearly distinguishable from the background when the measurements are made with the spectrophotofluorimeter. On the other hand, the visible fluorescence is clear and is brown-red, which is to be expected for a mixture of several wavelengths with broad peaks of equal intensities. It is considered that this phenomenon is due to the formation of different fluorophores from each substance. The difference between the fluorescence spectra reported earlier[13,15] and the present spectra is further evidence for the formation of several fluorophores. It has also been reported that changes in pH give additional fluorescent peaks with indoles[17]. If different fluorophores are formed, their formation might be explained in terms of polymerization products of dimers and trimers[17,18], in various combinations depending of the reaction conditions. One must also consider the degradation of the fluorophores with time and temperature.

Minor changes to the substituents on the indole nucleus cause shifts in excitation and emission wavelengths, which shows that spectra are easily affected by minor modifications of the fluorophores. For indole and gramines, no or extremely weak fluorescence is to be expected, as the side-chain is lacking or shorter than that for tryptophan derivatives. Thus β-carbolines cannot be formed. It has been shown that the gramines, however, become more fluorescent on prolonged oxidation, *e.g.*, on storage at room temperature for 1–2 days. The tryptamine and tryptophan derivatives, with the exception of N,N-dimethyltryptamine, readily form fluorophores at low concentrations.

The paraformaldehyde spray reagent has been used successfully in our laboratory for several years for the detection of tryptophan, tryptamine and 5-hydroxytryptamine in biological tissues, mainly from extracts of sea urchin larvae[19] and in model systems in which rat brain extracts were used. The sensitivity is in the nanogram range for indoles that fluoresce blue or yellow when activated in UV light. The method is a simple and useful complement to other analytical methods for the detection of indole derivatives in biological tissues.

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THE USE OF AN ENZACRYL AA DERIVATIVE FOR AFFINITY CHROMATOGRAPHY OF SEX HORMONE BINDING GLOBULIN

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SUMMARY

A polymer of the polyacrylamide type with side-chains bearing free amino groups on the benzene ring (Enzacryl AA) with covalently attached 3-hemisuccinate of 5α-androstane-3α,17β-diol was used for the affinity chromatography of sex hormone binding globulin.

After washing with buffer of increasing ionic strength, the release of protein was achieved by treatment with a buffered solution of testosterone. An approximately 63-fold enrichment of the required protein was achieved.

The purified protein was characterized by its binding properties and by isoelectric focusing.

INTRODUCTION

Studies on selective steroid-binding proteins such as transcortin or sex hormone binding globulin (SHBG) have shown that these high-affinity but low-capacity plasmatic carrier proteins can act as reversible regulators of hormone action[1].

SHBG has already been purified by classical means[1,2] (ammonium sulphate precipitation followed by repeated ion-exchange chromatography and molecular sieving) and its physico-chemical properties have been well established[1–5]; however, these techniques did not seem to be suitable for preparative-scale work. Affinity chromatography utilizing the unique selectivity of steroid–protein interactions appeared to be an ideal method for this purpose.

"Amino-Sepharose" coupled with cortisol 21-hemisuccinate has been used for the separation of transcortin from plasma[6], and excellent results have been obtained by affinity chromatography of soluble oestradiol receptors[7,8]. An attempt to separate SHBG by this method was also made as early as 1969, but the yields were very low (0.4%) owing to the failure to remove SHBG from the column without denaturation[9]. In each instance, cyanogen bromide-treated Sepharose 4B coupled with various steroid derivatives with or without a spacer was used.

As activated "amino-Sepharose" is now available commercially, the inconvenient work with cyanogen bromide can be avoided. However, when used for the

separation of steroid-binding proteins, these materials require extensive washing in order to remove the physically adsorbed affinant, which is limited by the resistance of the gel to organic solvents[8,10–12].

We searched for another matrix with good flow properties and resistant to organic solvents, which would moreover permit the direct attachment of suitable steroid derivatives. A cross-linked polymer of the polyacrylamide type containing sufficiently long side-chains (Enzacryl AA) seemed to fulfil these requirements. To the free amino group on the benzene ring of Enzacryl AA, a steroid derivative containing a carboxyl group, *e.g.*, hemisuccinate, could be attached. As a free 17β-hydroxy group is necessary for SHBG binding, a steroid derivative succinylated on a position other than 17β- had to be prepared[2,4].

MATERIALS AND METHODS

Steroids and chemicals

Radioactive steroids, *viz.*, [1,2-^{3}H]testosterone, specific activity 56 Ci/mmole, and [4-^{14}C]-3β-hydroxy-5-androsten-17-one (dehydroepiandrosterone), specific activity 52 mCi/mmole, purchased from the Radiochemical Centre, Amersham, Great Britain, were purified by paper chromatography in the system cyclohexane–toluene–methanol–water (9:1:8:2). The radiochemical purity (higher than 97%) was checked by chromatography in the same system using a non-labelled standard.

Non-radioactive steroids were obtained from Koch-Light (Colnbrook, Great Britain). All chemicals were of analytical grade and solvents were distilled before use.

5α-Androstane-3α,17β-diol 3-hemisuccinate was synthesized from androsterone by treatment with succinic anhydride in dry pyridine and by subsequent reduction of the resulting crystals (m.p. 183°) with sodium borohydride. The product was crystallized from acetone–*n*-hexane. The crystals, m.p. 221°, chromatographically homogeneous in the system Silica gel HF_{254}/ethyl acetate–methanol (92:8) (R_F = 0.36) and more polar than androsterone hemisuccinate (R_F = 0.58), were characterized as 5α-androstane-3α,17β-diol 3-hemisuccinate. The final yield was 31%.

5-Androstene-3β,17β-diol 3-hemisuccinate was prepared by the same method from dehydroepiandrosterone. The yield was 34%.

[4-^{14}C]-5-androstene-3β,17β-diol 3-hemisuccinate was prepared by the same method from [^{14}C]dehydroepiandrosterone (10 μCi) diluted with non-labelled steroid (100 mg). A product with a specific activity of 0.088 μCi/mg was obtained.

Preparation of Enzacryl–affinant

A 200-mg amount of 5α-androstane-3α,17β-diol 3-hemisuccinate and 200 mg of dicyclohexylcarbodiimide in 10 ml of dimethylformamide were stirred carefully at room temperature for 80 min, filtered, the filtrate was added to a suspension of 6 g of Enzacryl AA (Koch-Light) in 40 ml of dimethylformamide and the mixture was stirred gently for a further 3 h. The Enzacryl–affinant was then washed with dimethylformamide (5 × 100 ml), dioxane (3 × 100 ml), water (6 × 100 ml) and 0.015 *M* phosphate buffer (pH 7.2) with 1 m*M* EDTA.

After the affinity chromatography had been performed, the following sequence of solvents was used for regeneration of the Enzacryl–affinant: 6 *M* urea, water, dioxane, dimethylformamide, water and buffer.

Purification of SHBG

The serum obtained from pooled placental blood collected at full-term deliveries was first freed from endogenous steroids by treatment with charcoal[13]. As confirmed with trace amounts (0.1 μCi) of labelled testosterone, less than 0.014% of testosterone was retained after such a treatment. Steroid-free serum was then precipitated twice with equal volumes of saturated ammonium sulphate solution and the final precipitate was redissolved in 0.015 *M* phosphate buffer with 1 m*M* EDTA (one quarter of the serum volume). The globulins were then dialyzed with demineralized water on a Sephadex G-25 column (40 × 2.5 cm) and the 60-ml fraction following the void volume (70 ml) was taken for affinity chromatography.

The sample was suspended with Enzacryl–affinant (6 g) in phosphate buffer (10 ml) and, after gentle stirring for 1 h at 4°, was allowed to stand overnight at 4°. The mixture was then applied on a chromatographic column with a sinter (3 cm diameter) and, after the bed had settled, the proteins were eluted step by step with 0.015 *M* phosphate buffer containing 1 m*M* EDTA and increasing concentrations of potassium chloride (0, 0.2 and 1.0 *M*) at 4°. Concomitantly, an aliquot (10 ml) of a dialyzed globular fraction of serum was suspended with untreated Enzacryl AA (1 g) and then eluted with the same buffers as above as the "Enzacryl control". The flow-rate was 1 ml/min. The Enzacryl–affinant with bound protein was then transferred into a flask to which testosterone with a trace amount of radioactive testosterone in buffer was added (600 μg of testosterone and 0.6 μCi of [^{3}H]testosterone were dissolved in 60 μl of ethanol and 60 ml of 0.015 *M* phosphate buffer with 1 m*M* EDTA and 0.2 *M* potassium chloride). After stirring for 1 h at room temperature, the solution was allowed to stand overnight at 4° and then separated on a Büchner funnel. The steroid was removed by treatment with charcoal as described previously[13] and the solution was lyophylized. In an aliquot, the radioactivity before and after charcoal treatment was measured in order to check for the complete removal of the steroid.

Determination of binding capacity and binding affinity

Both the testosterone binding capacity (TeBC) and testosterone binding affinity (TeBA) were determined by Sephadex gel dialysis in a batchwise fashion according to Pearlman *et al.*[3]. In this method (Method I in ref. 3), a ten times larger amount of testosterone, *i.e.*, 10 ng, was used.

The method of Lowry *et al.*[14], with human serum albumin as a standard, was used for quantification of proteins.

Isoelectric focusing

After dialysis on Sephadex G-25 followed by lyophylization, an equivalent of 20 ml of purified SHBG (TeBC = 215 ng; protein content 3.3 mg) with 1 μCi of [^{3}H]testosterone in 1.5 ml of water was subjected to isoelectric focusing on a 5% polyacrylamide gel column (12 × 0.8 cm) containing 2% of Ampholine, pH 3–10 (LKB, Stockholm, Sweden). The initial voltage of 10 V/cm was gradually increased to 35 V/cm during 10 h; the current did not exceed 1 mA per column. The course of the pH gradient was determined according to Finalayson[15]. After staining with Coomassie Brilliant Blue R-250 (ICI, Dyestuffs Division, Manchester, Great Britain)[16], the pattern was cut into 3-mm segments, which were transferred into counting vials, the gel

was solubilized according to Caputo and Hosty[17] using NCS solubilizer (Amersham-Searle, Arlington Heights, Ill., U.S.A.) and the radioactivity was measured.

Determination of displacement of [^{3}H]testosterone from purified SHBG by various steroids

The relative displacement effect of various steroids was assessed according to Heyns *et al.*[4]. Briefly, the procedure was as follows. Increasing amounts (2–100 ng) of various steroids were added with stirring to the system containing a constant amount of purified protein (1 ml; TeBC = 10.73 ng), [^{3}H]testosterone (50 000 dpm) and Sephadex G-10 (100 mg) in a total volume 2 ml. After equilibrium had been achieved, the radioactivity in the supernatant was measured. The amount of each steroid producing the same displacement as 2 ng of testosterone was determined.

Radioactivity measurements

The radioactivities of both ^{3}H and ^{14}C were measured on a Betaszint BF 5000 liquid scintillation spectrometer (Berthold, Frieseke, Wildbad, G.F.R.) with a computing programme for the calculation of disintegrations per minute using the external standard channels ratio method. The scintillation fluid consisted of 4 g of PPO and 50 mg POPOP in 1 l of toluene. When aqueous samples (below 0.3 ml) were measured, a scintillation fluid containing 25 % (v/v) of ethylene glycol monomethyl ether was used.

RESULTS

Determination of the coupling of affinant to Enzacryl

As 5-androstene-3β,17β-diol 3-hemisuccinate did not differ from 5α-androstane-3α,17β-diol 3-hemisuccinate in its binding affinity to SHBG (see below), [^{14}C]-5-androstene-3β,17β-diol 3-hemisuccinate was used for the determination of coupling to Enzacryl. A 15-mg amount of labelled hemisuccinate with a specific activity of 0.088 μCi/mg was coupled with 100 mg of Enzacryl as described above. The Enzacryl–affinant complex was then washed thoroughly with dimethylformamide, dioxane and water until no radioactivity could be detected in the effluent. The remaining Enzacryl with covalently bound steroid was then subjected to hydrolysis with 10 % hydrochloric acid in methanol and the hydrolysate was extracted with dichloromethane. After evaporation of the solvent, the radioactivity in the dry residue was measured. The value of 34,000 dpm found corresponded to 195 μg, *i.e.*, 0.5 μmole of steroid bound to 100 mg of polymer.

In order to exclude the participation of physical adsorption, the same amount of labelled steroid was added to a suspension of Enzacryl AA in dimethylformamide without the addition of a condensation reagent (dicyclohexylcarbodiimide) and washed successively as above. The solid was hydrolyzed and the radioactivity in the hydrolyzate was measured; none was found.

Purification of SHBG

In a typical procedure, 40 ml of placental serum was worked up in one step. After removal of endogenous steroid with charcoal, the serum was precipitated twice with ammonium sulphate and the precipitate of globular proteins was dialyzed on

Sephadex G-25. The dialyzate was then applied in a batchwise fashion on Enzacryl–affinant and washed successively with 0.015 *M* phosphate buffer (pH 7.2) containing 1 m*M* EDTA and increasing concentrations of potassium chloride (0, 0.2 and 1.0 *M*). Simultaneously, an aliquot of the dialyzed protein mixture was applied on the column with untreated Enzacryl AA and worked up as above as an "Enzacryl control", in order to exclude non-specific adsorption. The bound protein was released by treatment with a concentrated testosterone solution containing trace amounts of labelled hormone. The steroid was removed again with charcoal. The completeness of the steroid removal was checked by measurement of tracer radioactivity; practically none could be detected after charcoal treatment.

In all fractions, including those of the "Enzacryl control", both the total protein concentration and the testosterone binding capacity were determined. The results obtained after individual purification steps are summarized in Table I. In the "Enzacryl control" pattern, almost complete recovery of TeBC was achieved within the first two fractions. As demonstrated in Table I, a product with a TeBC of 66 ng of bound testosterone per milligram of total protein was obtained, which corresponds to approximately 63-fold enrichment of the required protein.

TABLE I

SCHEME FOR PURIFICATION OF SEX HORMONE BINDING GLOBULIN

Treatment	*Volume (ml)*	*Total protein content (mg)*	*Testosterone binding capacity*	
			Total TeBC (ng)	*Specific TeBC (ng/mg protein)*
Initial serum	40	4068	4280	1.05
Ammonium sulphate precipitation (globular fraction)	10	1530	4210	2.75
Dialysis on Sephadex G-25	68	1000	4148	4.15
Fractionation on Enzacryl–affinant:				
fraction 1 (0.015 *M* phosphate buffer)	88	455	1804	3.96
fraction 2 (0.015 *M* phosphate buffer)	100	52	133	2.56
fraction 3 (0.015 *M* phosphate buffer with 0.2 *M* KCl)	100	195	386	1.98
fraction 4 (0.015 *M* phosphate buffer with 1.0 *M* KCl)	100	133	380	2.86
fraction 5 (0.015 *M* phosphate buffer with 1.0 *M* KCl)	100	83	35	0.42
fraction 6 (0.015 *M* phosphate buffer with 1.0 *M* KCl)	100	5	0	—
Elution with testosterone solution and removal of steroid with charcoal	80	13	858	66.0

Characterization of purified SHBG

The binding affinity expressed as an intrinsic association constant at 25° with testosterone as a tracer was determined in both a globular fraction of placental serum and purified material. The respective values (mean of five determinations, $\pm$ S.D.) were $3.4 \pm 0.43 \cdot 10^8$ and $1.92 \pm 0.28 \cdot 10^8$ l/mole. In both instances, complete loss of binding affinity after heating to 60° occurred.

The relative displacing effect of various steroids as a percentage (the testosterone effect being taken as 100%) is shown in Table II.

