ANALYTICAL CHEMISTRY

WALTER J. MURPHY, Editorial Director

Go West—Analysts—Go West

At least six innovations in the planning for the Summer Symposium on Analytical Chemistry are in evidence in the final program published in the April issue of this journal.

This annual event, cosponsored by the Division of Analytical Chemistry and Analytical Chemistry, for the first time will be held on the West Coast on the spacious campus of the University of California at Los Angeles. The ninth in the series of summer symposia, lengthened to a 3-day meeting, is a distinct departure from the pattern of former years. Instead of dealing with one subject, three topics are scheduled for the June 14 to 16 meeting: The Analysis of Industrial Wastes, Rapid Methods of Analyses, and Analytical Problems Encountered in Biological Systems.

The fourth innovation is the addition of an exhibit of scientific apparatus by local manufacturers and supply houses. Fifth in our list, we are happy to note, is that an introductory lecture will be given by the Fisher Award winner, Ernest H. Swift of California Institute of Technology and honorary chairman of the symposium. His subject will be coulometry.

Finally, a very interesting ladies' program has been added, something that has been talked about in connection with these meetings, but without tangible results until this year.

At first blush, it might seem ungracious to our hosts and hostesses to mention the subject of air pollution. However, smog is no longer wholly identified with the city of Los Angeles, although admittedly LA for a wide variety of reasons has been plagued by this nuisance more perhaps than any other city in this country. Smog conditions have been reported in other parts of the United States as well, and it seemed appropriate to the program committee to devote at least one full day to papers dealing with the analytical aspects of air pollution and industrial wastes.

A large turnout of analysts is expected for the 1956 summer symposium. We have been interested and especially pleased to note the record-breaking registration at the LSU symposium and the Pittsburgh Conference on Analytical Chemistry and Applied Spectroscopy, and the unexpectedly large attendance of analysts at the divisional sessions at the recent ACS national meeting in Dallas.

At the day-and-a-half symposium in Dallas on vapor phase chromatography, there were as many as 700 analysts in the lecture room at one time, and at least 300 attended the one-day session on the subject of thermogravimetry and differential thermal analysis. Large audiences such as these demonstrate conclusively that we still have not reached a saturation point in meetings devoted to the broad subject of analytical chemistry.

The addition of an exhibit of instruments to the summer symposium at UCLA next month is an experiment that will be watched with a great deal of interest. Unquestionably, the exposition staged in conjunction with the Pittsburgh Conference has helped materially to build up attendance at the conferences. Certainly, the exhibits now are a very integral part of these meetings.

Those who have been responsible for the planning and operation of the Pittsburgh conferences very wisely have seen to it that the scientific and technical sessions are not the "tail that wags the dog."

An unusually large committee has been involved for well over a year in planning for the summer symposium at UCLA. The general chairman, John Mitchell, Jr., Robert L. Pecsok, chairman of local arrangements, and their associates are to be congratulated. In addition to the innovations already mentioned, they have secured Herbert Meyer, chief chemist of the Motion Picture Research Council which serves nine of Hollywood's major studios, as the banquet speaker. The title of his address is a challenging one, "Hollywood Has Chemical Problems."

The California Chamber of Commerce assures us that every month is a good month to visit the state. We hope to take advantage of the symposium being held in LA by including a number of other interesting industrial and scenic areas in our tour. We strongly suspect that just about everyone who attends the symposium has the same objective in mind.

Potentiometric Titration of Very Weak Acids

Titration in Ethylenediamine Solution Using Platinum Electrodes

G. A. HARLOW, C. M. NOBLE,[1] and GARRARD E. A. WYLD

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Anodically polarized platinum wire acquires characteristics which greatly enhance its usefulness as an indicating electrode for the titration of very weak acids in ethylenediamine. Titration curves are obtained which span a potential range two to three times as great as those obtained with the glass electrode, and which are more reproducible than those obtained with the antimony electrode. Platinum wire inserted into the titrant stream serves as a satisfactory reference electrode.

THE use of alcoholic potassium hydroxide for the titration of very weak acids in ethylenediamine solvent has been described by Deal and Wyld (*2*). This titrant is referred to as a solution of potassium hydroxide, although it probably contains both hydroxyl and isopropylate ions in equilibrium with each other. Deal and Wyld also show that the glass electrode can serve as an indicating electrode in ethylenediamine if sodium ion is absent. This technique has the advantage of using a titrant (potassium hydroxide in isopropyl alcohol) which is already available in most petroleum laboratories for the determination of acidity of lubricating oils (ASTM D–664), and an electrode system which is commercially available and relatively stable.

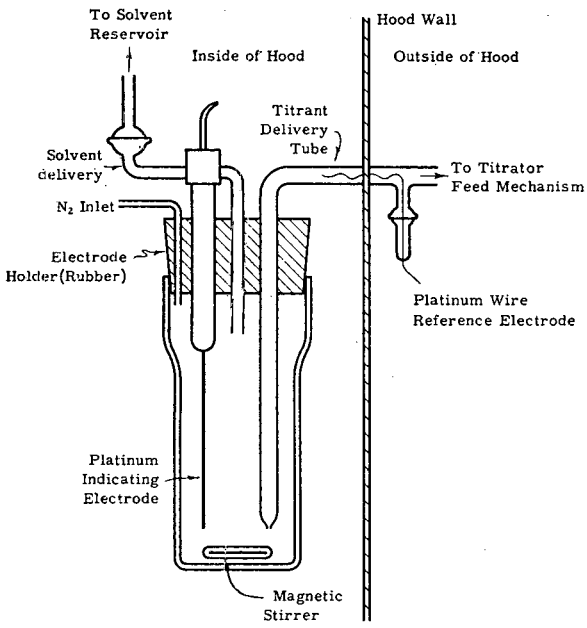

Figure 1. Titration cell assembly

Other workers, however, have reported larger inflections for the titration of phenols using an antimony or platinum indicating electrode (*4, 7*). In addition, they have found two inflections for salicylic acid (*4*). The second inflection is not obtained using the system described by Deal and Wyld (*2*).

The present investigation was undertaken to find an indicating electrode which produces the larger inflections without sacrificing the reproducibility of the glass electrode. Preliminary tests of antimony and platinum electrodes indicated that platinum was more promising.

The use of platinized platinum in the hydrogen electrode is, of course, well known. Platinized platinum has also been used as an oxygen or air electrode in acid-base titrations. In all of these platinum-gas electrodes, however, it is customary to pass a stream of the gas over the electrode surface during the titration. Utilization of the bare metal has not been thoroughly investigated. Popoff and McHenry (*8*) have used shiny platinum for the titration of alkaloids. Brunnich (*1*) reported the use of the platinum-graphite system but stated that small inflections were obtained. These and other applications have been discussed by Kolthoff and Furman (*5*).

The use of the platinum indicator electrode in the titration of very weak acids in nonaqueous solvents has been mentioned in several papers. Higuchi and others (*6*) used a platinum electrode for titrations in tetrahydrofuran with lithium aluminum hydride. A platinum electrode was also used by Gran and Althin (*3*) and by Katz and Glenn (*4*) for titrations in ethylenediamine. Very little detailed information is available from these papers on the characteristics of platinum as an indicator electrode for acid-base titrations in nonaqueous solvents. No data have been presented, for example, on the sensitivity of the platinum electrode compared with other electrodes or on the reproduciblity of the titration curves obtained.