Isoelectric focusing of purified protein showed only two bands: the first in the area between p*I* 5.2–5.5 and the second at the cathodic end area, corresponding to immunoglobulins. The radioactivity distribution showed two peaks, the first, probably the unbound testosterone, containing more than 70% of the total radioactivity was located as a diffuse band in the anodic area, and the second peak was associated with the band of p*I* between 5.2 and 5.5.

TABLE II

RELATIVE DISPLACEMENT OF [^{3}H]TESTOSTERONE BY VARIOUS STEROIDS

Testosterone effect is taken as 100%.

Steroid	*Relative displacement (%)*
Testosterone	100
5α-Androstane-3α,17β-diol	106
5-Androstene-3β,17β-diol	98
5α-Androstane-3α,17β-diol 3-hemisuccinate	2.6
5-Androstene-3β,17β-diol 3-hemisuccinate	2.5
17β-Oestradiol	58
4-Androstene-3,17-dione	1.8
Cortisol	<0.1

DISCUSSION

Using Enzacryl AA with covalently attached 3-hemisuccinate of 5α-androstane-3α,17β-diol, approximately 63-fold enrichment of SHBG was achieved in one step. Under the conditions used, approximately 84% recovery of the initial testosterone binding capacity was obtained and in the highly purified form about 20% of the binding capacity was found (Table I).

In order to establish a reliable technique for the release of SHBG from its binding to Enzacryl–affinant, various procedures involving changes of pH and ionic strength as well as elution with buffered solutions of various 17β-hydroxysteroids were tried. The most effective method appeared to be elution with a concentrated aqueous testosterone solution. A disadvantage of this procedure was the neccessity for intensive treatment with charcoal in order to remove the steroid moiety, in which a certain loss of binding capacity may occur.

The isoelectric focusing and the displacement experiments and the binding constants found confirmed the identity of SHBG in the purified fraction. The p*I* value of the band associated with radioactivity was in accordance with that reported previously for SHBG[5]. However, this method also revealed that some by-products with the mobility of immunoglobulins accompanied SHBG and could not be separated completely by the described method.

In addition to its convenient flow properties, another advantage of Enzacryl AA is its ability to give direct attachment of known amounts of steroid ligands. A ligand concentration of 30 μmole of 5-androstene-3β,17β-diol 3-hemisuccinate per 6 g of Enzacryl AA was obtained.

Although the displacing ability of androstanediol 3-hemisuccinate is not very high (2.5% relative to testosterone, see Table II), this derivative seems to be a reliable affinant, because, on the other hand, the binding protein can be released without great difficulty. In general, very strong binding of protein to the affinant is undesirable, as was found by experience with the affinity chromatography of oestradiol receptors on Sepharose derivatives[7,8].

Previously, Enzacryls had been used only for immobilization of biopolymers, especially enzymes. In the present study, Enzacryl AA was used for the affinity chromatography of selective steroid–carrier protein.

In comparison with other polymers used for affinity chromatography, Enzacryl seems to have several advantages, as follows. The synthesis of Enzacryl–affinant does not require previous activation of the carrier and the steroid is bound covalently in good yields. The resistance of Enzacryl AA to organic solvents permits the effective removal of physically adsorbed ligands without the use of large volumes of solvents. An enrichment of protein could be achieved by a relatively simple and rapid procedure.

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DETERMINATION OF METHYLMERCURY IN BLOOD BY GAS CHROMATOGRAPHY

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SUMMARY

A sensitive gas chromatographic method for the determination of methylmercury concentrations in milliliter samples of whole blood is described. The method employs oxalic acid as a hydrogen ion source, potassium iodide as a halide ion source, sodium hydroxide to remove unwanted impurities and radioactive ^{203}Hg for the precise calculation of recovery. Ethylene glycol succinate (2%) on a solid support of Chromosorb G or Chromosorb T was used as column packing. The advantages and disadvantages of both these packing materials are discussed.

INTRODUCTION

For some time we have employed gas chromatography for the determination of methylmercury in biological materials. There are a number of published methods for the measurement of methylmercury in samples where its concentration is of the order of 0.1 μg/g of sample; for such samples, we have normally used a simple modification of the Westöö method[1]. This proved to be adequate until we attempted to extend it to the measurement of methylmercury in blood of normal populations. By a series of modifications we can now make reliable estimations of methylmercury in samples containing as little as 1 ng. Although the method was originally developed for application to blood, it is also applicable to most other biological materials.

EXPERIMENTAL

Materials

The following material was used: Oak Ridge tubes, polypropylene, capacity 50 ml; each tube was discarded after a single extraction. Pyrex glass tubes (16 × 150 mm) equipped with PTFE-lined screw caps. Disposable glass pipettes of capacity 1 ml and 2 ml obtained from Kimble Glass, Inc.

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The following reagents were obtained from Fisher Scientific Co., Fair Lawn, N.J., U.S.A.: benzene, spectroanalyzed; urea, A.C.S. certified; oxalic acid, certified, anhydrous; potassium iodide, A.C.S. certified; 30% ammonium hydroxide, A.C.S. reagent grade.

L-Cysteine (free base) was obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A.; high-purity nitrogen (dry) from Linde Division, Union Carbide Corporation, New York, N.Y., U.S.A.; and methylmercury chloride from K and K Laboratories, Inc., Plainview, N.Y., U.S.A. The methylmercury salt was recrystallized from 100% ethanol before use.

Methyl[^{203}Hg]mercury chloride was obtained from New England Nuclear, Boston, Mass., U.S.A.; sepcific activities were in the range of 1.5 to 5.0 mCi/mg of mercury. If necessary, this material was purified by extraction into benzene.

Methods

All columns used were 6 ft. by 4 mm I.D. coiled glass. The columns may be packed with either of two packing materials that we have found to be suitable for methylmercury separation and analysis. The first of these is a liquid phase of 2% ethylene glycol succinate (EGS) on a solid support of Chromosorb G (AW-DMCS) 60–80 mesh (Hi-Eff 2B, Applied Science Labs., State College, Pa., U.S.A.). The second packing material is Chromosorb T (PTFE), 40–60 mesh (Applied Science). Both materials may be packed by the same basic method. The Hi-Eff 2B, however, is more convenient in that it flows freely and may be used easily at room temperature. The Chromosorb T, on the other hand, should be stored in a freezer and packed in a cold room at 0–5°. Packing material is added with the aid of vibration and vacuum until all but about 10 cm of the column has been filled. A small plug of quartz wool is placed directly over the packing, and approx. 6 cm of solid, ground potassium iodide sifted through an 80-mesh screen, is put on top of this plug and secured by another quartz wool plug. The columns are conditioned for 36 h at 30° above the normal operating temperature with a nitrogen flow-rate of 20–40 ml/min in the Packard, Model 7401, gas chromatograph. Detectors are left disconnected during the conditioning period.

After conditioning, the detector is connected and the chromatograph is adjusted to normal operating conditions. For the Hi-Eff 2B column, the detector and inlet temperatures are set at 180° while the oven is maintained at 150°. The gas flow-rate is 100–120 ml/min under a pressure of 36 p.s.i. The Chromosorb T column is operated at 100° for the inlet and detector, with the oven set at 70° and the gas flow-rate at 80 ml/min. Usually, an additional period of 24 h is required to establish equilibrium and a constant baseline. A series of injections of a methylmercury halide standard should complete the column preparation. It seems that, normally, 5–10 of these injections (1–5 ng per injection) are sufficient, but it is advisable to continue the injections of standard until a constant peak height is obtained. The electron-capture detector (150-mCi tritium foil) is operated at a suppression current of 10^{-7}–2×10^{-7} A at a potential of 3–7 V.

Sample preparation. The sample (normally 1.0 ml) of heparinized whole blood is placed in a 50-ml polypropylene Oak Ridge tube, and a 10-μl aliquot of spike (a solution containing less than 1 ng of methyl[^{203}Hg]mercury, approximately 3000 dpm) is added by means of an Eppendorf pipette. The spiked blood is mixed and

allowed to stand for 15 min, then 8 ml of 8 *M* urea, 2 ml of 1 *M* oxalic acid, 1 ml of 1 *M* potassium iodide and 15 ml of benzene are added and the tube is capped and shaken for 10 min on a Thomas–Boerner shaking apparatus; it is then centrifuged at 15,000 *g* for 10 min in a Sorval superspeed centrifuge. The benzene layer is transferred with Pasteur pipettes to a second Oak Ridge tube, and another 15 ml of benzene are added to the sample, which is shaken and centrifuged as before; the two benzene layers are combined. To the tube containing the 30 ml of benzene is added 1 ml of 0.1 *M* sodium hydroxide, then the tube is capped, shaken for 5 min and centrifuged for 5 min at 3000 *g*. The sodium hydroxide layer is removed with Pasteur pipettes and discarded. To the washed benzene is added 1 ml of 1.5% alkaline cysteine solution (adjusted to pH 10 with ammonium hydroxide), and the tube is capped, shaken for 10 min and centrifuged at 3000 *g*. The cysteine layer is removed and placed in a 15-ml glass tube equipped with a PTFE-lined screw cap. To this tube are added 1 ml of 1 *M* oxalic acid, 1 ml of 1 *M* potassium iodide and 1 ml of benzene, and the tube is capped, mixed in a vortex mixer for 10 min and centrifuged at 3000 *g* for 5 min. The benzene layer is then removed by Pasteur pipette and placed in a small culture tube, and this tube is corked. Finally, this benzene extract is counted in either a Packard Model 3002 Auto-gamma spectrometer, or in a Packard Tri-Carb liquid scintillation spectrometer.

Analysis and calculations. A standard curve is established for the present conditions of the column before each day's determinations. Depending on the status of the column, the methylmercury peak appears 1.5 to 2 min after injection on the EGS column and after about 30 sec on the PTFE column. The amount of methylmercury per injection is determined by reading the height of the peak directly from the chart paper and comparing the peak height of the unknown sample with the peak heights of the standard curve. The percentage recovery for each extraction is determined by measuring the cpm in the final benzene solution in comparison with that for the 10 μl of added spike. Relevant calculations for the method are as follows:

$$\text{Recovery of methylmercury, \%} = \frac{\text{cpm in final benzene} \times 100}{\text{cpm in 10-}\mu\text{l spike}} \quad (1)$$

$$\text{Total weight of methylmercury in sample, ng} = \text{methylmercury injected, ng}/\mu\text{l} \cdot \frac{1000\ \mu\text{l}}{1\ \text{ml}} \cdot \frac{1\ \text{ml} \times 100}{\text{recovery, \%}} = \frac{\text{methylmercury injected, ng}}{\text{recovery, \%}} \cdot 10^5 \quad (2)$$

$$\text{Methylmercury content in sample, ppb} = \frac{\text{total weight of methylmercury in sample}}{\text{weight of sample, g}} \quad (3)$$

RESULTS AND DISCUSSION

We have successfully developed a system for the analysis of methylmercury in blood. In order to do so, it was necessary to make some rather intricate modifi-

cations of earlier methods. Regardless of how trivial some of these changes may appear, no alterations were incorporated unless they were found to improve significantly the over-all method.

There are several methods for measuring methylmercury in fish and tissue samples where levels are of the order of 0.1 ppm. An original attempt to apply such a method to the determination of methylmercury in blood led to low recovery. Since concentrated urea solutions tend to uncoil proteins, it was thought that such treatment might increase methylmercury recoveries. Theoretically, uncoiled proteins would expose more of the methylmercury-binding sites for extraction; therefore, 8 *M* urea was used as the hemolyzing agent, rather than distilled water. Although recoveries were not greatly increased, the initial benzene extract from blood was much cleaner, and on this basis the use of urea was continued. For samples other than blood, however, water is still the preferred medium for homogenization.

In spite of the assistance of urea, the benzene after extraction contained numerous colored impurities. Interestingly, the brown impurities were carried through the remainder of the extraction procedure along with the methylmercury. From the first benzene solution they were extracted into the alkaline cysteine solution, and, on acidification of the cysteine layer, the impurities passed to the final benzene extract. The apparent affinity of the impurities for alkaline aqueous solutions suggested the use of an alkaline wash, and 1 ml of 0.1 *M* sodium hydroxide was found to remove practically all the interfering impurities. Unfortunately, a max. of about 10% of the methylmercury present was lost in the alkaline wash.

Mineral acids were an additional source of contamination. Commercially prepared halogen acids are routinely used for extraction of methylmercury from such biological samples as fish, but benzene extracts of hydrochloric, hydrobromic and hydriodic acids yield small interfering peaks on the gas chromatogram, especially at high sensitivity settings. In addition to interfering peaks, these acids give extensive late peaks. Commercially available mineral acids were thus unsatisfactory, and the use of a high-purity organic acid to adjust the pH seemed reasonable, as long as a source of halide ions was added to allow extraction of the methylmercury. Oxalic acid was chosen, primarily because of its insolubility in benzene, which prevented extraction of the acid into the benzene layer. Potassium iodide was superior to sodium chloride as a halide source in that it led to better recoveries; the use of sodium iodide was not attempted. Use of oxalic acid and potassium iodide as acid and halide sources has, in our hands, increased the average recovery by as much as 10%.

The extraction of methylmercury as the iodide form necessitates the measurement of standards in the iodide form as well. Consequently, a pre-pack of a few centimeters of finely ground potassium iodide was incorporated into all columns. Methylmercury chloride is presumably converted into the iodide form by ion exchange on the column; thus, the more stable methylmercury chloride can be used in standard solutions.

Usually, 80 to 90% of the methylmercury in the sample is extracted with the first portion of benzene, and the second benzene extraction removes the remainder. As previously stated, the sodium hydroxide wash removes about 10% of the methylmercury. The cysteine extraction effectively removes all the methylmercury from the benzene, but large recovery losses can be encountered during the final step. The addition of cadmium chloride or mercuric chloride to the cysteine to occupy the ex-

cess –SH binding sites may possibly facilitate the partition of methylmercury into the final extract, but the effect is not marked with the current protocol.

When dealing with volunteers or patients, the smallest workable sample of blood is desirable. Usually, 1 ml is enough for this method, but the sample size may easily be extended to 2 or 3 ml, and even 5-ml samples can be handled with little modification of the method.

Although recovery rates are improved by this method, the large number of steps and the extensive handling of the samples required for this type of procedure make partial recoveries almost inevitable. Recovery rates from blood average 60 to 70% on routine samples, but have reached as much as 95%. The potential scatter is large, and in consequence it was necessary to find a technique that allowed suitable corrections. Monitoring with methyl[^{203}Hg]mercury solved this problem, and periodic evaluation of each extraction step was possible. Normally, less than 1 ng is added to the sample before extraction. The specific activity is such that this amount corresponds to at least 3000 dpm. For samples of very low methylmercury content correction is made for the added spike.

The EGS columns, once conditioned and functioning properly, have proved somewhat superior to Chromosorb T in routine use. They are capable of absorbing large amounts of discolored or water-saturated solutions without extensive loss of sensitivity, and they generally give better peak separation than does PTFE. The PTFE columns, on the other hand, have several advantages over the EGS columns. Retention times are shorter, retention of methylmercury being approximately 30 sec as compared with 1.5 to 2 min for the EGS columns. Whereas EGS columns can handle only 2000 to 3000 sample injections before replacement becomes necessary, the PTFE columns may last indefinitely with cautious treatment and may be removed from the oven without deleterious effects. At optimal operating conditions, either column allows detection and measurement of organomercurials in blood samples from normal populations, but PTFE has the potential of providing twice the sensitivity of EGS. However, if the balancing current is not above 10^{-7} A at the start of the analysis, the foil and the anode probe must be cleaned with ferric oxide or replaced.