APPARATUS

A Precision-Dow Recordomatic Titrator was employed in conjunction with the specially designed cell assembly shown in

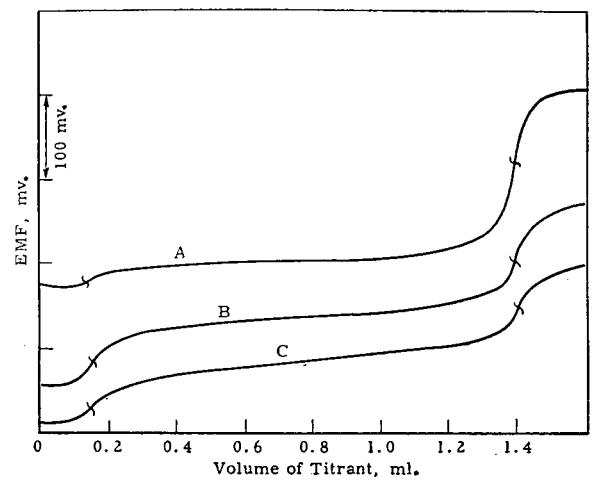

Figure 2. Comparison of electrode systems

0.24 meq. of phenol

Electrodes: *A*. Platinum-calomel; *B*. Glass-calomel; *C*. Antimony-calomel

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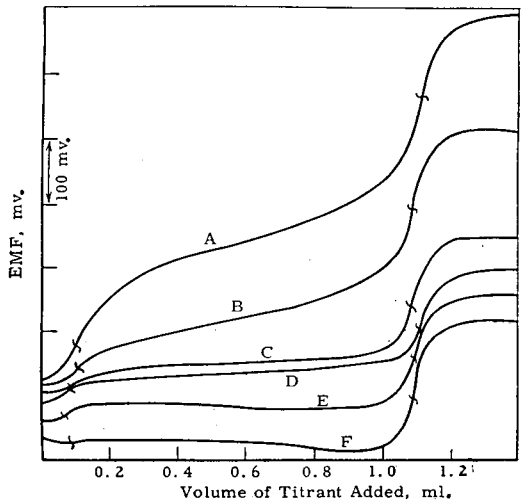

Figure 3. Effect of treatment of platinum indicating electrode

0.24 meq. of phenol
Electrode treatment:
A. Anodically polarized
B. Treated with cerium sulfate
C. Cathodically polarized
D. Treated with nitric acid
E. Untreated
F. Treated with hydrogen peroxide

Figure 1. The titration assembly was equipped with a 5-ml. syringe which permits the titrant to be delivered at one fifth of the usual rate. The scale of the titration was thus similar to the manual titrations previously described (*2*). The apparatus, except for the titrator, is contained in a fume hood. This arrangement protects both operator and instrument from toxic or corrosive fumes, such as those of ethylenediamine. The electrode holder consists of a rubber stopper drilled with holes of appropriate sizes. This permits effective sealing against atmospheric carbon dioxide and makes interchange of electrodes convenient. Solvent is added to the sample after the cell is in place by means of the solvent delivery tube. Agitation is accomplished with a magnetic stirrer, and the nitrogen inlet tube allows the sample to be covered with a blanket of inert gas just before the solvent is added and before the system is closed to the atmosphere.

PROCEDURE

The titration procedure employed was similar to that previously described (*2*), except that in this case an automatic titrator was used. The solvent was ethylenediamine (95 to 100%), Eastman Organic Chemicals. The titrant was 0.2*N* potassium hydroxide in isopropyl alcohol solution, except where otherwise indicated.

PRELIMINARY TESTS OF ELECTRODES

Antimony and platinum electrodes were tested as possible substitutes for the glass electrode. A comparison of their behavior with that of the glass electrode is given in Figure 2, which shows curves for the titration of phenol in ethylenediamine. The small first inflection is due to carbonate in the solvent, and the second inflection is due to the phenol. The curves produced by the antimony and the glass electrodes were similar. It was difficult, however, to obtain smooth curves with the antimony electrode, because the potentials tended to be erratic. In view of the more promising results obtained with commercially available platinum, the reasons for the instability of the antimony electrode were not investigated.

CHARACTERISTICS OF PLATINUM INDICATING ELECTRODE

Effects of Pretreatment. The behavior of the platinum electrode is very much dependent upon the condition of its surface. This is evidenced by the fact that subjecting it to various treatments prior to its use in a titration results in titration curves quite different in character. Figure 3 shows curves for the titration of phenol using platinum electrodes which were previously treated in several different ways. The small first inflection is due to carbonate in the solvent. The treatment referred to as polarization consisted of applying a potential of 3 volts to a pair of platinum electrodes immersed in dilute (1 to 100) sulfuric acid solution and allowing electrolysis to proceed for about 1 minute. The platinum wire which served as anode is referred to as being anodically polarized; similarly the one which served as cathode is cathodically polarized. It is not certain exactly what occurs on the surface of the platinum during this treatment, but apparently the surface is charged with hydrogen or oxygen, depending on whether the platinum was polarized at the cathode or anode.

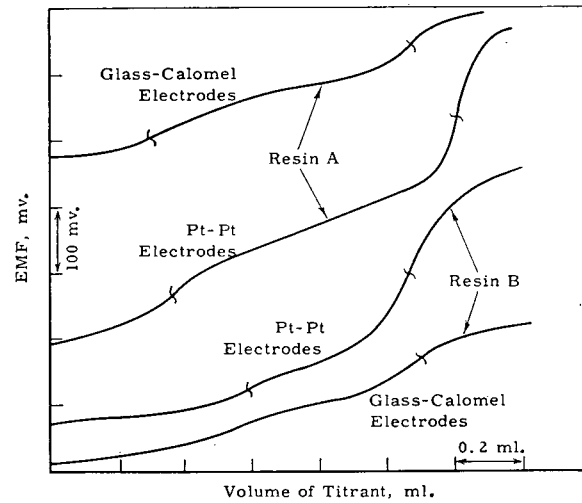

Figure 4. Titration of resins in ethylenediamine

Anodically polarized platinum produced curves having the greatest potential range and showing the longest and sharpest inflections. The treatment is not lasting, however, and must be repeated before every titration to gain any degree of reproducibility. After prolonged use platinum develops an insensitivity to the polarization process and the curves cover a progressively smaller potential range; however, the platinum may be rejuvenated by letting it stand in hot dilute (1 to 1) hydrochloric acid for 3 or 4 hours. The mechanism of operation of the electrode was not investigated, but it may be operating as an oxygen electrode of the type discussed by Kolthoff and Furman (*5*).

Anodic polarization largely erases the effects of other previous treatments. When electrodes which have been treated in various ways are anodically polarized, they yield titration curves which are very similar to one another.

The platinum electrode has from two to three times the voltage response of the glass electrode in ethylenediamine. The greater sensitivity is not limited to the most alkaline area of a titration but holds throughout the range of basicity encountered in this solvent.

Stability of Platinum Electrode Potentials. The anodic polarization of the platinum electrode, while causing it to span a large potential range throughout a titration, also causes it to exhibit an unstable initial potential. If the freshly polarized electrode is allowed to stand in a solution of an acid in ethylenediamine a few moments before any titrant is added, the initial potential may be observed to become slowly more positive. It was found necessary, therefore, to wait 2 to 3 minutes before starting the titration. When this was done, the potentials ob-

tained with the platinum electrode were comparable in reproducibility to those obtained with the glass electrode.

Applicability. The anodically polarized platinum electrode has proved applicable to the titration of a variety of acids including phenol, resorcinol, catechol, substituted catechols, and 4,4′-isopropylidenediphenol, both alone and in mixtures with stronger acids. In all of these cases larger inflections were obtained than in the corresponding titration using a glass electrode.

Curves for the titration of resinous materials are also improved by using the treated platinum electrode. Figure 4 shows comparative curves for the titration of two resins containing phenolic hydroxyl groups. The phenolic (second) inflections are particularly enhanced. The first inflections are due to unidentified acidic components which are stronger than phenol.

The undesirable effect of sodium ion on the behavior of the glass electrode, which is well known in water, is particularly marked in ethylenediamine. Figure 5 shows curves for the titration of salicylic acid with sodium aminoethoxide, using both the glass and the anodically polarized platinum electrodes. As can be seen, sodium has no apparent effect upon the potential range of the platinum electrode. Sodium aminoethoxide gives an inflection for the hydroxyl group of salicylic acid, whereas potassium hydroxide (not shown) does not.

PLATINUM REFERENCE ELECTRODE

Space is very limited in the special reduced scale titration assembly used for ethylenediamine titrations; therefore, it was desirable to remove the bulky calomel electrode from the titration cell if possible. In addition, the aqueous salt bridge used with the calomel cell is quickly contaminated with ethylenediamine, and what is more serious, it in turn contaminates the ethylenediamine with water. Moss and others (*7*) reported that a satisfactory reference electrode was obtained by inserting an antimony rod into the titrant stream. When this was tried, however, stable potentials could not be obtained.

The substitution of a short length of platinum wire into the titrant stream (*9*) was found to serve very satisfactorily. This yielded stable potentials for long periods of time and necessitated only infrequent cleaning. The removal of the reference electrode from the titration cell in this way allows a greater degree of freedom in the design of titration assemblies; it permits the apparatus to be scaled down, if desired, for titrations on a microscale.

DISCUSSION AND CONCLUSIONS

The anodically polarized platinum electrode is a more sensitive indicator electrode than is the glass electrode when potassium hydroxide is used as the titrant in ethylenediamine titrations. When sodium aminoethoxide is used as the titrant, the platinum electrode retains its sensitivity, but the glass electrode becomes practically useless as an acidity indicator, although it may be useful as a reference electrode. The two electrodes are roughly comparable regarding durability, availability, and reproducibility of potentials.

There seems to be no ready explanation for the difficulty which was experienced with the stability of the antimony electrode. It is possible that impurities either in the ethylenediamine or the antimony may have been the cause of the instability.

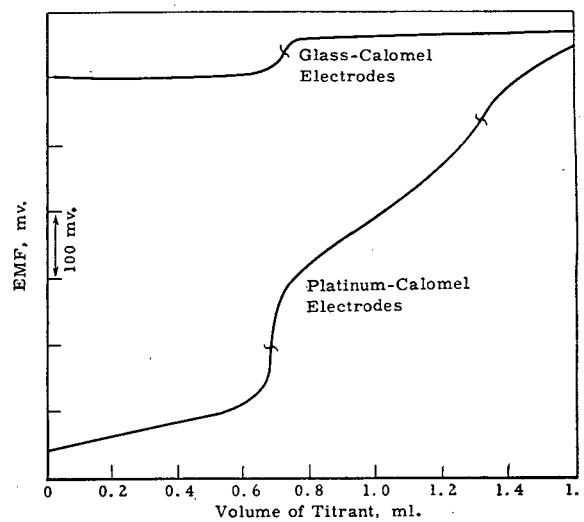

Figure 5. Comparison of electrode behavior with sodium aminoethoxide

0.30 meq. of salicylic acid in 2 ml. of isopropyl alcohol, titrated with 0.2*N* sodium aminoethoxide in isopropyl alcohol

Because the main advantage of the platinum electrode over the glass electrode is its freedom from alkali ion errors, the two electrodes should be more comparable in sensitivity when alkali ions are absent. A study of quaternary ammonium titrants is now in progress.

LITERATURE CITED

(1) Brunnich, J. C., *Ind. Eng. Chem.* **17**, 631 (1925).
(2) Deal, V. Z., Wyld, G. E. A., ANAL. CHEM. **27**, 47 (1955).
(3) Gran, G., Althin, B., *Acta Chem. Scand.* **4**, 967 (1950).
(4) Katz, M., Glenn, R. A., ANAL. CHEM. **24**, 1157 (1952).
(5) Kolthoff, I. M., Furman, N. H., "Potentiometric Titrations," p. 215, Wiley, New York, 1931.
(6) Lintner, C. J., Schleif, R. H., Higuchi, T., ANAL. CHEM. **22**, 534 (1950).
(7) Moss, M. L., Elliott, J. H., Hall, R. T., *Ibid.*, **20**, 784 (1948).
(8) Popoff, S., McHenry, M. J., *J. Am. Pharm. Assoc.* **14**, 473 (1925).
(9) Willard, H. H., Boldyreff, A. W., *J. Am. Chem. Soc.* **51**, 471 (1929).

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Potentiometric Titration of Very Weak Acids

Tetrabutylammonium Hydroxide as Titrant in Nonaqueous Media

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Tetrabutylammonium hydroxide in nearly anhydrous isopropyl alcohol solution has a number of advantages over more conventional titrants for very weak acids in nonaqueous solvents. The tetrabutylammonium salts of most weak acids are more soluble in organic solvents than are the corresponding sodium or potassium salts; thus, difficulties due to precipitation are minimized. The titrant can be used with the glass electrode without the pronounced loss in sensitivity in the highly alkaline region which is encountered with titrants containing sodium and potassium. The titrant can be prepared by passing a solution of tetrabutylammonium iodide in isopropyl alcohol through an anion exchange column which has been converted to the hydroxide form with potassium hydroxide.

THE first successful potentiometric titration of very weak acids (those comparable in strength to phenol) was reported by Moss, Elliot, and Hall (*6*) in 1948. These workers used ethylenediamine as the solvent and a solution of sodium aminoethoxide in ethylenediamine as the titrant. In later publications other compounds have been suggested for use as titrants. Sodium methoxide (*3*) and potassium methoxide in methanol-benzene (*2*) were employed by Fritz and others. Deal and Wyld (*1*) reported that potassium hydroxide in isopropyl alcohol solution could be used for the titration of very weak acids in ethylenediamine and dimethyl formamide. It was also shown that tetrabutylammonium hydroxide in isopropyl alcohol containing 10% water gave inflections for the titration of very weak acids in the above solvents which were similar in appearance to those obtained with potassium hydroxide in anhydrous isopropyl alcohol. These results were obtained with a glass indicating electrode which loses sensitivity in the presence of potassium ions. Because quaternary ammonium ions do not reduce the sensitivity of the glass electrode, tetrabutylammonium hydroxide should have given considerably larger inflections than potassium hydroxide. There was reason to believe that the relatively high water content of the quaternary ammonium hydroxide titrant was counteracting the advantage of greater electrode sensitivity.

TETRABUTYLAMMONIUM HYDROXIDE TITRANT

Tetrabutylammonium hydroxide is available commercially in 1*M* aqueous solution. Unfortunately, water has a detrimental effect on the titration of very weak acids and the presence of only a few per cent in the solvent is sufficient to reduce markedly the size and sharpness of the inflections obtained. If the solution is diluted with a less acidic solvent such as isopropyl alcohol to give a 0.2*N* solution for titration purposes, the water content is still 20%. It was felt, therefore, that a satisfactory evaluation of tetrabutylammonium hydroxide as a titrant could be made only after a method had been developed for preparing it in a nearly anhydrous solvent which is less acidic than water.

Preparation. The first attempts to prepare nonaqueous quaternary ammonium hydroxide titrants were along conventional lines. A quaternary ammonium halide was dissolved in a nonaqueous solvent and the solution was shaken with finely powdered silver oxide. Although this method has been previously used with success to prepare these hydroxides in aqueous solution (*7*), it was completely unsuccessful in the present case. Attempts to concentrate the commercially available 1*N* solution of tetrabutylammonium hydroxide by evaporation and crystallization were also unsuccessful.

An ion exchange process utilizing an exchange resin and an isopropyl alcohol solution of tetrabutylammonium iodide was found to be very satisfactory. The saturated alcoholic solution was slowly passed through a column containing Amberlite IRA-400 resin which had previously been converted to the hydroxide form by washing with aqueous potassium hydroxide solution, then rinsed with isopropyl alcohol to remove the water. This process resulted in the conversion of the quaternary ammonium iodide to quaternary ammonium hydroxide.

An exchange column 4.5 cm. in diameter and 52 cm. long was found to be sufficiently large for the preparation of 1400 ml. of the hydroxide. Potassium hydroxide rather than sodium hydroxide was chosen as the strong alkali to convert the exchange resin to the basic form because the presence of small amounts of potassium ion in the titrant would be less objectionable than the same amount of sodium ion. No tests were conducted to determine the minimum amount of potassium hydroxide required, but 10 liters of 1*N* aqueous solution appeared to give satisfactory conversion. Six liters of distilled water was used to rinse the column free of potassium hydroxide and 5 liters of anhydrous isopropyl alcohol was used to remove the excess water. A saturated solution of tetrabutylammonium iodide in isopropyl alcohol was prepared and passed through the column very slowly (not more than 5 ml. per minute). More rapid throughput resulted in incomplete conversion. The effluent was collected in a receiver which was protected from atmospheric carbon dioxide.