It is important to emphasize that the extraction procedure is not specific for methylmercury, but works equally well for other organomercury compounds; indeed, ethylmercury is detectable at somewhat lower levels than methylmercury. In addition, although the method was developed for application to blood, it is equally suitable for measurement of organomercury compounds in any biological sample that lends itself to a benzene extraction. It has been successfully applied to wheat, fish and a variety of other tissue samples, and comparison with the values for "organic" mercury obtained as the difference between total and inorganic mercury as determined by atomic absorption shows good correspondence. In samples from a number of populations studied jointly with Dr. T. W. Clarkson[2], it appears that the amount of methylmercury determined by gas chromatography is sufficient to account for all organic mercury in the blood.

ACKNOWLEDGEMENT

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CHROM. 7578

Note

A syringe holder modification to the Hewlett-Packard 7671A Autosampler to allow the use of more robust syringes

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(Received May 13th, 1974)

A problem which has been experienced with the Hewlett-Packard Model 7671A Autosampler is the tendency for syringe needles and/or plungers to be bent during operation. This can be due to a variety of causes, including incorrect alignment of the injection unit, a too viscous sample, a too rapid plunger speed and a dirty syringe needle or barrel. After attempting the remedies suggested in the appropriate manual[1] without success, it was decided to modify the syringe holder assembly to take Scientific Glass Engineering (S.G.E., Melbourne, Australia) Type A 5- or 10-μl fixed needle syringes. These have stronger plungers and needles than the syringes normally supplied with the Autosampler.

The modified syringe holder is shown in Fig. 1. The body of the holder is made from brass tubing, 62 mm × 11 mm O.D. × 8.5 mm I.D. A 30-mm length of brass

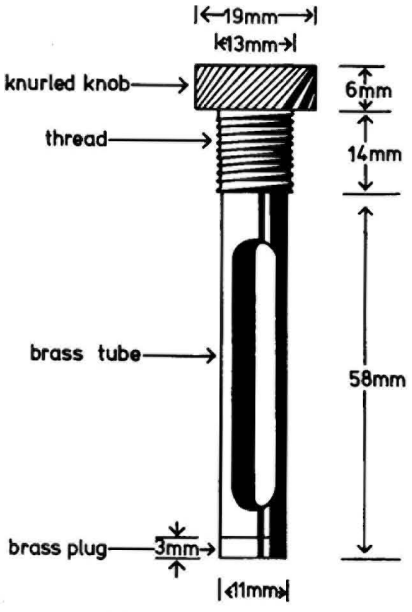

Fig. 1. Modified syringe holder.

rod of 20 mm diameter was turned on a lathe to provide the knurled knob at the top and the threaded portion was drilled out to 8.5 mm I.D. The lower 7 mm was then drilled out to 11 mm to take the brass tubing and the two pieces were sweated together.

A brass plug with a 1-mm hole in the centre was then fitted to the bottom of the brass tube as shown. Finally, a section of the brass tube was cut out to enable the graduations on the syringe to be read once placed in the holder.

To instal the modified holder in the Autosampler requires expansion of the hole in the syringe casting (Part 07671-20160, Fig. 6–7, ref. 1). This was easily accomplished with a 1-cm circular file. The holder is then held in place with a washer and hexagonal brass nut.

The syringe is held firm in the holder by inverting the syringe bracket (Part 07670-20650), placing a rubber or asbestos washer on top of the syringe and screwing the bracket down tightly.

To enable the plunger to reach the zero mark on the syringe once clamped in the plunger holder, the top 1 cm of the syringe must be broken off. This is easily accomplished with a triangular file and glass-blowing torch.

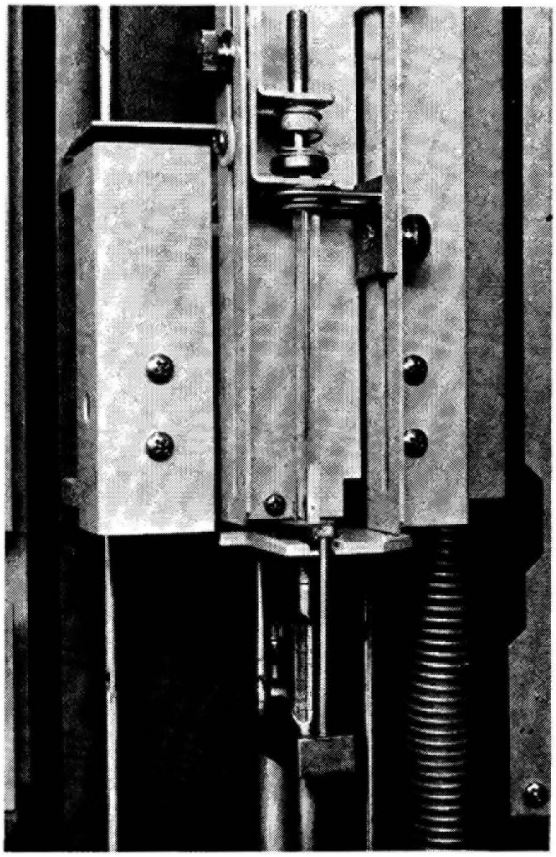

Fig. 2. Modified syringe holder and S.G.E. 10-μl syringe installed in a Hewlett-Packard Model 7671 Autosampler.

The modified syringe holder and syringe are shown installed in the Hewlett-Packard 7671 Autosampler in Fig. 2.

Since this modified syringe holder has been in use with S.G.E. 10-μl syringes (about 4 months) we have had no bent or broken plungers or needles.

S.G.E. have recently developed a series of syringes (0.4–5-μl plunger-in-needle and 10-μl removable needle) designed for use with the Hewlett-Packard 7670 and 7671 Autosamplers using the 1-μl adaptor supplied with the sampler. We have tried one of these (a 5-μl No. 5BL-FV23 kindly donated by S.G.E.) with satisfactory results, but we feel that the modification described in this paper offers greater support for the syringe and needle than when the 1-μl adaptor is used and also allows the use of cheaper syringes, thus offsetting the cost of the modification.

REFERENCE

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CHROM. 7580

Note

Use of a Peltier unit as a solid-state heat of sorption detector

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(Received May 10th, 1974)

The thermal conductivity detector has been a standard in the area of measurement of fixed gases and is still the method of choice in a wide number of inorganic separation techniques. This detector is relatively simple, rugged and easy to operate and at the same time provides reasonable sensitivity. We have been interested in gas phase detectors that were capable of operating under adverse conditions in either continuous or discrete sampling modes. We have found that a thermal detector may be built using a commercially available Peltier unit (Asarco Intermetallics, New York, N.Y., U.S.A.). The detector is based upon the heat of sorption between the sample in a flowing gas stream and the sorptive material packed into the detector volume. The principle is somewhat analogous to the Varian Associate's liquid chromatography detector which uses thermistors as a detector element.

MATERIALS AND PROCEDURES

Apparatus

A Varian dual-column gas chromatograph with dual thermal conductivity (TCD) and flame ionization detectors was used. The chromatograph was equipped with two 6-ft. × 1/4-in.-O.D. Teflon (DuPont, Wilmington, Del., U.S.A.) columns packed with Poly Pack 2 (Hewlett-Packard, Avondale, Pa., U.S.A.).

Operating conditions were as follows: carrier gas, helium at 50 ml/min; column temperature, 90° (isothermal); detector temperature (TCD), 90°; detector temperature (sorption), ambient; injection port temperature, 140°; attenuation

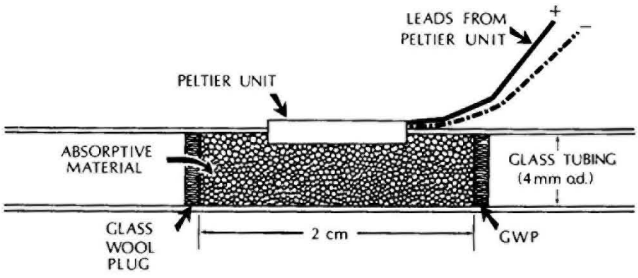

Fig. 1. Construction of the sorption detector.

(T.C.), ×156; attenuation (sorption), direct to recorder; chart speed, 1 in./min; recorder (Texas Instruments, dual pen), 1 mV.

The sorption detector was constructed as shown in Fig. 1. The device is obviously extremely simple and care need only be taken to assure a leak-free connection between the Peltier unit and the glass tube and to be sure that cadmium solder (low noise) is used in all electrical connections.

In the operation shown, the glass tube of the detector was attached to the effluent of the TCD such that samples leaving the TCD passed immediately into the sorptive detector.

RESULTS AND DISCUSSION

Two applications of the detector were attempted. In the first a 5A molecular sieve material was used to pack the detector volume. The use of this arrangement to detect water vapor in air is shown in Fig. 2. The usual adsorption–desorption curve for the water vapor was observed with reasonable sensitivity when one considers that the cost of the detector materials is less than $ 20.00, exclusive of the recorder.

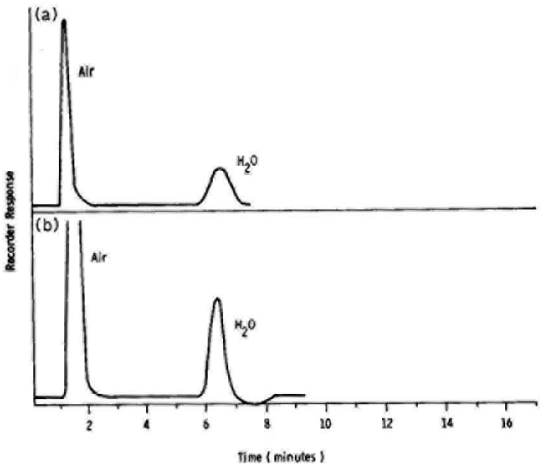

Fig. 2. Separation of water vapor in air. The column used was as described in the text. (a) Response of the TCD. (b) Response of the sorption detector packed with 5A molecular sieve.

In the second application, shown in Fig. 3, a more unique application of the detector is shown using the same samples. In this case the detection volume is packed with activated alumina. It is apparent that in this mode the detector responds only to the water vapor and thus the same determination may be made without the use of a column.

In addition, the use of alumina to detect water eliminates the bothersome desorption curve.

It is thus felt that the design offers certain advantages in applications where extreme simplicity and rugged design, coupled to reasonable sensitivity, are required. In addition, the sensitivity of the device could be enhanced by the use of a d.c.

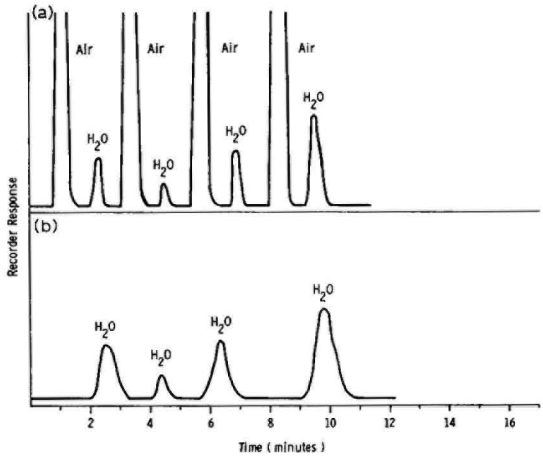

Fig. 3. Selective response of the sorption detector. (a) Response of the TCD. (b) Response of the sorption detector (alumina). It is apparent that only water vapor gives a response in the sorption detector.

amplifier in front of the recorder; however, this would greatly increase the cost and complexity of the system.

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Note

Gas chromatography of higher polyamines on Tenax-GC columns

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(Received May 13th, 1974)

In this journal Casselman and Bannard[1] have reported the separation of polyamine mixtures by gas chromatography using three polymer packings. Specifically, aliphatic amines up to but not including tetraethylenepentamine (TEPA) were separated without the problems associated with coated diatomaceous earths and PTFE packings. However, TEPA was not eluted from those workers' columns of Porapak Q, Porapak Q impregnated with KOH, and Chromosorb 103.

This note reports the successful elution of TEPA and the resolution of several components in samples of TEPA and triethylenetetramine (TETA) using the chromatographic packing Tenax-GC, a porous polymer based on 2,6-diphenyl-*p*-phenylene oxide developed by Van Wijk[2].

EXPERIMENTAL

Tenax-GC, 60–80 mesh (Applied Science Labs., State College, Pa., U.S.A.), was packed by conventional means into a 5-ft. length of 1/8-in.-O.D. (0.055-in.-I.D.) stainless-steel tubing. The column was coiled and installed in a Model 1520 flame ionization chromatograph (Varian, Walnut Creek, Calif., U.S.A.) with one end of the column fitted within the injector to allow for on-column injection. Injector and detector temperatures were maintained at 300° and the carrier gas (helium) at 50 ml/min. The polyamines TEPA and TETA were obtained from Dow Chemical (Midland, Mich., U.S.A.).

RESULTS AND DISCUSSION

A chromatogram (Fig. 1) of TEPA obtained by temperature programming showed several peaks which are thought to be position isomers[3]. Similar results were obtained for TETA without overlapping of any of the major peaks' retention times in the two chromatograms.

At higher temperatures and under isothermal conditions, TEPA and TETA gave individual sharp peaks which may exhibit only very small skewness similar to that obtained by Casselman and Bannard for later eluting polyamines.

Even when the temperature program was extended to 350° there was no column bleed detected under the most sensitive detector conditions.

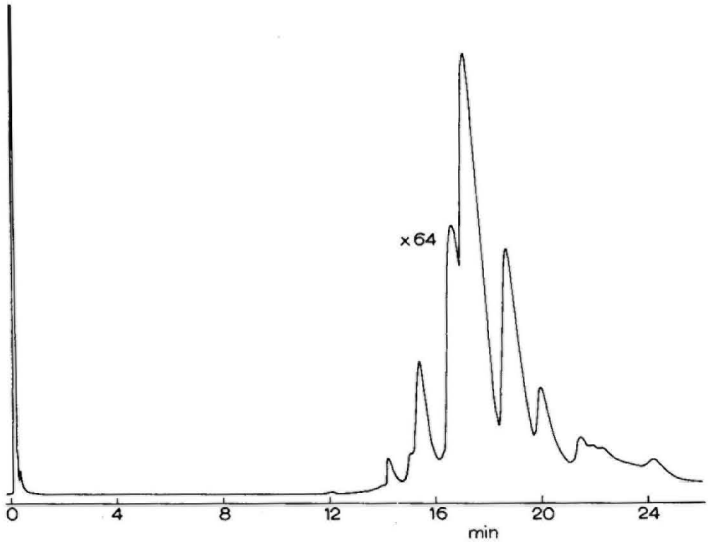

Fig. 1. Temperature-programmed chromatogram of TEPA. Injection, 1 μl of a 10% solution. Temperature program, 10°/min for 13 min, then 6°/min to 300°.

Tenax-GC offers a thermally stable column for gas chromatographic separation of higher polyamines and also may be used to examine and compare individual polyamines.

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CHROM. 7560

Note

Quantitative determination of semivolatile compounds in cigarette smoke

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The term "semivolatiles" is used to describe the volatile fraction of the particulate phase of smoke[1], and this fraction includes compounds with boiling points up to 250°. Graham[2,3] used a fraction trapping and transfer device with two gas chromatographic (GC) columns to separate fractions of the semivolatiles. Six fractions of this portion of smoke were analyzed by Neurath *et al.*[4] using column chromatography followed by gas chromatography–mass spectrometry (GC–MS). Grob and Völlmin[5] analyzed the semivolatiles on a single chromatographic column without prior fractionation. The objective of this work was to develop a GC analysis that would serve as a fingerprint of this fraction of smoke and which could be used for the quantitative determination of certain semivolatile compounds.

EXPERIMENTAL

Sample preparation

An internal standard was needed to perform the comparative and quantitative analyses. Undecane (99% pure, Chemical Samples Co., Columbus, Ohio, U.S.A.) was used as the internal standard because it did not interfere with any peak of measurable size in the chromatogram. The internal standard solution was 14.8 μg of undecane/ml of a 1:1 mixture of acetone–methylene chloride.