In order to determine the efficiency of the conversion of the iodide to the hydroxide, 400 ml. of the saturated iodide solution

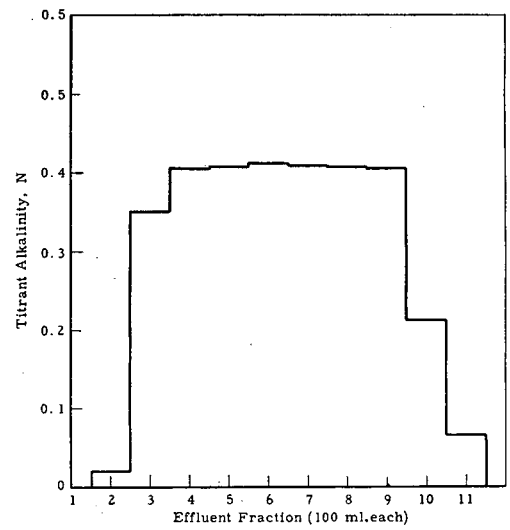

Figure 1. Ion exchange conversion of tetrabutylammonium iodide to hydroxide in isopropyl alcohol

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was passed through the column and the effluent collected in 100-ml. fractions. The column was then rinsed with isopropyl alcohol and further fractions were collected. Each fraction was analyzed to determine the alkalinity and iodide content. Results of the determination of hydroxide content are shown in Figure 1. The iodide content, which amounted to no more than a trace in the fraction, indicated that the conversion was nearly complete. The first 1000 ml. of solution to come through the column had an average free hydroxide content of 0.303*N* and represented over 90% of the theoretical yield.

Not all of the commercially available tetrabutylammonium iodide was satisfactory as the starting material. However, that obtained from Matheson, Coleman and Bell, Inc., and from Southwestern Analytical Chemicals, Austin, Tex., was satisfactory.

Attempts were also made to prepare tetrabutylammonium hydroxide in pyridine, but were not successful. The pyridine effluent from the ion exchange column appeared to be very unstable, rapidly growing dark in color and losing its strong alkali content.

Characteristics. The water content of the 0.2*N* tetrabutylammonium hydroxide was determined by means of the Karl Fischer reagent and found to be about 0.5%. It is likely that the water content could be reduced by more thorough dehydration of the ion exchange column during its preparation if this should prove desirable.

The stability of the titrant compares favorably with that of solutions of similar strength of potassium hydroxide in isopropyl alcohol. Over a period of 6 weeks the normality of the tetrabutylammonium hydroxide dropped from 0.2349 to 0.2331; during this same period a solution of potassium hydroxide changed from 0.1893 to 0.1821. As in the case of the potassium hydroxide titrant, care must be taken during storage to prevent the absorption of carbon dioxide from the air. Some batches of the titrant appeared to decompose slowly upon standing. The titration curves obtained with these solutions show the presence of a base weaker than tetrabutylammonium hydroxide, probably the tertiary amine. Although this component does not interfere with the titration of very weak acids when the titrant is standardized against benzoic acid, it complicates the determination of mixtures of strong and weak acids.

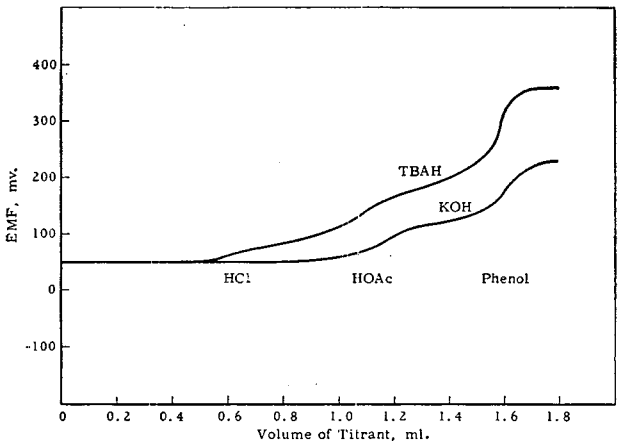

Figure 2. Titration of phenol-acetic acid-hydrochloric acid mixture in ethylenediamine

Glass-platinum electrodes

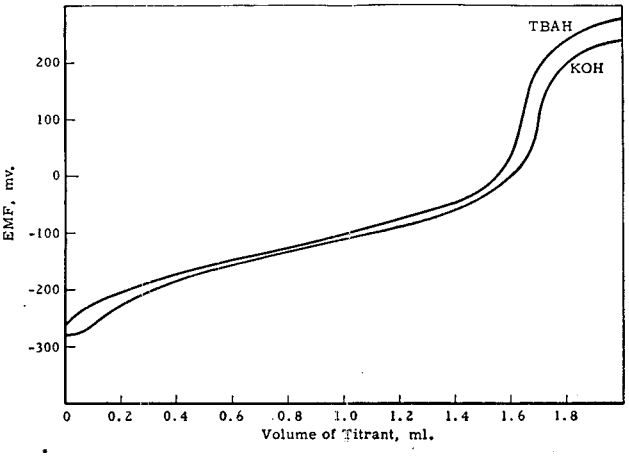

Figure 3. Titration of phenol in ethylenediamine

Platinum electrodes

APPARATUS AND PROCEDURE

Most of the titrations were performed automatically with the aid of the modified Precision-Dow Recordomatic Titrator. The titration assembly employed for these titrations was similar to the one previously described (*4*) in which the cell, the solvent reservoir, the electrodes, and the stirrer are all housed in a fume hood while the titrator and the titrant reservoir are outside of the hood area. In cases where the titration was performed manually, a Precision-Shell Dual AC Titrometer was used in conjunction with a titration assembly which has been described elsewhere (*1*). The platinum-oxygen indicating electrode was prepared by the polarization procedure (*4*).

The procedure used for the titrations consisted of dissolving the sample in 20 ml. of the solvent and titrating with approximately 0.2*N* titrant (*4*). The solvents used were of the best grade available commercially and were used directly in cases

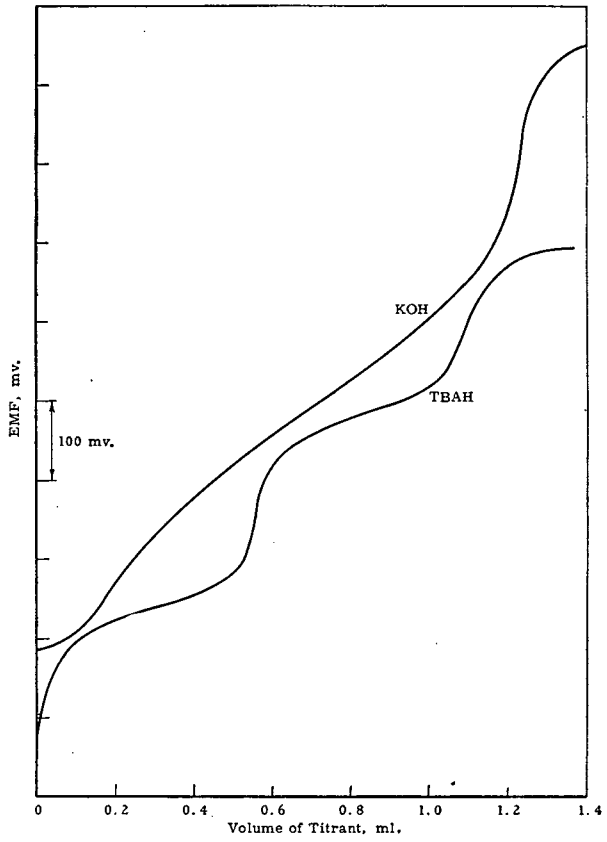

Figure 4. Titration of *p,p'*-dihydroxydiphenylmethane in ethylenediamine

Platinum electrodes; curves shifted vertically for clarity

where blank titrations indicated the absence of acidic impurities. This was the case with the alcohols, acetone, and pyridine. Piperidine and ethylenediamine were found to contain acidic impurities and these solvents were purified by passing them through a column of activated alumina (Alcoa F-20).