One brand of domestic filter blend cigarettes was used to obtain the quantitative data. A cold trap[6] containing 4 ml of the internal standard solution was immersed in liquid nitrogen. The cigarettes were smoked under standard conditions (one 35-ml, 2-sec puff/min) to a 27-mm butt length on a Filtrona 20-port smoking machine connected directly to the trap. The whole smoke condensed as it came into contact with the cold glass. After forty cigarettes had been smoked into the trap, it was disconnected from the smoking machine and was warmed to room temperature. The whole smoke mixture was diluted to 5 ml with the internal standard solution.

Gas chromatographic conditions

An open tubular column was used for the separation of the semivolatiles. The 525-ft. × 0.012-in. I.D., glass capillary was coated with methyl silicone OV-101 liquid phase. The carrier gas, nitrogen, was regulated at a flow-rate of 1.85 ml/min

with a split ratio of 1:1. The column temperature was maintained at 30° for 12 min and was then programmed to 200° at 2°/min. A flame ionization detector was used to obtain the chromatogram shown in Fig. 1.

Mass spectrometric conditions

Mass spectra were recorded for the separated components of the complex semivolatile mixture with the Finnigan Model 1015 S/L gas chromatograph–mass spectrometer. The mass spectrometer serves as the detector for the gas chromatograph, and the signal for the total ion monitor is displayed on a strip chart recorder to obtain the chromatogram.

Quantitative data

Known amounts of the semivolatile compounds were added to portions of the internal standard solution. Three solutions, having approximately one-half the amount, equivalent amounts, and twice the amount of these semivolatile compounds present in forty cigarettes per 5 ml, were prepared. Then forty cigarettes were smoked into 4 ml of each of the three spiked solutions.

A peak height ratio between the peak height of each semivolatile compound and that of the internal standard was determined. A calibration curve was obtained from a plot of the peak height ratio *versus* the amount of the compound added. The point for the peak height in a control sample is on the ordinate, at 0 μg spiked. The amount of the compound in the control sample is the point where the calibration curve crosses the abscissa[7]. The amounts of sixteen of these semivolatile compounds identified are given in Table I.

TABLE I

QUANTITATIVE DETERMINATION OF SEMIVOLATILE COMPOUNDS IN A DOMESTIC FILTER BLEND CIGARETTE

Compound	*Amount (μg/cigarette)*
Benzene	23.0
Dihydropyran	0.6
2,5-Dimethylfuran	58.0
3-Heptyne	2.0
Ethylcyclopentene	0.3
Toluene	44.0
Pyrrole	4.0
m- and *p*-Xylenes	19.0
Styrene	18.0
o-Xylene	6.0
Cumene	0.8
Benzaldehyde	48.0
Benzonitrile	20.0
Limonene	64.0
Indene	2.0
p-Cresol	14.0

RESULTS AND DISCUSSION

The objective of this work was not to identify new compounds in cigarette smoke but to develop a rapid method to obtain an overall chromatographic picture of the semivolatile fraction of smoke. The analysis of the semivolatiles requires approximately 2 h and includes compounds with boiling points of 80° (benzene) to 246° (nicotine). An elaborate system of traps, extractions, valves, and chromatographic columns was avoided to give a simple analysis.

The compounds identified in the semivolatile fraction and the methods of identification are given in Fig. 1 and Table II. Certain of these compounds were quantitatively determined on a popular domestic filter blend cigarette (Table I). This GC method can be used to compare the amounts of these compounds in various cigarettes.

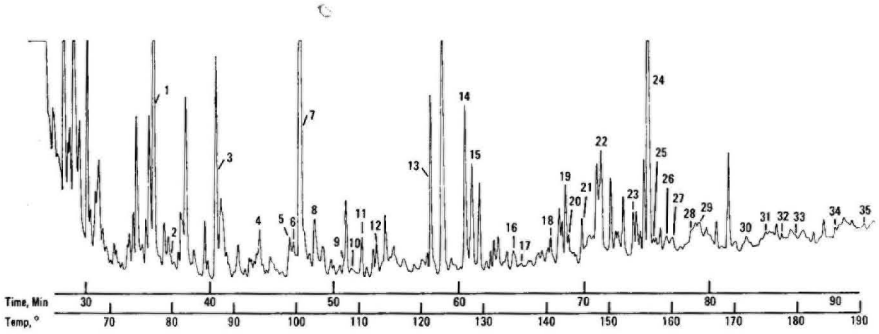

Fig. 1. Gas chromatogram of semivolatiles of a domestic blend filtered cigarette. See Table II for peak identification.

TABLE II

SEMIVOLATILE COMPOUNDS IDENTIFIED IN CHROMATOGRAM IN FIG. 1

No.	*Compound*	*Method of identification*	*No.*	*Compound*	*Method of identification*
1	Benzene	GC,MS	18	Benzaldehyde	GC,MS
2	Dihydropyran	GC	19	C_3-Benzene	MS
3	2,5-Dimethylfuran	GC,MS	20	Benzonitrile	GC,MS
4	Vinylcyclopentene	MS	21	C_3-Benzene	MS
5	3-Heptyne	GC,MS	22	C_3-Benzene	MS
6	Ethylcyclopentene	GC	23	Diethylbenzene	MS
7	Toluene	GC,MS	24	Limonene	GC,MS
8	Paraldehyde	GC	25	Indene	GC,MS
9	Pyrrole	GC,MS	26	Phenol	MS
10	Dimethylhexadiene	MS	27	C_4-Benzene	MS
11	2-Methylpyrazine	MS	28	Methoxyphenol	MS
12	Furfural	GC,MS	29	*p*-Cresol	GC,MS
13	*m*- and *p*-Xylenes	GC,MS	30	Methylindane	MS
14	Styrene	GC,MS	31	Methylindene	MS
15	*o*-Xylene	GC,MS	32	C_5-Benzene	MS
16	Cumene	GC,MS	33	Naphthalene	GC,MS
17	5-Methyl-2-furaldehyde	GC,MS	34	Ethylphenol	MS
			35	Trimethylphenol	MS

Most of the values given in the literature for the amounts of these compounds in cigarette smoke are for unfiltered cigarettes[3,8]. Both limonene and 2,5-dimethylfuran (Table I) were present in the domestic filter blend cigarettes in considerably larger quantities than reported previously. Smaller amounts of other compounds were found because they were selectively removed by cellulose acetate filters.

A highly precise method was sacrificed for one that could be performed routinely. The compounds that are determined quantitatively are representative of different classes of compounds containing various functional groups. Correlations can be made between the presence and amounts of these compounds and the presence and amounts of similar compounds known to be present in cigarette smoke.

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CHROM. 7575

Note

A new spray reagent for organosulfur compounds

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(First received January 14th, 1974; revised manuscript received May 14th, 1974)

In recent years a widespread interest in the preparation, properties and chemistry of α-polyhalosulfoxides has developed[1–12]. During a recent study of the oxidative chlorination of some organosulfur compounds[11,12] we encountered a number of α-polychlorosulfoxides. Attempts to characterize these compounds by thin-layer chromatography (TLC) were initially frustrated by the failure of both phosphomolybdic acid[13] and tetracyanoethylene[14] sprays to visualize the spots.

Thiophenes, thiols, sulfides and disulfides have been reported[15,16] to give colored complexes when added to ammonium hexanitratocerate(IV) solution. Although the thiophene complexes have some stability, the others undergo rapid oxidation, giving rise to colorless solutions.

The addition of an alcohol to ammonium hexanitratocerate(IV) solution results in the formation of a deep red stable complex which is the basis of a qualitative test for organic hydroxyl groups[17,18]. The formation of complexes has been rationalized as depicted below, on the basis of titrimetric evidence.

$$ROH + Ce(NO_3)_6^{2-} \rightarrow Ce(OR)(NO_3)_5^{2-} + H^+ + NO_3^-$$

EXPERIMENTAL

Chemicals

Compounds 1 and 2 (*cf.* Table I) were supplied by Dr. R. W. Frei; compounds 3, 4, 6 and 10 were purchased from Eastman-Kodak (Rochester, N.Y., U.S.A.); compounds 11 and 17 from Fluka (Buchs, Switzerland); and compound 16 was obtained from Fisher Scientific (Fair Lawn, N.J., U.S.A.). Ammonium hexanitratocerate(IV) was obtained from the BDH (Poole, Great Britain). Silica gel HF_{254} was purchased from Merck (Darmstadt, G.F.R.).

Compound 5 was prepared as described by Tsung and Chi[19], compounds 7–9, 12–14 and 17 were prepared as described by Bordwell and Pitt[20], or Truce *et al.*[21]; compounds 19–24 were prepared as we have described elsewhere[12] and compound 18 was prepared as described by Tsuchihashi and Ogura[22].

The appropriate sulfinyl chlorides may be prepared as we have described elsewhere[12]. The sulfinate esters 25 and 26 are prepared by the dropwise addition of the sulfinyl chloride to an excess of cold methanol, followed by stirring at room temperature for 1 h, and distillation at reduced pressure.

General procedure

The plates were made with silica gel HF_{254} (750-μm thickness) using a Desaga TLC applicator. Plates were air dried for 24 h before use. A standard solution of ammonium hexanitratocerate(IV) solution was prepared following the procedure of Shriner *et al.*[18]. Ammonium hexanitratocerate(IV) (40 g) was dissolved in 2 *N* HNO_3 (100 ml). Plates were spotted with the appropriate amount of compound (see Table I), developed 15 cm, air dried, and sprayed with ammonium hexanitratocerate(IV) solution. Spots for the non-sulfoxides appeared almost immediately as colorless areas on a yellow background. The α-polychlorosulfoxide spots required vigorous heating on a hot plate in order to be visualized as yellow-brown areas on a colorless background.

TABLE I

RESULTS FOR THIOLS, SULFIDES, SULFOXIDES, AND SULFINATE ESTERS

No.	*Compound*	*Solvent*	*Color**	*Temp.***	R_F	*µg/spot*
	Thiols					
1	2-Benzimidazolethiol	EtOAc	C	RT	0.89	80
2	2-Mercaptonaphthalene	$CHCl_3$	C	RT	0.83	92
3	3-Mercaptopropionic acid	$CHCl_3$	C	RT	0.18	70
4	Benzenethiol	CCl_4	C	RT	0.62	80
5	Benzylthiol	$CHCl_3$	C	RT	0.91	82
	Sulfides					
6	Di-*n*-butyl	CCl_4	C	RT	0.60	85
7	$CH_3(CH_2)_3 \cdot S \cdot CHCl(CH_2)_2CH_3$	CCl_4	C	RT	0.72	88
8	Phenyl trichloromethyl	CCl_4	C	RT	0.79	90
9	Phenyl chloromethyl	CCl_4	C	RT	0.49	87
10	Phenyl methyl	CCl_4	C	RT	0.74	89
11	Thiacyclopentane	CCl_4	C	RT	0.45	90
12	Bischloromethyl	C_6H_6	C	RT	0.82	100
13	Methyl trichloromethyl	C_6H_6	C	RT	0.89	95
14	Methyl dichloromethyl	C_6H_6	C	RT	0.72	90
15	Methyl chloromethyl	C_6H_6	C	RT	0.67	90
	Sulfoxides					
16	Dimethyl	Acetone	YB	H	0.20	90
17	Di-*n*-propyl	Acetone	YB	H	0.54	92
18	Methyl chloromethyl	EtOAc	YB	H	0.48	200
19	Methyl dichloromethyl	Et_2O	YB	H	0.49	600
20	Methyl trichloromethyl	$CHCl_3$–Et_2O(4:1)	YB (wk)	H	0.62	1,630
21	Chloromethyl trichloromethyl	CHCl	YB (wk)	H	0.63	2,820
22	Chloromethyl dichloromethyl	$CHCl_3$–Et_2O(4:1)	YB	H	0.55	1,630
23	Phenyl trichloromethyl	$CHCl_3$	YB	H	0.61	1,300
24	Bischloromethyl	Et_2O	YB	H	0.51	600
	Sulfinate esters					
25	Methyl chloromethane	MeOH–$CHCl_3$(2:1)	C	RT	0.75	250
26	Methyl benzene	$CHCl_3$	C	RT	0.39	200

* Abbreviations of colors: C = colorless; YB = yellow brown; (wk) = weakly developed.
** H = vigorous heating; RT = room temperature.

RESULTS AND DISCUSSION

Table I depicts the results obtained on a variety of sulfur functional groups including the α-polychlorosulfoxides, which were of primary interest to us.

In contrast to these functional groups, sulfones and sulfonyl chlorides could not be visualized with ammonium hexanitratocerate(IV) spray. Sulfinyl chlorides could be readily visualized if sprayed prior to developing; however, development of the sulfinyl chlorides led to decomposition so that no useful data could be obtained.

Results on TLC parallel those in solution. Unchlorinated sulfoxides give intensely red, stable complexes when added directly to ammonium hexanitratocerate(IV) solution. On the plates unchlorinated sulfoxides give orange-brown spots upon spraying. However, α-chlorinated sulfoxides (with the exception of chloromethyl methyl sulfoxide) fail to give the red complex or any sign of reaction at room temperature. The same results are realized on TLC unless the plates are vigorously heated (after spraying). After vigorously heating the plates yellow-brown spots appear on a colorless background.

Sulfides, thiols and sulfinate esters give colorless spots on a yellow background without heating. In solution the yellow reagent goes colorless in a vigorously exothermic reaction immediately upon addition of a sulfide, thiol or sulfinate ester. Such an observation, coupled with the absence of change when sulfones or sulfonyl chlorides are added to a solution of the reagent, is consistent with the proposal of an oxidation–reduction reaction as originally advanced by Hartough[16].

In general, one would anticipate that oxidation would proceed with more difficulty as the electron density on the sulfur atom was decreased, thereby impairing the sensitivity of an oxidizing spray such as ammonium hexanitratocerate(IV). Apparently, simply incorporating chlorine atoms on the carbon atom α to the sulfur atom (see sulfides in Table I), or an oxygen atom on the sulfur atom (see dimethyl sulfoxide and di-*n*-propyl sulfoxide in Table I) does not sufficiently reduce the electron density on the sulfur atom to alter the sensitivity of the spray. However, the presence of both chlorine atoms on the α carbon atoms and an oxygen atom on sulfur significantly reduces the electron density on the sulfur atom, thus substantially impairing the sensitivity of the spray, as expected (see compounds 18–24 in Table I).

CONCLUSIONS

The development of ammonium hexanitratocerate(IV) solution as a spray reagent for the detection of selected organosulfur compounds has been reported. It is believed to oxidize the organosulfur compounds which is more difficult in the case of sulfoxides due to the formation of stable complexes. Vigorous heating decomposes the complexes and permits the oxidation to occur, thereby providing a means to detect α-polyhalosulfoxides, a relatively new and interesting class of compounds.

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Note

δ-Aminolaevulinic acid synthetase

Synthesis of δ-aminolaevulinic acid pyrroles and their separation by thin-layer chromatography

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δ-Aminolaevulinic acid (ALA) synthetase is the rate-limiting enzyme of porphyrin biosynthesis in animal, bacterial and plant cells[1–10]. It catalyses the synthesis of δ-aminolaevulinic acid by the condensation of glycine and succinyl coenzyme A[1,11]. The activity of this enzyme is increased in the livers of patients with acute intermittent porphyria[12], a genetic abnormality of the regulation of heme synthesis, and in the livers of animals rendered porphyric by means of variety of drugs, referred to as experimental porphyria[13].

Granick and Urata[14] induced an experimental porphyria in guinea pigs by the administration of 3,5-diethoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine (DDC) and showed that there is a large increase in porphyrin synthesis resulting from a greater increase in the ALA-synthetase activity in liver mitochondria. Later, working with the elegant chick embryo liver cells technique, Granick[15] showed that the increased activity of this enzyme, induced by DDC, results from an increased synthesis of this enzyme, rather than from activation of the inactive enzyme. ALA-synthetase activity is usually measured by converting the ALA produced by the enzyme during incubation into a pyrrole by condensation with either ethyl acetoacetate or acetylacetone[16]. The pyrrole formed is reacted with Ehrlich's reagent and measured. This interest in ALA-synthetase has prompted us to study ALA pyrroles. The purpose of this paper is to describe the synthesis of ALA pyrroles from δ-ALA acid hydrochloride and β-keto esters and the use of TLC to characterize these compounds. The separation by TLC of ALA pyrroles does not seem to have been studied. This study has given important information regarding the effect of various ester and alkyl substituents in the 3- and 2-positions on the mobility of the ALA pyrroles during TLC separation.

MATERIALS AND METHODS

Benzyl acetoacetate was obtained by the method described in the literature[17]. Methyl, ethyl and *tert.*-butyl acetoacetate and ethyl propionyl and ethyl butyryl acetate were purchased from Aldrich (Milwaukee, Wisc., U.S.A.). δ-Aminolaevulinic acid hydrochloride was synthetized according to the method of Shemin *et al.*[5]. Ultraviolet absorption spectra were determined in absolute ethanol in a Unicam SP-800

TABLE I

UV AND IR DATA FOR ALA PYRROLES

Compound	R_1	R_2	*Yield (%)*	*M.p. (°C)*	*Infrared spectrum (cm^{-1})*		*Ultraviolet spectrum*				*Elemental analysis (%)*					
					NH	*CO*	$\lambda_{max.}$ *(nm)*	$\varepsilon \times 10^{-3}$	$\lambda_{max.}$ *(nm)*	$\varepsilon \times 10^{-3}$	*C*		*H*		*N*	
											Calculated	*Found*	*Calculated*	*Found*	*Calculated*	*Found*
1a	CH_3	CH_3	61	178–180	3300	1710 1665	232	8.37	260	5.8	56.86	56.62	6.20	6.15	6.63	6.50
2a	CH_3	C_2H_5	50	164–166	3300	1710 1665	233	8.25	260	5.79	58.65	58.55	6.71	6.56	6.22	6.17
3a	CH_3	$CH_2C_6H_5$	50	143–145	3280	1705 1665	233	11.8	260	7.42	66.88	66.65	5.96	5.80	4.88	4.92
4a	CH_3	$C(CH_3)_3$	67	144–146	3320	1705 1665	232	8.97	260	6.02	61.64	61.57	7.56	7.63	5.53	5.44
5a	C_2H_5	C_2H_5	45	153–155	3300	1710 1660	233	13.01	263	9.7	60.24	60.26	7.16	7.23	5.85	5.74
6a	C_3H_7	C_2H_5	37	123–125	3300	1715 1660	233	8.52	263	6.1	61.64	61.49	7.56	7.47	5.53	5.50

spectrophotometer. Infrared spectra were obtained in Nujol on a Perkin-Elmer Model 137E infrared spectrometer. All melting points are uncorrected.

Standard conditions for the synthesis of ALA pyrroles

A solution of β-diketone (0.01 mole) in 20 ml of phosphate buffer (0.25 *M*; pH 6.6) was treated with equimolar amount of δ-aminolaevulinic acid hydrochloride (0.01 mole) and refluxed for 15 min. The solution was cooled and pH was adjusted to 7. The pyrrole formed was separated by filtration, washed and crystallized from aqueous ethanol.

Thin-layer chromatography (TLC) was carried out according to Roomi[18,19]. A suspension of 30 g of silica gel G (E. Merck, Darmstadt, G.F.R.) in 60 ml of water was spread on glass plates (20 × 20 cm) to a thickness of 250 μm with a Desaga applicator. The plates were dried at 105–110° for 30 min and stored in a desiccator. A 0.1% solution of the compounds in methanol was prepared and 2 μg of each compound was spotted 2 cm from the edge of the plate. The plate was then developed with 150 ml of the solvent system diethyl ether–*n*-hexane containing 2% glacial acetic acid (1:1). Usually 45 min were required for the solvent to travel a distance of 12–15 cm. The plates were then dried, sprayed with Ehrlich reagent[20] and, after keeping for 5 min at 100°, the pyrroles appeared as blue spots.

RESULTS AND DISCUSSION

Fig. 1 lists the various β-diketo esters used and ALA pyrroles formed and their separation by TLC. The UV and IR spectra of these compounds are recorded in Table I. The IR spectra of all the compounds showed bands around 3300 cm^{-1} due to the N–H and at 1710 and 1660 cm^{-1} due to C=O. The UV spectra of these compounds showed a maximum around 233 nm and a minimum around 260 nm. Reaction of these pyrroles with modified Ehrlich's reagent[16] gave a pink solution, which showed a maximum at 552 nm and a shoulder at 525 nm. Fig. 2 shows the absorption spectra of ALA pyrrole obtained with *tert.*-butoxy ester, which has

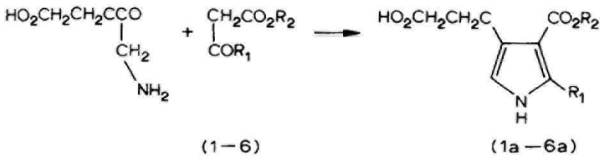

Fig. 1. Reaction scheme for synthesis of ALA pyrroles and their separation by TLC. Solvent system used: diethyl ether–*n*-hexane containing 2% glacial acetic acid (1:1).

No.	*Pyrrole*		*R_F × 100*
1a	$R_1 = CH_3$	$R_2 = CH_3$	24
2a	$R_1 = CH_3$	$R_2 = C_2H_5$	29
3a	$R_1 = CH_3$	$R_2 = CH_2C_6H_5$	33
4a	$R_1 = CH_3$	$R_2 = C(CH_3)_3$	43
5a	$R_1 = C_2H_5$	$R_2 = C_2H_5$	38
6a	$R_1 = C_3H_7$	$R_2 = C_2H_5$	43

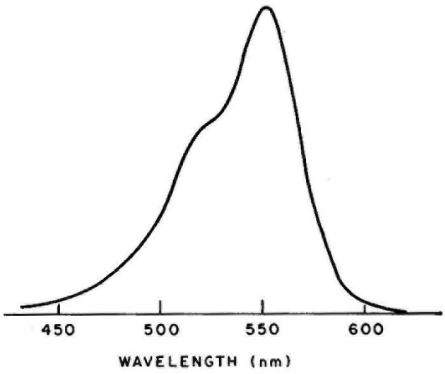

Fig. 2. Absorption curve for *tert.*-butoxy ALA pyrrole.

absorption maximum at 552 nm. The mechanism and the steps involved in the colour reaction of ALA pyrroles with Ehrlich's reagent are shown in Fig. 3.

The separation of ALA pyrroles (1a–6a) by TLC is shown in Fig. 1. 2-Methyl-3-carbethoxy-4-propionic acid pyrrole (2a) has higher R_F value than the 2-methyl-3-carbomethoxy-4-propionic acid pyrrole (1a) and are readily separated. Replacement of 2-methyl in 2a by either ethyl (5a) or propyl (6a) makes it less polar and it therefore then moves faster.

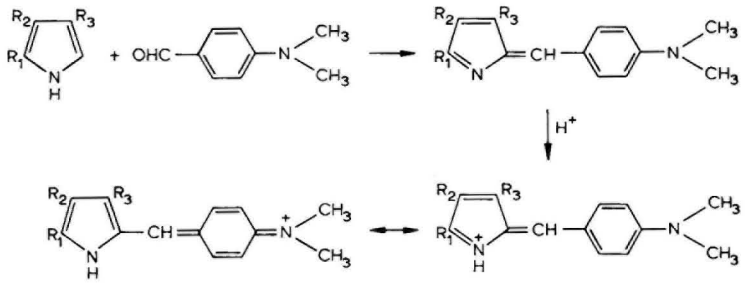

Fig. 3. Mechanism of colour reaction of ALA pyrroles with Ehrlich's reagent.

Similar substitution of 3-carbethoxy in 2a by more bulky groups, *viz.*, *tert.*-butoxy (4a) and benzyl (3a), also decreases the polarity of the compound and results in an increase R_F value. Hence the ALA pyrroles with different esters groups (methyl (1a), ethyl (2a), benzyl (3a) and *tert.*-butoxy (4a)) in the 3-position are separated from each other. Similar resolution was achieved among the ALA pyrroles having different alkyl substituents (methyl (2a), ethyl (5a) and propyl (6a)) in the 2-position. Thus all the ALA pyrroles synthesized in the present investigation can be separated from each other with the exception of 4a and 6a, which have the same R_F value.

Recently, ALA-synthetase activity has been measured in tissue obtained from liver biopsies[21] and in cultured cells[22,23]. The small amounts of tissue obtained by these procedures generate a small amount of ALA. The assay procedure utilizes ethyl acetoacetate to form the pyrrole prior to addition of Ehrlich's reagent. As *tert.*-butyl

acetoacetate ester gives a better yield than ethyl acetoacetate when used to form the ALA pyrrole, this compound may be more desirable to use in assays of ALA-synthetase.

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Note

Séparations en chromatographie sur couches minces des dérivés chlorophylliens tétrapyrroliques et application à la recherche de ces dérivés dans un fruit en cours de maturation*

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La chromatographie ascendante sur couches minces est fréquemment employée pour la séparation de faibles quantités de pigments liposolubles[1]. Si certains auteurs[2] distinguent jusqu'à 18 pigments dans des extraits d'algues ou de fruits en cours de maturation, d'autres[3–4] pensent que beaucoup de ces taches sont des produits de transformation des chlorophylles ou des caroténoïdes apparus *in vitro* au contact des adsorbants inorganiques.

Nous nous proposions de caractériser d'éventuels dérivés chlorophylliens de nature tétrapyrrolique pouvant apparaitre *in vivo* au cours de la maturation du fruit de *Lycopersicum esculentum* var. *cerasiforme* (tomate "cerise"). Pour cela nous avons défini des conditions assurant la non dégradation *in vitro* des pigments étudiés, puis avons appliqué, à un extrait volontairement enrichi en dérivés tétrapyrroliques artificiels, divers types de chromatographie sur couche mince. Nous avons alors sélectionné celui qui donnait la meilleure séparation d'un nombre maximum de taches, donc qui pouvait nous permettre de détecter les produits cherchés.

MATÉRIEL ET MÉTHODES

Matériel végétal et extraction des pigments

La mise au point des systèmes chromatographiques a été faite à l'aide d'extraits pigmentaires de feuilles fraîches de *Symphytum officinale*, réalisés sous conditions ambiantes de température, lumière et atmosphère, de façon à obtenir un maximum de dérivés tétrapyrroliques. Après broyage au broyeur à billes (type Dangoumau) les pigments sont extraits par de l'acétone aqueuse (concentration finale 80:20) puis transférés dans de l'éther de pétrole (30–50°). L'extrait ainsi obtenu est évaporé à sec et les pigments repris dans l'acétone. Lors de l'étude physiologique portant sur des fruits lyophilisés de tomate "cerise", pour éviter les dégradations *in vitro* des pigments, le broyage a été fait en présence de borate d'ammonium et d'azote liquide, l'extraction à une température voisine de 4° et à l'abri de la lumière.

* La conception de ces méthodes revient à Noël Delaporte, décédé.

Méthodes chromatographiques

Les essais, réalisés en chromatographie monodimensionnelle à l'aide de différents systèmes de solvants, ont porté systématiquement sur la qualité des revêtements (leur nature organique ou inorganique, leur épaisseur, leur polarité et l'importance du liquide de dispersion). Les revêtements servent soit de support à une phase stationnaire solide ou liquide, soit de phase stationnaire. Dans le premier cas, si la phase stationnaire est apolaire, il s'agit d'un système à *phases inversées*, par rapport aux systèmes courants pour lesquels la phase stationnaire est polaire, la phase mobile apolaire. La nature du revêtement a, dans ce dernier cas, beaucoup d'importance: ainsi les adsorbants inorganiques, type gel de silice, peuvent entraîner des transformations de pigments[3,4]. Certains de nos essais ont cependant porté sur de tels revêtements souvent utilisés[5,6], afin de comparer les résultats avec ceux obtenus sur des revêtements de nature organique préférés par quelques auteurs[7,8]. Nous avons combiné en systèmes bidimensionnels les solvants de développement les plus satisfaisants.

Techniques

Les revêtements sont déposés, sur des plaques de verre (20 × 20 cm), à l'aide d'un étaleur manuel (Shandon Unoplan), après homogénéisation au mixer (Omnimixer, Serval) dans un liquide que nous nommons liquide de dispersion. L'épaisseur du revêtement est généralement de 0.4 mm, sauf indication dans le texte. Les cuves sont hermétiques, certaines équipées pour admettre un courant d'azote et un enrichissement progressif en un solvant déterminé. D'autres cuves sont à front perdu. Il faut en moyenne 16 h pour saturer les cuves dans le cas de systèmes à phase stationnaire polaire, 1 h si les phases sont inversées ou s'il s'agit d'une chromatographie pour laquelle les forces d'adsorption sont dominantes. Dans tous les cas les développements se déroulent à l'obscurité à température ambiante et leur durée n'excède jamais 1 h 30 min. Le repérage des taches se fait à la lumière du jour et en lumière ultra-violette.

Produits utilisés

Revêtements organiques. Les revêtements organiques utilisés sont: Poudres de cellulose ordinaires, de grain 15–40 μm (CC31 Whatman) ou 10–25 μm (CC41 Whatman); cellulose imprégnée de polyéthylène-imine (PEI Merck). Poudre de saccharose du commerce, renfermant 3% de matières amylacées, tamisée au laboratoire pour obtenir des grains de 60–80 μm.

Revêtements inorganiques. Les revêtements inorganiques utilisés sont: Poudre de borate d'ammonium (Merck) tamisée (grains 60–80 μm). Poudre d'alumine (Merck). L'ascorbate de sodium (Merck) est employé comme adjuvant des différents revêtements.

Liquides de dispersion. Les liquides de dispersion sont indiqués dans le Tableau I pour chaque type de couche mince ainsi que les rapports volume/poids de poudre (ml/g).

Solvants de développement. Pyridine, acétonitrile, pentane, hexane, heptane et octane sont des produits Carlo Erba R.P.; les alcanes supérieurs des produits Schuchardt.

TABLEAU I

LIQUIDES DE DISPERSION EMPLOYÉS POUR LE DÉPÔT DES COUCHES MINCES ET RAPPORTS VOLUME DE LIQUIDE (ml)/POIDS DE POUDRE (g)

	CC31	*CC41*		*Saccharose*			*Borate d'ammonium*	*Alumine*
	Méthanol (Merck)	*Méthanol (Merck)*	*Azéotrope chloroforme–méthanol (78:22)*	*Acétone (Merck)*	*Chloroforme (Merck)*	*Chloroforme–hexane (50:50)*	*Azéotrope chloroforme–méthanol (78:22)*	*Chloroforme (Merck)*
Rapports (volume de liquide)/(poids de la poudre) ml/g	2.1	1.8	1.5	1.5	1.6	1.7	1.1	2.3

RÉSULTATS

Étude des qualités des revêtements et des solvants de développement pour les essais monodimensionnels

Systèmes à phase stationnaire polaire. L'acétonitrile, la pyridine et leurs azéotropes avec l'eau ont permis des séparations nettes seulement si les cuves sont à front perdu. Sur certains revêtements (saccharose, cellulose, borate d'ammonium, alumine) la pyridine et son azéotrope avec l'eau donnent des résultats médiocres si la phase mobile est du pentane ou de l'éther de pétrole (30–50°), et des résultats satisfaisants si la phase mobile utilisée est un alcane (de C_6 à C_8). Ainsi nous avons séparé neuf taches bien distinctes à l'aide des systèmes suivants: CC41/pyridine/hexane; saccharose/azéotrope pyridine–eau/hexane; borate d'ammonium/azéotrope pyridine–eau/heptane, et même dix taches sur saccharose si nous substituons l'octane à l'hexane*.