TITRATION OF VERY WEAK ACIDS

In Ethylenediamine. Tetrabutylammonium hydroxide in isopropyl alcohol was compared with potassium hydroxide in isopropyl alcohol as a titrant for the determination of very weak acids in ethylenediamine. The titration curves obtained from a mixture of phenol, acetic acid, and hydrochloric acid titrated with the aid of a glass indicating electrode are shown in Figure 2. The difference in the size of the inflections for phenol in the two curves is due largely to the effect of the potassium ion on the response of the glass electrode in strongly basic media. When an indicating electrode which is not sensitive to the potassium ion is used for the titration of phenol the two titrants yield curves which are similar in appearance. This can be seen from Figure 3, which shows curves obtained with a platinum-oxygen electrode.

The titration curves in Figure 2 show another characteristic difference in the two titrants. The curve obtained with tetrabutylammonium hydroxide has an incipient inflection for hydrochloric acid, whereas no sign of such an inflection appears in the curve obtained with potassium hydroxide. This difference in the degree of resolution which can be achieved with the two titrants is even more noticeable in some other solvents.

The titration of *p,p'*-dihydroxydiphenylmethane, a very weak dibasic acid, illustrates another influence of the titrant cation on the appearance of the titration curve. The differentiation of the two replaceable hydrogens of *p,p'*-dihydroxydiphenylmethane when this compound is titrated in ethylenediamine with tetrabutylammonium hydroxide is shown in Figure 4. A similar titration with potassium hydroxide (also shown) yields a curve with a single inflection. The resolution obtained with the tetrabutylammonium hydroxide is due to the fact that the salt formed when the first hydrogen of the *p,p'*-dihydroxydiphenylmethane is titrated is insoluble in the ethylenediamine, while the completely neutralized compound is soluble. This titration was one of the rare cases in which a precipitate formed during titration with quaternary ammonium hydroxide titrant. Even in this case, however, the final product of the titration was soluble.

In Pyridine. A comparison of potassium hydroxide and tetrabutylammonium hydroxide in pyridine solvent was made by titrating very weak acids with the aid of both glass and platinum indicating electrodes (Figures 5 and 6). As can be seen from Figure 5, obtained with a glass electrode, good resolution was achieved with both titrants, but a much larger inflection was obtained with the tetrabutylammonium hydroxide. The difference in the size of the phenol inflection is too great to be explained simply on the basis of the potassium ion effect on the response of the glass electrode. The fact that some other factor is also

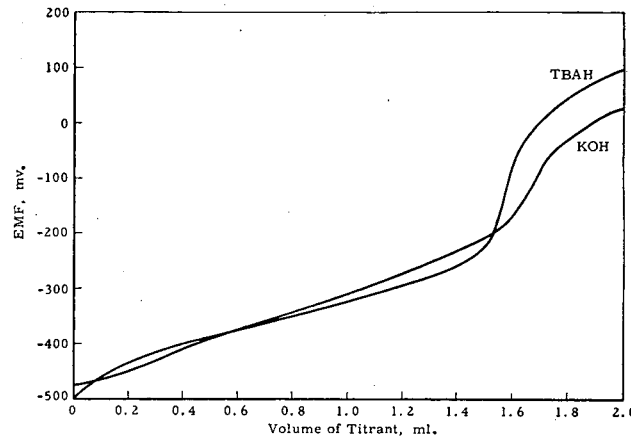

Figure 6. Titration of phenol in pyridine

Platinum electrodes

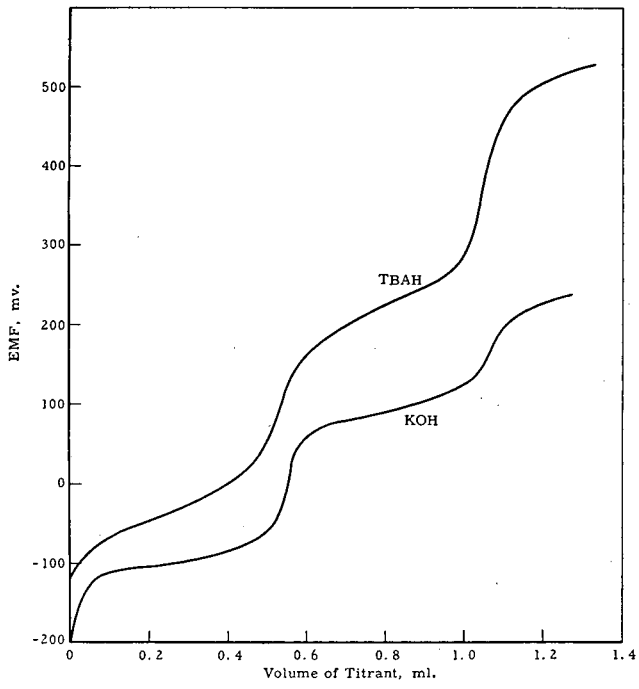

Figure 5. Titration of phenol–acetic acid mixture in pyridine

Glass-calomel electrodes

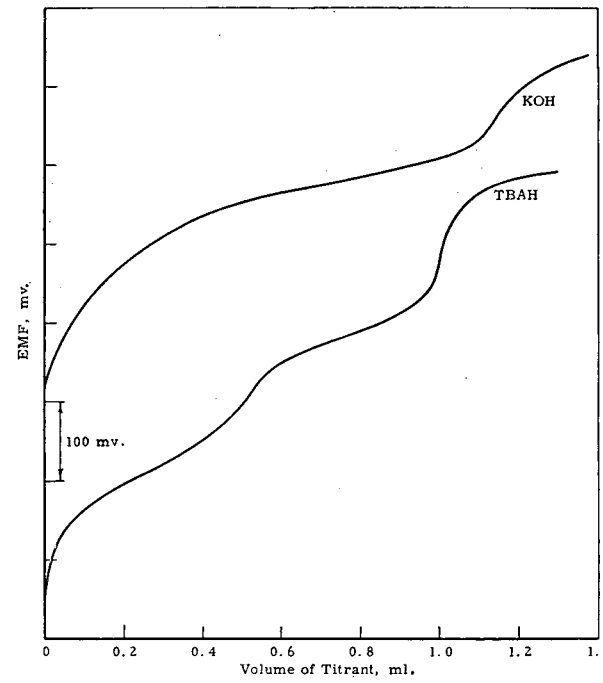

Figure 7. Titration of *p,p'*-dihydroxydiphenylmethane in pyridine

Platinum electrodes; curves shifted vertically for clarity

involved is apparent from the titration curves for phenol (Figure 6), which were obtained with the aid of a platinum-oxygen indicating electrode.

Some solubility difficulties are encountered when potassium hydroxide is used as a titrant in pyridine. There is a tendency for a precipitate to form when the first few drops of titrant are added to the pure solvent, as in the case of a blank titration. Apparently a certain amount of isopropyl alcohol is necessary to keep the potassium hydroxide in solution, because the precipitate redissolves upon the addition of more titrant. No difficulties of this type are encountered when tetrabutylammonium hydroxide is used as the titrant.