Systèmes à phases inversées. Ces essais ont été réalisés dans des cuves hermétiques avec les phases stationnaires suivantes: huile de vaseline, pentadécane, hexadécane, octadécane, la phase mobile pouvant être l'acétonitrile, la pyridine, leurs azéotropes avec l'eau, l'azéotrope hexane–propanol, des mélanges pentane–propanol ou des mélanges acétone–méthanol–eau. Le système saccharose/hexadécane (à 10% dans l'hexane)/méthanol–eau (93:7) et les systèmes borate d'ammonium/hexadécane (à 10% dans l'éther de pétrole, 30–50°)/acétone–méthanol–eau (40:40:8 ou 20:80:10) permettent de séparer dix taches dont la répartition et la netteté sont excellentes.

Au cours de ces essais, nous avons par ailleurs montré que la présence dans la phase mobile d'un alcane permettant la conservation à 4° des taches de pigments sur les plaques, ne gêne ni la migration de cette phase, ni la séparation des pigments.

Revêtements constituant eux-mêmes une phase stationnaire. La cellulose CC41, le saccharose et le borate d'ammonium ont maintenant servi de phases stationnaires dans des systèmes chromatographiques à élutions successives. Les résultats obtenus, portés dans le Tableau II, montrent que, sur saccharose, ces élutions séparent très nettement un grand nombre de pigments.

Nous avons tenu à vérifier que certaines de ces taches ne correspondaient pas à des dérivés apparus, par oxydation des pigments, lors des nombreux séchages à l'air séparant les opérations d'élutions successives, ou bien à des dérivés dûs à une attaque acide au cours des élutions elles-mêmes. Pour celà nous avons adjoint aux revêtements de l'ascorbate de sodium comme antioxydant et du borate d'ammonium qui évite toute attaque acide des chlorophylles ou de leurs dérivés. Sur saccharose la migration et la séparation des pigments a été trouvée inchangée, à condition de ne pas dépasser des teneurs de 0.4% en ascorbate de sodium, de 4% en borate d'ammonium et d'éluer avec de l'hexane enrichi à chaque fois de 0.3% en propanol au lieu de 0.4%.

Essais de chromatographie bidimensionnelle

Sur CC41, les résultats sont améliorés si le développement est effectué avec le système azéotrope pyridine–eau/hexane en première dimension et le système à quatre élutions successives, hexane enrichi à chaque opération de 0.6% de propanol (*cf.*

* Au cours de cette étude nous avons montré l'importance capitale du liquide de dispersion. Ainsi, lorsque le saccharose est déposé à l'aide du méthanol il n'y a aucune séparation avec le système pyridine–eau/hexane. Pour avoir de bonnes séparations des pigments le saccharose doit être déposé à l'aide d'acétone, de chloroforme ou d'un mélange chloroforme–hexane (50:50).

TABLEAU II

NOMBRE DE PIGMENTS SÉPARÉS À PARTIR D'UN EXTRAIT DE FEUILLES DE SYMPHYTUM PAR ÉLUTIONS SUCCESSIVES SUR DES REVÊTEMENTS CONSTITUANT EUX MÊMES LA PHASE STATIONNAIRE

L'azéotrope chloroforme–méthanol (78:22) est utilisé pour déposer la cellulose CC41 et le borate d'ammonium; le chloroforme pur pour déposer le saccharose. Dans tous les cas, pour une chromatographie effectuée sur une hauteur h par n éluants successifs: le premier éluant parcourt h/n en 5 à 10 min, le second $2\,h/n$ en 15 à 20 min et le $n$ième h. La durée totale des développements n'excède pas 1 h 30 min, le sèchage de la plaque entre deux élutions ne nécessite que quelques minutes.

Nature des revêtements	*Éluants successifs*	*Séparation des pigments Remarques*
CC41 (0.3 mm d'épaisseur)	(1) Hexane + 0.6% de propanol (2) Hexane + 1.2% de propanol (3) Hexane + 1.8% de propanol (4) Hexane + 2.4% de propanol	10 à 12 taches assez bien séparées
Saccharose	(1) Hexane (2) Hexane + 0.4% de propanol (3) Hexane + 0.8% de propanol (4) Hexane + 1.2% de propanol (5) Hexane + 1.6% de propanol	14 à 16 taches bien séparées
Borate d'ammonium	(1) Hexane (2) Hexane + 0.19% de propanol (3) Hexane + 0.38% de propanol (4) Hexane + 0.57% de propanol	7 à 10 taches maximum

Tableau II), en deuxième dimension. En lumière naturelle, treize taches vertes et trois taches jaunes, toutes bien séparées, sont dénombrées; en lumière ultra-violette nous distinguons deux nouvelles taches. Soit au total dix-huit taches.

Sur saccharose et borate d'ammonium les résultats sont encore plus satisfaisants. Vingt taches, dont cinq visibles en lumière ultra-violette seulement, sont séparées sur borate d'ammonium dans le système azéotrope pyridine–eau/heptane en première dimension et le système hexadécane à 7.5% dans l'hexane/acétone–méthanol–eau (20:80:10) en deuxième dimension. Quant au saccharose il peut permettre la séparation de vingt-quatre taches avec les deux systèmes suivants: azéotrope pyridine–eau/octane et hexane enrichi en propanol (système à élutions successives, *cf.* Tableau II). Le schéma de cette séparation est donné dans la Fig. 1.

Application à la recherche de dérivés chlorophylliens dans des extraits de fruits de tomate "cerise" en cours de maturation

Trois lots de fruits sont étudiés. Le premier est constitué de fruits verts adultes, c'est-à-dire cueillis au stade[9] marquant la fin de la croissance et le début de la maturation et où la teneur en pigments chlorophylliens est maximale[10]. Le deuxième est constitué de fruits cueillis rose-oranges ou rouges. Le troisième de fruits verts adultes laissés à lumière et température ambiantes jusqu'à ce qu'ils virent au rose ou rouge orangé.

L'application, aux trois lots de fruits, des meilleurs systèmes chromatographiques définis précédemment et en particulier du système saccharose/azéotrope pyridine–eau/octane en première dimension, hexane enrichi progressivement en propanol

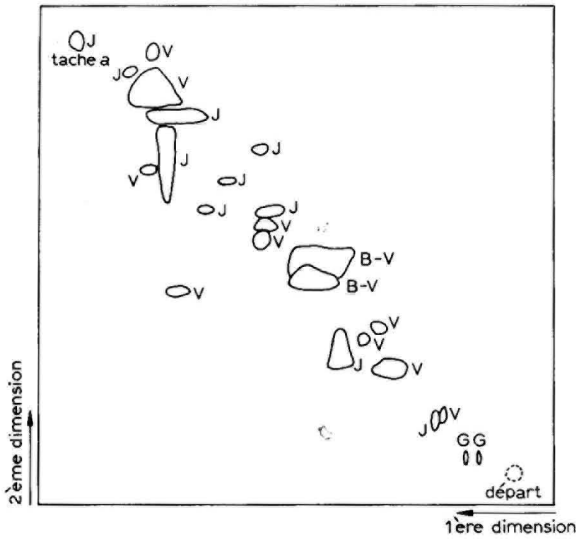

Fig. 1. Schéma du développement bidimensionnel, sur couche mince de saccharose, d'un extrait de feuilles de Symphytum. Le saccharose contient 4% de borate d'ammonium et 0.3% d'ascorbate de sodium. Première dimension: azéotrope pyridine–eau/octane; deuxième dimension: quatre élutions successives dans hexane puis hexane enrichi trois fois de propanol (0.3% à chaque fois). Abbréviations: B-V = bleu-vert; G = gris; J = jaune; V = vert. Au niveau de la tache *a* se retrouvent les carotènes non oxygénés les plus apolaires (*β*-carotène dans le cas d'un extrait de feuille ou de fruit de tomate verte; mélange phytofluène, lycopène et *β*-carotène dans le cas de fruits rouges).

en deuxième dimension, n'ont alors permis de séparer que deux pigments verts correspondant aux deux chlorophylles *a* et *b* en plus des caroténoïdes déjà définis[10].

CONCLUSION

Nous définissons un système de chromatographie sur couches minces de saccharose qui permet une séparation fine des carotènes oxygénés (époxy- et hydroxy-carotènes) et des pigments tétrapyrroliques d'origine chlorophyllienne. Sur un extrait pigmentaire obtenu en conditions telles, qu'il y a dégradation *in vitro* des chlorophylles, nous avons à l'aide de ce système pu dénombrer jusqu'à vingt-quatre taches dont une quinzaine correspondait à des composés tétrapyrroliques. Dans le cas des extraits de fruits mûrs préparés en présence d'azote liquide, à l'abri de la lumière et au froid, ce même procédé n'a jamais permis de détecter la présence de dérivés chlorophylliens tétrapyrroliques. Nous pensons donc, en accord avec certains auteurs[11], qu'au cours de la maturation les produits de dégradation des chlorophylles n'apparaissent pas sous forme de composés de nature tétrapyrrolique.

Des contrôles utilisant une distribution automatique d'éluant en gradient continu de polarité, sous atmosphère d'azote, ou l'adjonction d'antiacides ou d'antioxydants dans le revêtement des plaques, ont montré qu'il ne se produisait pas de dégradation des pigments séparés au cours des développements successifs.

Enfin, la présence d'alcane dans la phase mobile a pu permettre la conservation à 4° des pigments en place après chromatographie.

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Note

Micropreparative gas–liquid chromatography of methylated sugars

I. Preparative separation of partial methylation products from methyl β-D-xylopyranoside

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Recently, we have shown[1] that, depending on the procedure and conditions used for the partial methylation of methyl β-D-xylopyranoside, different methylated derivatives are produced. Analytical gas–liquid chromatography (GLC) was successfully used for the quantitative and qualitative analysis of methylated methyl glycosides as the corresponding acetates. These mixtures may serve as starting materials for obtaining authentic methylated sugar samples which are essential for a structural study of the carbohydrate chain by methylation.

While we were completing this work, Fournet and Montreuil[2] reported that GLC can be used for the preparative separation of small amounts of products obtained by partial methylation of methyl α-D-mannopyranoside.

In this work, methyl β-D-xylopyranoside was used to show the possibility of and to select suitable conditions for the separation on a preparative scale of considerable amounts of methylated methylglycoside acetates (MMGA) obtained by partial methylation.

Previous results from the study of the partial methylation of methyl β-D-xylopyranoside by different methods[1] were used to obtain the various methyl ethers.

EXPERIMENTAL

Partial methylation of methyl β-D-xylopyranoside was achieved by the procedures of Kuhn and Trischman[3], Haworth[4] and Purdie and Irvine[5].

Kuhn and Trischman methylation

The starting xyloside (4 g) was dissolved in dimethylformamide (120 ml) with subsequent addition of barium oxide (24 g), barium hydroxide octahydrate (1.9 g) and methyl iodide (24 ml). The solution was stirred for 1.5 h in the dark. The reaction mixture was treated as described previously[1] to yield 5.1 g of MMGA.

Haworth methylation

The starting xyloside (2 g) was dissolved in water (10 ml) and stirred on a

TABLE I

CHARACTERIZATION OF METHYLATED METHYL β-D-XYLOPYRANOSIDE ACETATES (MMGA) AS PRODUCTS OF PARTIAL METHYLATION FOLLOWED BY GLC SEPARATION*

Methyl ether	$T_{2,4\text{-}xyl}$**	*M.p. (°C)***	$[\alpha]_D^{20}$ *in CHCl* *	*Yield in accordance with procedure of*								
				Kuhn and Trischman[3]			*Haworth*[4]			*Purdie and Irvine*[5]		
				Preparative data		*Analytical data*	*Preparative data*		*Analytical data*	*Preparative data*		*Analytical data*
				mg	*%*	*%*	*mg*	*%*	*%*	*mg*	*%*	*%*
2, 3, 4	0.19	48.5–49.5° (49–50°, ref. 6)	−68.3° [3.0] (−73°, ref. 6)	27	4.5	4.0	110	18.3	22.2	2	0.3	1.4
2, 3	0.64	51.5–52.5°	−79.4° [3.6]	56	9.3	21.9	31	5.2	10.1	8	1.3	2.3
2, 4	1.00 (5.8 min)	Syrup	−60.9° [3.6]	105	17.5	32.0	151	25.2	42.8	20	3.3	8.8
2	1.72	94–95°	−35.3° [3.6]	53	8.8	14.0	46	7.7	12.7	68	11.3	21.0
4	2.28	119–121°	−80.2° [1.5]	19	3.2	7.3	30	5.0	10.9	43	7.2	12.3
3	2.83	86–88°	−77.5° [2.6]	40	6.7	14.3	3	0.5	1.3	36	6.0	13.9
Parent	4.72	118–119.5° (115°, ref. 7)	−60.1° [3.1] (−60.8°, ref. 7)	27	4.5	6.5	—	—	—	186	31.0	40.3
			Total yield	327	54.5	100.0	371	61.9	100.0	361	60.4	100.0

* 600 mg of MMGA were separated by GLC.

** Retention times relative to peracetate of methyl 2,4-di-O-methyl-β-D-xylopyranoside (analytical column).

*** Reference data are given in parentheses. Concentrations are given in brackets.

magnetic mixer in an argon flow with subsequent dropwise addition of dimethyl sulphate (20 ml) and 30% aqueous sodium hydroxide solution (34 ml) for 2 h. Following degradation of dimethyl sulphate and neutralization of the solution, its volume was made up to 100 ml with water and extracted with chloroform (2 × 200 ml). The mixture of methyl ethers obtained was acetylated as usual to yield 1.5 g of MMGA.

Purdie and Irvine methylation

Methyl β-D-xyloside (5 g) was dissolved in methanol (100 ml) with subsequent addition of methyl iodide (20 ml) and inactivated silver oxide (30 g). The solution was stirred on a magnetic mixer for 2 h in the dark. The reaction mixture was treated as described previously[1] to yield 8.2 g of MMGA.

Gas–liquid chromatography

A Tsvet-106 (U.S.S.R.) chromatograph with dual columns (100 × 0.3 cm) and flame ionization detectors was used for analysis. The columns were packed with butanediol succinate (10%) on 80–100 mesh Chromosorb W. The thermostat and batcher temperatures were 160° and 300°, respectively. The carrier gas was argon at a flow-rate of 30 ml/min.

A Tsvet-3-66 (U.S.S.R.) chromatograph with U-shaped stainless-steel columns, A (100 × 0.8 cm) and B (200 × 1.4 cm), equipped with a preparative attachment and flame ionization detector was used for GLC on a micro-preparative scale. The columns were packed with butanediol succinate (10%) on 60–80 mesh Chromosorb A. The temperature in the evaporator and collector was 240°. The thermostat temperature was 150° when using column A and 170° when using column B. The argon flow-rates with columns A and B were 200 and 300 ml/min, respectively. Straight glass tubes (6 × 0.5 cm) having narrow openings and connected with receivers served as traps. When operating column A, up to 100 mg of the mixture of MMGA were introduced into the chromatograph as a concentrated solution in chloroform. When operating column B, up to 600 mg of the MMGA mixture were introduced into the chromatograph as a concentrated solution in chloroform (*ca.* 0.8 ml). The specific rotation of MMGA (in chloroform) was measured on a Perkin-Elmer 141 instrument.

RESULTS AND DISCUSSION

When the 100-cm column packed with 10% butanediol succinate on 60–80 mesh Chromosorb W was used for analysis, complete separation of the methylated derivatives of methyl β-D-xylopyranoside as the corresponding acetates was achieved. It should be noted that the 3,4-dimethyl ether does not result from partial methylation.

The conditions for the micro-preparative GLC were virtually the same as those in the analytical version. The load on column A was *ca.* 100 mg of the mixture of MMGA, and the yield of individual methyl ethers acetates was about 5–10 mg, depending on their contents in the mixture. High loads resulted in a considerable decrease in column efficiency and did not permit effective separation of the methyl ether mixture. In order to compensate for the loss in efficiency, the length of the column was increased to 200 cm and the I.D. to 1.4 cm, and it was then possible to increase the load on the column to 600 mg of mixture without a noticeable decrease in efficiency.

At present, it is known that the principal factor responsible for decreased efficiency is the component displacement rate profile over the column section, this rate profile increasing as the column diameter increases. Hence the efficiency of the analytical column used is equal to 400 theoretical plates while for the 100- and 200-cm preparative columns, it is 90 and 150 theoretical plates, respectively. Thus, when the column diameter was increased from 0.8 to 1.4 cm, *i.e.*, 1.7-fold, and the column length 2-fold, the efficiency of a 2-m column increased 1.7-fold compared with a 1-m column. At the same time, the cross-sectional area increased 3-fold thus allowing the overall load to be increased at least 3-fold and the same specific load to be retained. The possibility of using even longer columns is handicapped by the increased time required in order to give the same component yield and the pressure differential in the column, which naturally results in a lower efficiency. Table I shows the yields and characteristics of methylated methyl β-D-xylopyranoside acetates obtained with partial methylation by the Kuhn and Trischman[3], Haworth[4] and Purdie and Irvine[6] procedures with subsequent separation by GLC on a micro-preparative scale. In each instance, the load on the column was 600 mg.

The MMGA isolated were individual compounds, and under analytical GLC conditions resulted in a single peak, the analytical and theoretical data virtually coinciding. It is noteworthy that all of the individual components obtained, except for the 2,4-dimethyl ether, instantly crystallized in a trap. We used small glass tubes as traps, bearing in mind that MMGA are high-boiling liquids, which do not require special cooling of receivers. The total recovery was about 70%, the chromatographic recovery factor for mono- and dimethyl ethers being about 60%.

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Note

A multi-residue extraction procedure for the gas chromatographic determination of the herbicides dichlobenil, dinitramine, triallate and trifluralin in soils

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Analytical procedures for the simultaneous extraction and gas chromatographic determination of residue combinations of several insecticides in soils are well established[1–3]. Although procedures for the multi-residue extraction of herbicides from soils for gas chromatographic analysis have been reported, these methods are mainly for the assay of herbicides belonging to specific groups, such as the triazines[4,5] or ureas[6].

In Saskatchewan, the carry-over of some soil-based herbicide residues from one growing season to the next has been reported[7]. The procedure to be described here was developed for the routine extraction and gas chromatographic estimation of four such persistent chemicals in soils, either alone or in combination.

MATERIALS AND METHODS

Soils

The composition and physical characteristics of the soils used in these studies are shown in Table I.

TABLE I

CHARACTERISTICS OF SOILS

Soil	*Clay (%)*	*Silt (%)*	*Sand (%)*	*Organic carbon (%)*	*pH in water (1:1)*
Jameson sandy loam	6	9	85	3.2	7.5
Regina heavy clay	69	26	5	4.2	7.7
Melfort silty clay	30	38	32	11.7	5.2

Herbicides

The following technical-grade herbicides were used in these studies: dichlobenil (2,6-dichlorobenzonitrile), dinitramine (N,N-diethyl-α,α,α-trifluoro-3,5-dinitrotoluene-2,4-diamine), triallate (S-2,3,3-trichloroallyl diisopropylthiocarbamate)

and trifluralin (α,α,α-trifluoro-2,6-dinitro-N,N-dipropyl-*p*-toluidine). A combined stock solution was prepared containing 50 μg of each herbicide per milliliter of methanol.

Soil fortification

A portion (20 or 200 μl) of the mixed herbicide solution was added to each of several 20-g samples of the sieved air-dried soils in screw-capped glass bottles (capacity 70 ml). Thus, the soils were fortified with 1 or 10 μg of each individual herbicide to give a soil concentration of 0.05 or 0.5 ppm. After mixing to ensure distribution of the chemicals throughout the soil samples, the bottles containing the fortified soils were capped and frozen at $-5°$ for 8 weeks, after which four replicate samples for each soil type and herbicide concentration were extracted and analysed.

Extraction procedure

Each fortified soil sample (20 g) was placed in a 100-ml beaker together with 60 ml of acetonitrile–water (9:1) and extracted for 2 min using a Sonic Dismembrator (Artek Systems Corp., Farmingdale, N.Y., U.S.A.) at maximum power. After settling, 30 ml of the acetonitrile solution, corresponding to 10 g of soil, were added to 150 ml of distilled water plus 20 ml of saturated aqueous sodium sulphate solution, and the mixture was shaken twice with 25-ml portions of *n*-hexane. Following extraction, the aqueous phase was discarded, the combined *n*-hexane layers were dried over anhydrous sodium sulphate, and 3 or 4-μl aliquots were examined by gas chromatography.

Gas chromatographic analysis

A Hewlett-Packard 5713 A gas chromatograph equipped with means for on-column injection and a nickel electron-capture detector (operated at 300°) was used. The glass column (1.5 m $\times$ 6.0 mm O.D.) was packed with 10% of OV-1 on Chromosorb G-HP (80–100 mesh), and the carrier gas was argon containing 5% of methane at a flow-rate of 40 ml/min. With a column temperature of 190° the retention times for dichlobenil, trifluralin, dinitramine and triallate were 0.95, 2.85, 4.5 and 5.0 min, respectively.

Chromatographic standards were prepared by adding 20 or 200 μl of the mixed herbicide solution to 100 ml of *n*-hexane, to give solutions containing 0.01 or 0.10 ng of the four chemicals per μl. The concentrations of the various herbicides present in the samples were calculated by comparing the sample peak heights with those of the appropriate standard.

RESULTS AND DISCUSSION

Typical gas chromatograms obtained from the standard solution containing 0.01 ng/μl of each herbicide, and from Jameson sandy loam fortified at the level of 0.05 ppm, indicated (see Fig. 1) that the 10% OV-1 column gave a good resolution of the individual herbicides; although resolution between dinitramine and triallate was not complete, adequate quantitative measurements could be made. Recoveries of the four herbicides from treated soils (see Table II) were all greater than 90%, and reproducibility was excellent. Separate experiments showed that no interfering sub-

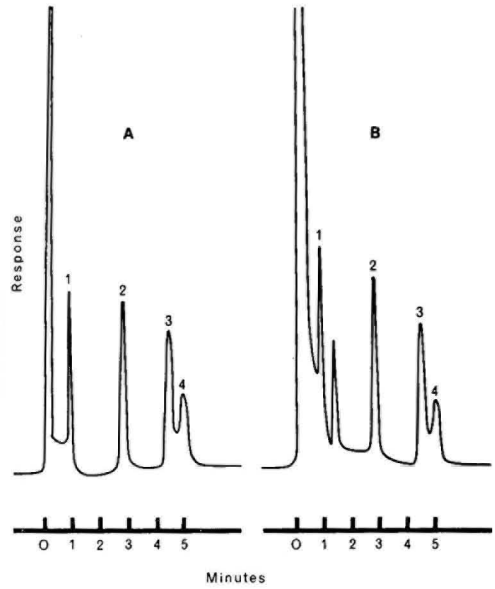

Fig. 1. Chromatogram of (A) 4 μl of standard solution containing 0.04 ng of dichlobenil (1), trifluralin (2), dinitramine (3), and triallate (4); and (B) Jameson sandy loam fortified with 0.05 ppm of the four herbicides.

stances were detected in any of the untreated soils, even at the high attenuations required for the analysis of the lower concentrations of herbicides in the soils.

The soil samples were frozen for 8 weeks before analysis, as it is the practice in this laboratory to air-dry field soils containing herbicide residues to constant weight at room temperature and then to store them at $-5°$ while awaiting extraction and analysis.

A mixture (2:1) of benzene and isopropanol has been used for the extraction dichlobenil[8], triallate[9] and trifluralin[10] from soils. For dinitramine, this solvent mixture gave recoveries of less than 50% from all three soil types. Acetonitrile–water (9:1)

TABLE II

RECOVERY OF DICHLOBENIL, TRIFLURALIN, DINITRAMINE AND TRIALLATE FROM FORTIFIED SOILS

Soil	*Amount added (ppm)*	*Recovery (%)**			
		Dichlobenil	*Trifluralin*	*Dinitramine*	*Triallate*
Regina heavy clay	0.05	92 ± 5	96 ± 5	96 ± 8	96 ± 6
	0.5	93 ± 1	104 ± 4	106 ± 4	101 ± 3
Jameson sandy loam	0.05	92 ± 9	92 ± 11	93 ± 9	95 ± 5
	0.5	93 ± 2	106 ± 4	107 ± 4	100 ± 1
Melfort silty clay	0.05	94 ± 5	106 ± 12	90 ± 8	112 ± 12
	0.5	97 ± 3	101 ± 3	102 ± 3	105 ± 4

* Mean and standard deviation from four determinations.

was finally selected as extracting solvent with excellent results (see Fig. 1 and Table II).

The proposed procedure for the recovery of triallate at the level of 0.5 ppm from the three soils was compared with methods using benzene and isopropanol[9] or trimethylpentane and isopropanol[11] as extractants. The last two solvent systems gave recoveries of less than 60% from dry soils. However, recoveries were almost 100%, and thus comparable to those obtained by using aqueous acetonitrile, when the soils were moistened to the wilting point and equilibrated overnight before extraction. Thus, an advantage to using acetonitrile as extracting solvent is that samples containing triallate do not have to be moistened with water before extraction[12].

With the present procedure, residues of dichlobenil, dinitramine, triallate and trifluralin (singly, or in the presence of each other) can be detected in a variety of soils simply, rapidly and accurately at levels down to 0.05 ppm. The herbicide recoveries obtained (see Table II) are also comparable to, or better than, those attained by using methods[8,13–16] outlined for their individual analyses.

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Note

Conditions for the use of Merck silica gel 60 F_{254} plates in the standardized thin-layer chromatographic technique for lichen products

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Thin-layer chromatography is an important tool for the identification of secondary products in lichens. A standardized method using three solvent systems has been developed[1,2] to facilitate the recognition of these compounds, and R_F data, obtained by using this method, have been reported for well over 200 natural products and their derivatives.

The standardized method involves running the samples in three solvent systems, determining R_F classes for each spot from the positions of control compounds on each plate, and sorting a deck of punched cards for the three R_F classes found for each unknown. An essential feature of this system is that the R_F classes are determined on every plate by the positions of two substances (atranorin and norstictic acid), so that moderate changes in absolute R_F values do not interfere with the identification. Although the method works even if R_F values change, it fails if relative R_F values change.

Recently, the Merck Company reduced the concentration of binder in the silica gel F_{254} plates (catalog number 5765) that were used to determine R_F values reported for the standardized method of identifying lichen products. Although the reduced concentration of binder in the new plates, now called silica gel 60 F_{254} (catalog number unchanged), improves the quality of the chromatograms, it also drastically changes some relative R_F values. Since the standardized method depends upon relative R_F values in three solvent systems, the new plates cannot be used under the conditions originally described. Compounds chromatographed on the new plates show changes in R_F values that depend upon the solvent system and the chemical nature of the substance. These effects are most serious for compounds that run with R_F values greater than 0.5 and when comparing substances of differing acidity. This note describes methods for improving the results obtained by using the new plates.

Of the three solvent systems used for the standardized method only solvent A (benzene–dioxane–acetic acid, 180:45:5) shows R_F values and R_F classes reasonably close to those previously reported. Chromatograms on the new plates can be run in this solvent without any change in procedure.

When chromatograms are prepared on the new plates using solvent B (*n*-hexane–ethyl ether–formic acid, 130:80:20), R_F values and classes change for compounds that normally run between atranorin and norstictic acid. The values for compounds with a free carboxyl group are depressed as compared with those for

similar compounds that lack this functional group. In solvent C (toluene–acetic acid, 200:30), the chromatograms now show a pronounced secondary solvent front between the positions of the control substances atranorin and norstictic acid. Many compounds previously well separated in this solvent system now seem to be identical because they run with the secondary front. Most of the compounds so affected are carboxylic acids that originally gave R_F classes of 5 or 6. Compounds with R_F classes from 1 to 4 are generally not changed on the new plates.

The silica gel 60 F_{254} plates give better results in solvents B and C if they are equilibrated for 5 min in an acidic atmosphere just before being placed in the developing tanks. For chromatograms to be run in solvent B, the spotted plate is equilibrated in an atmosphere of 60% aqueous formic acid. The pre-treatment should be checked by comparing perlatolic acid with atranorin; these two compounds should have nearly identical R_F values in solvent B if the original R_F classes of most of the other lichen products are to be maintained. Without the pre-treatment, perlatolic acid runs below atranorin, and pre-treatment for 5 min in the atmosphere over undiluted formic acid (99%) raises the spot for perlatolic acid above that of atranorin. Dilution of the formic acid to approximately 60% lowers perlatolic acid to the same R_F value as atranorin. To equilibrate the spotted plate, it is placed in a tank over the formic acid solution, but the plate should not touch the solution at any time; the plate is removed after 5 min and run immediately in the usual way. If the R_F values of the atranorin and norstictic acid spots are consistently low in solvent B, they can be raised by increasing the proportion of ethyl ether and decreasing that of hexane, so that the solvent has the composition *n*-hexane–ethyl ether–formic acid, 120:90:20, for example.

For chromatograms to be run in solvent C, the spotted plate should be equilibrated for 5 min in an atmosphere over glacial acetic acid; this treatment eliminates the secondary solvent front. In both solvents B and C, the pre-treatments described above will restore most of the R_F classes observed for lichen products to those reported in the literature, but spots in R_F classes 5 and 6, especially those near the boundary between these two classes, should be searched for in both classes.

The reduction in the concentration of binder used in the commercial silica gel plates effectively changes the activity of these plates for certain compounds chromatographed in certain solvent systems. Although it is unfortunate to encounter a change in these plates once a standardized method had been adopted for screening a large number of compounds, the uniformity of the coating on these plates makes them superior for this work to plates that are prepared in the laboratory.

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CHROM. 7664

Note

High-voltage paper electrophoresis for the separation of 1,8-dihydroxyanthracene derivatives in senna and rhubarb

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A large number of 1,8-dihydroxyanthracene derivatives are present in senna leaf (*Cassia senna* L. or *Cassia angustifolia* Vahl) and rhubarb root (*Rheum palmatum* L.)[1]. The activities of all of these constituents have not yet been investigated, but it is known that particularly dianthrone glycosides based on rhein (the sennosides) and rhein glycosides have a high purgative activity[2].

Numerous methods have been described for the separation of these glycosides[1]. Low-voltage paper electrophoresis has been applied before[3–10], but no reports are available concerning the application of high-voltage paper electrophoresis in this field.

This paper describes our studies on low-voltage paper electrophoresis and the results obtained by using high-voltage paper electrophoresis for the separation of dianthrone glycosides and rhein glycosides in senna and rhubarb.

EXPERIMENTAL

Apparatus and materials

A Camag high-voltage electrophoresis apparatus, consisting of an electrophoretic cell, protection chamber and power supply (0–5000 V d.c., 200 mA) was used, with Schleicher and Schüll 2043 bMgl (40 × 20 cm) paper.

For the separation of anthraquinone aglycones, the following buffers were used:

I: pH 6.0; 0.1 *M* citric acid, 36.85 ml; 0.2 *M* sodium phosphate to 100 ml.

II: pH 7.0; 0.1 *N* sodium hydroxide, 29.63 ml; 0.2 *M* sodium diphosphate, 25.00 ml; water to 100 ml.