The titration of *p,p'*-dihydroxydiphenylmethane in pyridine with the two titrants results in curves which are very different in appearance. Figure 7 shows that two well-defined inflections are obtained when tetrabutylammonium hydroxide is used, but only a single inflection when potassium hydroxide is used. These

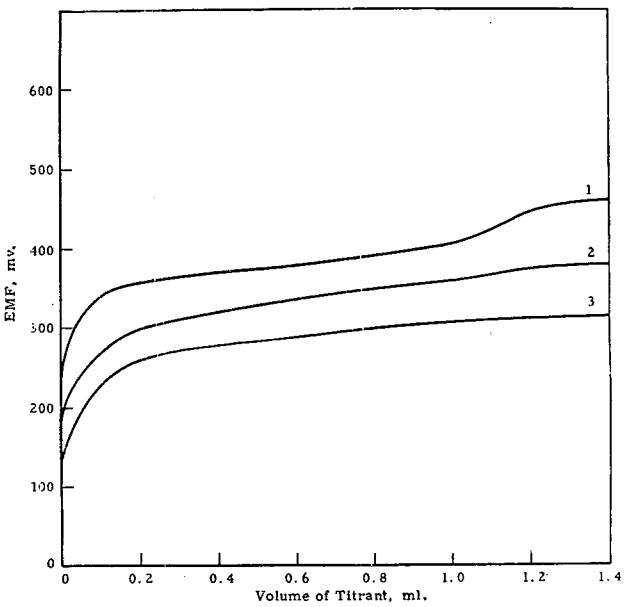

Figure 8. Titration of phenol in alcohols

0.2*N* tetrabutylammonium hydroxide titrant in isopropyl alcohol, glass-calomel electrodes
1. Isopropyl alcohol
2. Ethyl alcohol
3. Methanol

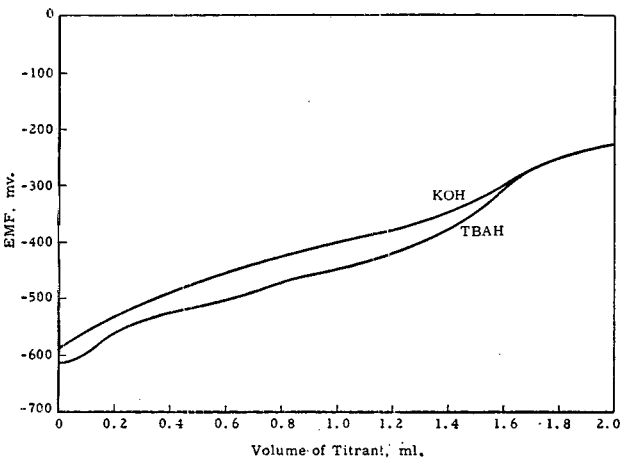

Figure 9. Titration of phenol in isopropyl alcohol

Platinum electrodes

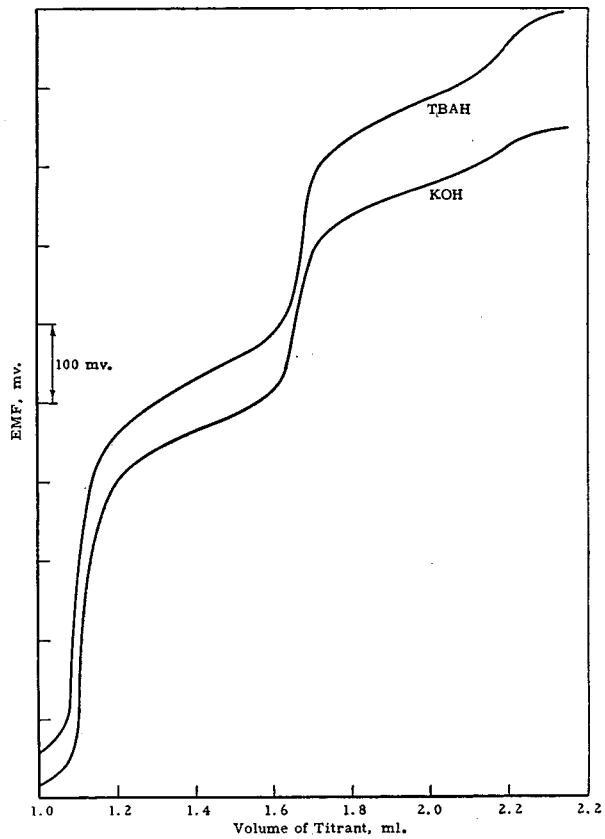

Figure 10. Titration of phenol–acetic acid–hydrochloric acid mixture in isopropyl alcohol

Glass-platinum electrodes; curves shifted for clarity

titration curves cannot be explained on the basis of solubility as were the curves which were obtained in ethylenediamine (Figure 4), because precipitation does not occur in pyridine.

In Alcohols. Alcohols are not generally considered as suitable solvents for the titration of very weak acids, although they have found considerable application for the titration of stronger acids. Very little information can be found in the literature which would permit the titration characteristics of different alcohols to be compared.

An attempt was made to titrate phenol in methanol and ethyl and isopropyl alcohols with tetrabutylammonium hydroxide. The resulting curves, shown in Figure 8, indicate that only the isopropyl alcohol gives a detectable inflection. Apparently both methanol and ethyl alcohol are more acid than isopropyl alcohol and thus less suited for the titration of very weak acids. This view is supported by the results of Hine and Hine (*5*), who found the order of increasing acidity to be isopropyl alcohol, ethyl alcohol, and methanol, as measured by a colorimetric procedure.

A comparison of the titration curves obtained when phenol is titrated in isopropyl alcohol with potassium hydroxide and tetrabutylammonium hydroxide with the aid of a platinum indicating electrode is shown in Figure 9. Because the platinum electrode is not subject to alkali ion error, the differences between the two curves must be due to some other effect. The low dielectric constant of isopropyl alcohol (D = 18.3) suggests the possibility of ion pair formation.

The curves for the titration of mixtures of a strong, weak, and very weak acid with tetrabutylammonium hydroxide and potassium hydroxide are shown in Figure 10. Separate inflections are obtained for hydrochloric acid, acetic acid, and phenol,

although the inflection for phenol is not sharp. These curves illustrate the value of a solvent such as isopropyl alcohol which is not strongly basic for the resolution of acid mixtures.

In Other Solvents. The curves obtained when phenol is titrated with tetrabutylammonium hydroxide in chloroform, ethyl ether, acetone, methyl ethyl ketone, and piperidine are shown in Figure 11. The most promising of these solvents is methyl ethyl ketone, because it not only gives good inflections for phenol but permits excellent resolution of acid mixtures. Although potassium hydroxide can be used as a titrant in this solvent, tetrabutylammonium hydroxide is preferable because it gives better inflections when the glass electrode is used and because of the greater solubility of its salts. Titration curves for a mixture of hydrochloric acid, acetic acid, and phenol in methyl ethyl ketone are shown in Figure 12. Curve 1, in which tetrabutylammonium hydroxide was used as titrant, and curve 2, in which potassium hydroxide was used, were both obtained with the platinum-oxygen indicating electrode. These curves are similar to one another in shape and in voltage span and quite different from curve 3, which was obtained with the potassium hydroxide titrant and a glass indicating electrode. Apparently the reduced span and small phenol inflection of curve 3 are due to the effect of potassium ion on the glass indicating electrode.

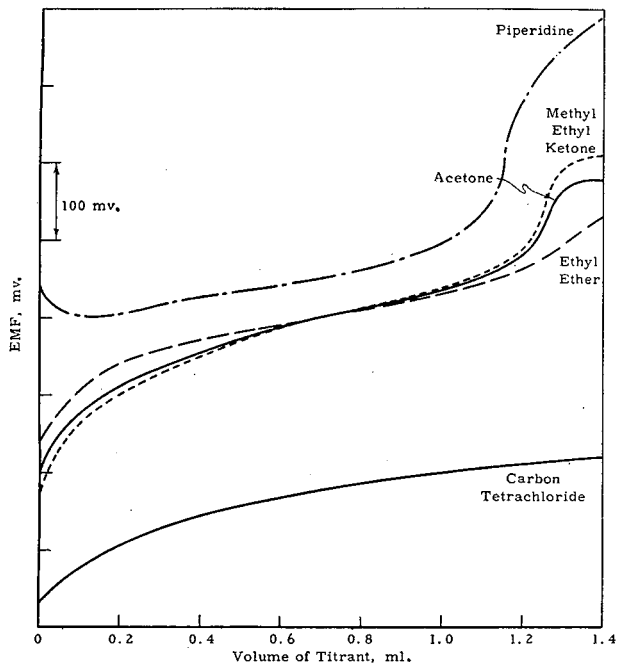

Figure 11. Titration of phenol in various solvents with tetrabutylammonium hydroxide in isopropyl alcohol