III: pH 8.0; 0.1 *N* sodium hydroxide, 46.80 ml; 0.2 *M* sodium diphosphate, 25.00 ml; water to 100 ml.

IV: pH 9.0; 0.1 *N* sodium hydroxide, 21.30 ml; boric acid–potassium chloride (12.37 g of boric acid and 14.91 g of potassium chloride per litre), 25.00 ml; water to 100 ml.

For the separation of anthraglycosides, the following buffers were used:

A: pH 9.4; 0.05 *M* sodiumdiborate, 64.3 ml; 0.05 *M* sodium carbonate, 35.7 ml.

B: pH 6.0; 0.1 *N* sodium hydroxide, 5.0 ml; 0.1 *M* potassium diphosphate, 50 ml.

C: pH 8.6; barbital, 0.184 g; sodium barbital, 1.03 g; water to 100 ml.

Operating conditions

For high-voltage paper electrophoresis, the following conditions were used:
Buffers I–IV: 3500–4000 V; 100–120 mA; V4A steel electrode; 30 min.
Buffers A and B: 3700 V; 160 mA; V4A steel electrode; 45 min.
Buffer C: 4500 V; 35 mA; platinum electrode; 50 min.
For low-voltage paper electrophoresis, the following conditions were used:
Buffers A and B: 130 V; 10 mA; V4A steel electrode; 24 h.
Buffer C: 220 V; 10 mA; platinum electrode; 16 h.

Detection

When buffers with a pH greater than 7 are used, spots of anthraquinone aglycones are red and spots of anthraquinone glycosides are orange on the electropherograms. Dianthrone glycosides give a yellow colour, which turns brown after 24 h. Under UV light (360 nm), anthraquinone aglycones and glycosides show an orange fluorescence and dianthrone glycosides a dull ochre colour. Using buffer B, all anthracene derivatives give a yellow colour; after spraying with a 5% solution of potassium hydroxide in 50% methanol, anthraquinones change to red and dianthrones to yellow.

RESULTS AND DISCUSSION

The investigation was started with low-voltage paper electrophoresis. Anthraquinone aglycones could not be separated completely in this way. Rhein migrated 6.7–12 cm, depending on the buffer used, emodin migrated about 1 cm in all buffers, while the other aglycones (chrysophanol, physcion and aloe-emodin) did not migrate.

The dianthrone glycosides sennoside A, B and C could be separated from each other and also from rhein and rhein mono- and diglycoside. All of these compounds contain a carboxyl group in their molecule. Anthraglycosides without a carboxyl group did not migrate. However, the spots obtained by low-voltage electrophoresis are not clearly defined because of diffusion which is produced by the long duration of the runs. In particular, ions with a small molecular weight (< 1000) show this phenomenon of diffusion. In order to reduce this undesirable transport of ions, the duration of the separation should be shortened. This can be achieved by applying high-voltage electrophoresis, as the electrophoretic mobility is a linear function of the field strength. When high-voltage paper electrophoresis was applied to the separation of the anthracene derivatives, no diffusion of the spots occurred. However, with this technique, again no complete separation of the anthraquinone aglycones was obtained. In buffers I–IV, physcion and chrysophanol did not migrate. Also with buffers I, II and III aloe-emodin remained at the start. Rhein showed the greatest migration in all of the buffers used.

The best separation of anthraquinone aglycones was obtained in buffer IV; the migration distances were rhein 8.5 cm, emodin 1.7 cm, aloe-emodin 0.7 cm and

TABLE I

MIGRATION DISTANCES OF 1,8-DIHYDROXYANTHRACENE DERIVATIVES FROM SENNA AND RHUBARB

Migration distances are given in centimetres.

Compound	*Buffer*		
	A	*B*	*C*
Rhein diglycoside	19.5	17.5	13.5
Sennoside B	15.0	14.5	11.0
Sennoside A	15.0	14.5	9.0
Rhein monoglycoside	12.5	10.0	6.0
Sennoside C	8.5	7.0	5.0
Rhein	4.0	2.5	4.0

physcion and chrysophanol 0.0 cm.

The separation of anthraglycosides gave better results. From Table I, it can be seen that these glycosides and the aglycone rhein can be separated in buffers A and B, with the exception of sennosides A and B. When buffer C is used, these two stereoisomeric glycosides also can be separated. Although anthraglycosides without a carboxyl group do not migrate, it is possible to separate the glycosides that contain a carboxyl group from senna and rhubarb by high-voltage paper electrophoresis.

These last named anthracene derivatives are also the constituents which show a high laxative activity.

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Note

Separation of four components of the antibiotic EM49 by partition chromatography on paper

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The new antibiotic, EM49 (ref. 1), is a complex of cyclic, homodectic octapeptides, monoacylated with β-hydroxy fatty acids. It consists of four components, α, β, γ, and δ, differing from each other in their contents of phenylalanine and in the chain length of their fatty-acid substituents. The four components have been separated by ion-exchange column chromatography on CM-cellulose[2]. For routine analysis of fermentation samples, a simpler chromatographic procedure was sought for the separation of these four entities. Whereas thin-layer chromatography employing plates pre-coated with CM-cellulose did not reproduce the result of the column chromatography, a "real-partition chromatographic" technique on paper[3–5] proved successful for this purpose.

The solvent system consists of a mixture of *n*-amyl alcohol–amyl acetate–propionic acid–water (6:9:5:15), this mixture separates into two phases. The lower phase is mixed with an equal volume of acetone and the paper strip (Whatman No. 1), on which sample spots have been applied, is drawn through a bath of this mixture. It is not necessary to wet the end of the paper that will be placed in the solvent trough, but the origin at which the sample spots have been applied must be wetted. After evaporation of the acetone, achieved by hanging the paper strip in air for 3 min, the paper strip, while still wet, is transferred to a jar of a height greater than 24 cm and a descending chromatogram is developed with the upper phase of the solvent mixture. The jar is lined with paper that has been wetted with the lower phase. The chromatogram is allowed to develop for 16 h at room temperature of 21.5–24°. After the chromatogram has been dried in air, the spots are detected either by bioautography on an *Escherichia coli* plate or by the chloramide reaction[6,7].

A photograph of a papergram developed in this manner is shown in Fig. 1, the four components (α, γ, β, δ) having migrated 5.0, 6.2, 8.5, and 11.5 cm, respectively. The R_F values of the components measured when the solvent front had moved 20 cm in about 3 h were: α, 0.13; γ, 0.16; β, 0.22; and δ, 0.30. Fig. 1 also shows that a mixture of these four components was resolved to give four discrete spots with mobilities characteristic of the four individual components. The chromatogram of a fermentation-broth sample is also shown in Fig. 1. This sample was prepared by extracting 10 ml of the fermentation broth, adjusted to pH 12, with 4 ml of *n*-butanol–ethyl acetate (1:3, v/v). An aliquot of the solvent phase, representing approximately

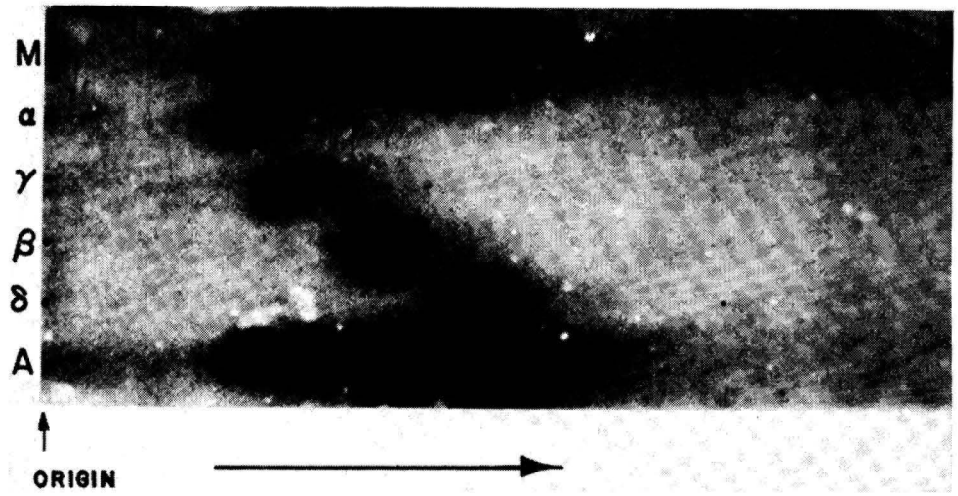

Fig. 1. Paper chromatographic separation of four components of EM49. Sample M = a mixture of α, β, γ, and δ; sample A = fermentation-broth extract.

10 μg of the antibiotic mixture, was chromatographed. The presence of the four components was clearly demonstrable and visual examination permitted easy estimation of their relative quantities.

The technique described above, which is essentially the same as Zaffaroni's technique[8,9] for steroids, is not the only way to introduce the stationary phase. Equally satisfactory results can be obtained if the paper strip is drawn through a bath of the undiluted lower phase, then blotted thoroughly with a few layers of Whatman No. 1 filter paper. This is the technique for "real-partition chromatography" introduced by Tschesche *et al.*[4] and used by Tuzson[10]. Introduction of the stationary phase by vapor-phase equilibration overnight in a jar lined with paper that had been wetted with the lower phase proved unsatisfactory. The spots appeared as streaks and no separation was observed when a mixture was chromatographed. These results clearly demonstrated that "real-partition chromatography" is a very effective tool for the separation of compounds of closely related structure.

Although the antibiotic mixture appeared as a single spot on papergram developed with several different solvent mixtures or on a silicic acid thin-layer chromatogram[1], it was resolved into four discrete spots by the technique reported here. We conclude that by the use of a real-partition chromatographic technique and a properly chosen solvent system, paper chromatography can achieve a separation as satisfactory as that achieved, for example, by CM-cellulose column chromatography[2].

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Gas Chromatography

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21. PURINES, PYRIMIDINES, NUCLEIC ACIDS AND THEIR CONSTITUENTS

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31. PLASTICS AND THEIR INTERMEDIATES

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35. SOME TECHNICAL PRODUCTS AND COMPLEX MIXTURES

35a. Surfactants

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35b. Antioxidants and preservatives

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Paper Chromatography

1. REVIEWS AND BOOKS

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7. PHENOLS

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11. ORGANIC ACIDS AND LIPIDS

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11b. Lipids and their constituents

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18. AMINO ACIDS

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19. PEPTIDES; CHEMICAL STRUCTURE OF PROTEINS

19b. Elucidation of structure of proteins

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21. PURINES, PYRIMIDINES, NUCLEIC ACIDS AND THEIR CONSTITUENTS

21a. Purines, pyrimidines, nucleosides, nucleotides

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24. ORGANIC SULPHUR COMPOUNDS

See 4896.

25. ORGANIC PHOSPHORUS COMPOUNDS

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28. ANTIBIOTICS

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33. INORGANIC SUBSTANCES

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34. RADIOACTIVE AND OTHER ISOTOPE COMPOUNDS

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Thin-Layer Chromatography

1. REVIEWS AND BOOKS

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21. PURINES, PYRIMIDINES, NUCLEIC ACIDS AND THEIR CONSTITUENTS

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PUBLICATION SCHEDULE FOR 1974

Journal of Chromatography (incorporating *Chromatographic Reviews*)

MONTH	J	F	M	A	M	J	J	A	S	O	N	D
JOURNAL	88/1 88/2	89/1 89/2	90/1	90/2 91	92/1 92/2	93/1 93/2	94 95/1	95/2 96/1	96/2	97/1 97/2	99 100/1 100/2	
REVIEWS*			98/1						98/2	98/3		

* Volume 98 will consist of *Chromatographic Reviews*. The issues comprising this volume will not be published consecutively, but will appear at various times in the course of the year.

GENERAL INFORMATION

(A leaflet *Instructions to Authors* can be obtained by application to the publisher.)

Types of Contributions. (a) Original research work not previously published in a generally accessible language in other periodicals (Full-length papers). (b) Review articles. (c) Short communications and Notes. (d) Book reviews; News; Announcements. (e) Bibliography of Paper Chromatography, Thin-Layer Chromatography, Column Chromatography, Gas Chromatography and Electrophoretic Techniques. (f) Chromatographic Data.

Submission of Papers. Three copies of manuscripts in English, French or German should be sent to: Editorial office of the Journal of Chromatography, P.O. Box 681, Amsterdam, The Netherlands. For *Review articles*, an outline of the proposed article should first be forwarded to the Editorial office for preliminary discussion prior to preparation.

Manuscripts. The manuscript should be typed with double spacing on pages of uniform size and should be accompanied by a separate title page. The name and the complete address of the author to whom proofs are to be sent should be given on this page. Authors of papers in French or German are requested to supply an English translation of the title. A short running title of not more than 50 letters (including spaces between the words) is also required for Full-length papers and Review articles. All illustrations, photographs, tables, etc., should be on separate sheets.

Heading. The title of the paper should be concise and informative. The title should be followed by the authors' full names, academic or professional affiliations, and addresses.

Summary. Full-length papers and Review articles should have a summary of 50–100 words. In the case of French or German articles an additional summary in English, headed by an English translation of the title, should also be provided. (Short communications and Notes will be published without a summary.)

Illustrations. The figures should be submitted in a form suitable for reproduction, drawn in Indian ink on drawing or tracing paper. Particular attention should be paid to the size of the lettering to ensure that it does not become unreadable after reduction. Sharp, glossy photographs are required to obtain good halftones. Each illustration should have a legend, all the *legends* being typed together on a *separate sheet*. Coloured illustrations are reproduced at the author's expense.

References. References should be numbered in the order in which they are cited in the text and listed in numerical sequence on a separate sheet at the end of the article. The numbers should appear in the text at the appropriate places using superscript numerals. In the reference list, periodicals[1], books[2], and multi-author books[3] should be cited in accordance with the following examples:

1 A. T. James and A. J. P. Martin, *Biochem. J.*, 50 (1952) 679.
2 L. R. Snyder, *Principles of Adsorption Chromatography*, Marcel Dekker, New York, 1968, p. 201.
3 R. D. Marshall and A. Neuberger, in A. Gottschalk (Editor), *Glycoproteins*, Vol. 5, Part A, Elsevier, Amsterdam, 2nd ed., 1972, Ch. 3, p. 251.

Abbreviations for the titles of journals should follow the system used by *Chemical Abstracts*.

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Publication. The *Journal of Chromatography* (including *Chromatographic Reviews*) appears fortnightly and has 13 volumes in 1974. The subscription price for 1974 [Vols. 88–100] is Dfl. 1066.00 plus Dfl. 65.00 (postage). Subscribers in the U.S.A., Canada and Japan receive their copies by air mail. Additional charges for air mail to other countries are available on request. Back volumes of the *Journal of Chromatography* (Vols. 1 through 87) are available at Dfl. 92.00 (plus postage).

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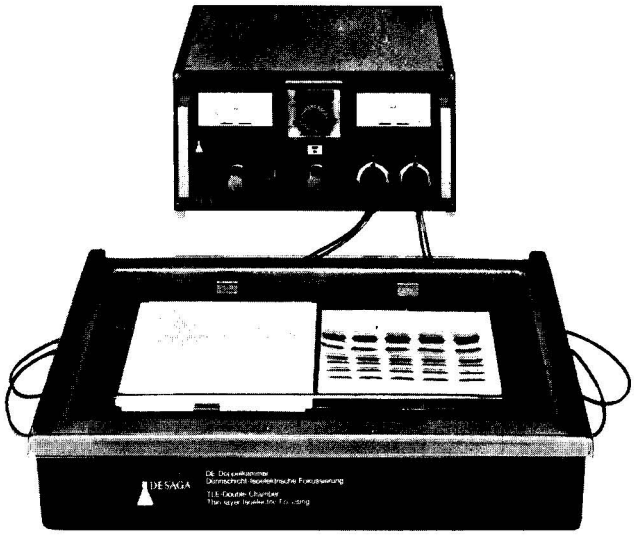

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