Glass-calomel electrodes; curves shifted for clarity

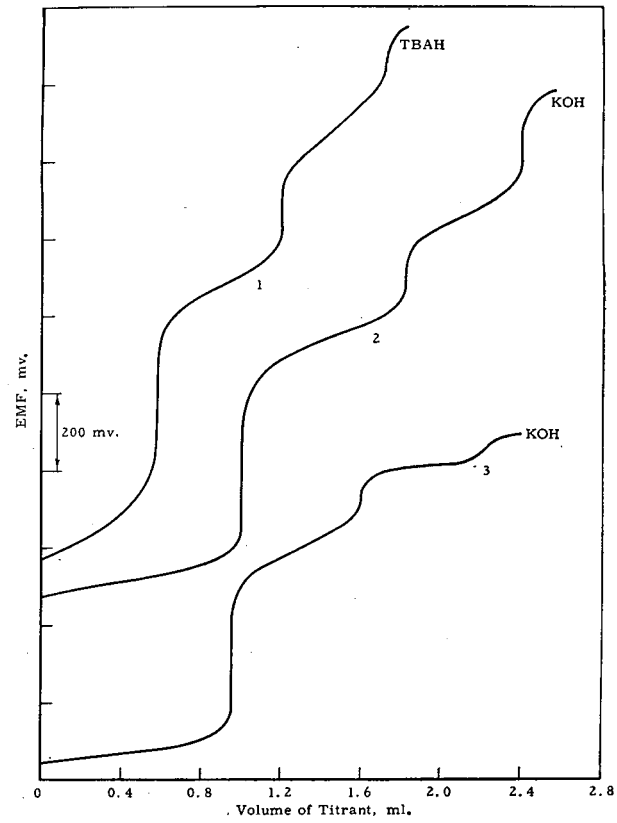

Figure 12. Titration of phenol-acetic acid-hydrochloric acid mixture in methyl ethyl ketone

1, 2. Platinum electrodes
3. Glass-platinum electrodes
Curves shifted vertically for clarity

DISCUSSION

The use of tetrabutylammonium hydroxide in the present investigation in no way implies that it is the best quaternary ammonium hydroxide for nonaqueous titrations. It was chosen as a convenient starting point for the exploratory study of these compounds. There is also reason to believe that more convenient and less expensive methods of preparing such titrants can be developed.

Because of the wide variety of quaternary ammonium hydroxides, specific effects of structure may be pronounced in some cases. This should be especially true in titrations conducted in solvents of very low dielectric constant where ion association is important. The possibility of applying such specific effects to differentiate acids should not be overlooked.

The curves obtained when phenol is titrated in nonaqueous solvents with tetrabutylammonium hydroxide support the contention that the hydroxide ion is a sufficiently strong titrant for very weak acids. It has also been shown that very weak acids can be successfully titrated with tetrabutylammonium hydroxide in solvents such as pyridine, isopropyl alcohol, ethyl ether, acetone, and methyl ethyl ketone, which are not generally considered as very basic solvents. The main purpose of this investigation was to obtain information on the general characteristics of tetrabutylammonium hydroxide; therefore, no single titration solvent was studied in detail. It is apparent, however, that some of the solvents tested possess such interesting and useful properties that further study should prove profitable. This is especially true of pyridine and methyl ethyl ketone which combine three very important qualities of a good titration medium: (1) They are good solvents for a wide variety of materials; (2) they are sufficiently weakly acidic to permit the titration of very weak acids; and (3) they are sufficiently weakly basic to be good differentiating solvents.

LITERATURE CITED

(1) Deal, V. Z., Wyld, G. E. A., ANAL. CHEM. 27, 47 (1955).
(2) Fritz, J. S., Keen, R. T., *Ibid.*, 25, 179 (1953).
(3) Fritz, J. S., Lisicki, N. M., *Ibid.*, 23, 589 (1951).
(4) Harlow, G. A., Noble, C. M., Wyld, G. E. A., *Ibid.*, 28, 784 (1956).
(5) Hine, J., Hine, M., *J. Am. Chem. Soc.* 74, 5266 (1952).
(6) Moss, M., Elliot, J., Hall, R., ANAL. CHEM. 20, 784 (1948).
(7) Weaver, J. R., Lykken, L., *Ibid.*, 19, 372 (1947).

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Tetrabutylammonium Hydroxide as Titrant for Acids in Nonaqueous Solutions

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Tetrabutylammonium hydroxide in benzene-methanol is a stable titrant which yields constant and reproducible potentials with an improved glass-calomel electrode system, and forms no precipitate with any of the compounds tested. Thus, it may overcome a number of limitations of bases previously suggested. In conjunction with amine-type or neutral solvents, this base can be used for the determination of a wide variety of acidic compounds, including phenols, amino acids, imides, thiols, enols, mono-, di-, and tribasic acids, amine salts of weak and strong acids, and mixtures of very weak, weak, and strong acids. In a number of solvents, the titrant is sufficiently basic to distinguish both hydrogens of such acids as oxalic, tartaric, malic, succinic, fumaric, phthalic, and sulfuric. Many of the acidic compounds may also be titrated visually using thymol blue or azo violet as indicators.

THE first comprehensive review of acid-base titrations in nonaqueous solutions can be found in the booklet by Fritz (*4*), in which the author compiled not only references on the theory and scope, but also some of the applications of this subject. Pifer, Wollish, and Schmall (*14*) give an excellent summary of the practical applications of nonaqueous titrations to pharmaceutical compounds and their intermediates. Riddick (*15, 16*) lists comprehensive bibliographies of the theoretical aspects and applications of nonaqueous titrations. Recently, Deal and Wyld (*3*) presented an excellent survey of the application of basic titrants for the determination of weak acids in nonaqueous solutions; the literature up to the beginning of 1954 is summarized by this paper.

Heretofore, the alkali metal alcoholates have usually been used as basic titrants in nonaqueous solutions (*4*). These include lithium, sodium, and potassium methoxides, which are prepared by reaction of the respective alkali metal with methanol and dilution of the alcoholates with benzene. Other basic titrants are sodium aminoethoxide, prepared by reaction of sodium metal with ethanolamine and dilution with ethylenediamine (*12*), and sodium triphenylmethane in 1 to 1 benzene-ether solution (*4*).

Basic solvents which have been employed are dimethyl formamide, pyridine, *n*-butylamine, and ethylenediamine. Some of the relatively neutral solvents which have been used successfully in acidic titrations include acetone, acetonitrile, alcohols, benzene-alcohol solutions, and chloroform.

Indicators which have proved satisfactory include thymol blue, azo violet (*p*-nitrobenzeneazoresorcinol), and *o*-nitroaniline.

Electrode systems which have been used in acid determinations in nonaqueous solutions include antimony-calomel (*4*); antimony-glass (*5*), antimony-antimony, hydrogen-antimony, hydrogen-calomel (*12*), glass-calomel (*3*), and platinum-calomel, where the calomel electrode was prepared with ethylenediamine (*7*).

This investigation on tetrabutylammonium hydroxide was initiated to find a nonaqueous titrant which is more easily manipulated than those reported previously. There are several factors which impair the utility of these earlier procedures: The stability of the alkali metal alcoholates is not as satisfactory as is desirable; it is difficult to obtain reproducible potentials with some of the electrode systems recommended, and with some electrode pairs the end point potential in the titration is slowly reached; in the titration of some organic acids the formation of gelatinous precipitates seriously affects the discernment of the true potentials; and although not necessarily a disadvantage, most titrimetric methods are general and do not ordinarily differentiate different types of acids.

These disadvantages were partially overcome by the method of Deal and Wyld (*3*), who employed potassium hydroxide in isopropyl alcohol as a titrant, substituting tetrabutylammonium hydroxide in water–isopropyl alcohol where precipitation interfered with the potassium hydroxide titrant. They used glass-calomel electrodes for the potentiometric determination of weak acids in ethylenediamine or dimethyl formamide solution. Contrary to the present work, their study was limited in that the tetrabutylammonium hydroxide solution was not strictly nonaqueous, and because only a selected number of weakly acidic compounds were tested. In addition they reported on only two solvents, dimethyl formamide and ethylenediamine, and gave no information as to the applicability of indicators. Harlow, Noble, and Wyld (*8*) have recently extended the work of Deal and Wyld on the use of tetrabuylammonium hydroxide for the potentiometric titration of very weak acids.

The work on the procedure reported herein was started more than 2 years ago, before the authors were aware of the above-mentioned work of the Shell Development group. The method described here uses a more strongly basic titrant of tetrabutylammonium hydroxide in benzene-methanol to increase greatly the scope of the method. Other quaternary ammonium hydroxides were investigated in addition to tetrabutylammonium hydroxide; however, from the standpoint of basicity of the titrant and the availability and purity of the quaternary ammonium halides, the tetrabutylammonium hydroxide was found to be the most satisfactory. Several additional anhydrous solvents, including acetone, acetonitrile, benzene-methanol, benzene-ethanol, benzene–isopropyl alcohol, pyridine, and *n*-butylamine, as well as dimethylformamide and ethylenediamine, were found applicable. Glass-calomel electrodes were satisfactory for potentiometric titrations, but a much improved electrode system was obtained by a slight modification of the calomel electrode. Constant potentials were reached rapidly, remained steady, and were reproducible. No precipitate formed in the titration of any acidic compound tested, and indicators could be used with many test compounds. In addition much better differentiation between various acidic groups within the same molecule or in mixtures was realized with this titrant than has been reported for other basic titrants.

In this work acids similar in strength to the mineral acids are designated "strong acids," those similar in strength to the unsubstituted monocarboxylic acids are designated "weak acids," and those similar in strength to phenol are designated "very weak acids."

PREPARATION OF TETRABUTYLAMMONIUM HYDROXIDE

The starting compound for the preparation of tetrabutylammonium hydroxide (Bu_4NOH) is tetrabutylammonium iodide (Bu_4NI). This compound, although available commercially, may be prepared by refluxing butyl iodide with *n*-tributylamine

(*11*). Recrystallization from benzene yields a material of sufficiently high purity.

In some of the preliminary work tetrabutylammonium hydroxide was prepared by passing a benzene-methanol solution of tetrabutylammonium iodide through an Amberlite IRA-411(OH) anion exchange resin. A satisfactory titrant was obtained in this manner, but it was not too suitable a preparation because the exchange capacity of the resin for iodide was poor, and it was exceedingly difficult to regenerate the hydroxide from the iodide form of the resin.

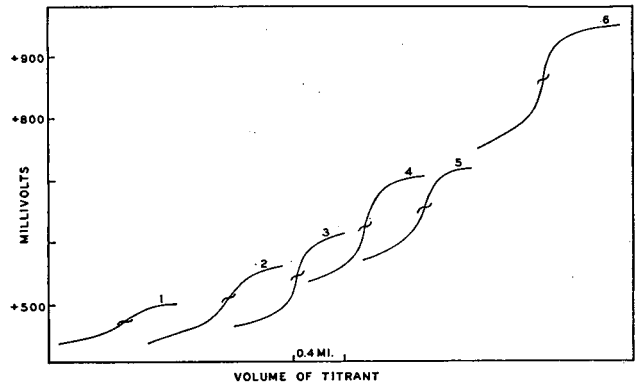

Figure 1. Effect of solvent, titrant, and electrode system on titration of phenol

0.1*N* Tetrabutylammonium hydroxide titrant

Curve	Solvent	Solvent for Titrant	Electrodes
1	Dimethyl formamide	Water	Glass-calomel
2	Benzene–isopropyl alcohol	Benzene-methanol	Glass-modified calomel
3	Dimethyl formamide	Water–isopropyl alcohol	Glass-calomel
4	Acetonitrile	Benzene-methanol	Glass-modified calomel
5	Pyridine	Benzene-methanol	Glass-calomel
6	Pyridine	Benzene-methanol	Glass-modified calomel

The classical preparation of the quaternary ammonium hydroxides is by reaction of an aqueous solution of a quaternary ammonium halide with an aqueous suspension of silver oxide. It was found that tetrabutylammonium hydroxide could be prepared in either methanol or ethanol by reaction of tetrabutylammonium iodide solution with an excess of silver oxide. The reaction proceeds readily at room temperature. The preparation of 0.1*N* tetrabutylammonium hydroxide is as follows.

Dissolve 40 grams of tetrabutylammonium iodide in 90 ml. of absolute methanol. Add 20 grams of finely ground purified silver oxide, stopper the flask, and agitate vigorously for 1 hour. Centrifuge a few milliliters of the mixture and test the supernatant for iodide. If the test is positive, add 2 grams more of silver oxide and reagitate 30 minutes; if negative, filter the mixture through a sintered-glass funnel of fine porosity. Rinse the reaction flask and funnel with three 50-ml. portions of dry benzene, and add to the filtrate. Dilute the filtrate to 1 liter with dry benzene. Flush this solution for 5 minutes with prepurified nitrogen and store in a reservoir protected from carbon dioxide and moisture. The titrant remains stable on extended storage. Standardize against benzoic acid by the visual titration procedure.

Ethanol may be substituted for methanol in the above procedure, but tetrabutylammonium iodide is more soluble in methanol, and there is little difference in the strength of the resulting titrants.

APPARATUS AND REAGENTS

The following are needed:

Beckman general purpose glass electrode, No. 4990-80.

Beckman sleeve-type calomel electrode, No. 1170-71, modified by replacing the saturated aqueous potassium chloride solution in the outer jacket with a saturated solution of potassium chloride in methanol; designated hereafter as methanol-modified calomel electrode.

Buret, 10 ml.

Tetrabutylammonium hydroxide, 0.1*N*, in 10 to 1 benzene-methanol, prepared as described above.

Acetonitrile, technical grade.

Pyridine, technical grade.

Dimethyl formamide, technical grade (Du Pont).

Benzene–isopropyl alcohol, 10 to 1. Mix 100 ml. of isopropyl alcohol with 1 liter of benzene.

Thymol blue indicator solution. Dissolve 0.3 gram of thymol blue in 100 ml. of isopropyl alcohol.

Azo violet indicator solution, a saturated solution of *p*-nitrobenzeneazoresorcinol in benzene.

Nitrogen, prepurified.

POTENTIOMETRIC TITRATIONS

Procedure. Accurately weigh a sample which will require a titration of 2 to 10 ml. into a 250-ml. electrolytic beaker. Add 50 ml. of solvent and place in position under the glass and methanol-modified calomel electrodes. Fill the buret with 0.1*N* tetrabutylammonium hydroxide and cover the buret top with an Ascarite tube. Best results are obtained if the titrations are performed under a nitrogen blanket when basic solvents, such as dimethyl formamide or pyridine, are used. Add the titrant in 0.1-ml. increments until just before the equivalence potential is indicated, then in 0.05-ml. increments. Continue the titration until the cell potential reaches a maximum and remains relatively constant on addition of titrant. Because most of the solvents contain acidic impurities, it is necessary to perform a blank titration.

Prepare a titration curve by plotting volume of titrant against millivoltage and determine the equivalence point or points from this curve. When two or more inflections are present in the curve, use the difference between successive equivalence points to calculate the volume of titrant equivalent to the acids represented.

Titration of Known Acids. A number of acidic compounds were titrated following the potentiometric method. Pyridine was the best basic solvent for use with 0.1*N* tetrabutylammonium hydroxide titrant. The cell potentials were steadier and were reached more rapidly in this solvent than in others tested. Dimethyl formamide was also used, but was not as satisfactory as pyridine. Acetonitrile was the best of the neutral solvents tested, although other neutral solvents including benzene-methanol, benzene-ethanol, benzene–isopropyl alcohol (all in 10 to 1 volume ratios), and acetone were usable.

The strongly basic solvents, *n*-butylamine and ethylenediamine, have not been studied exhaustively as yet because the potential range was so greatly compressed in these solvents. In dimethyl formamide and pyridine the e.m.f. range is approximately 900 mv., whereas in ethylenediamine and *n*-butylamine this range is compressed to approximately 300 mv. In addition, the titrations in pyridine or dimethyl formamide were much more definitive.

Good titration curves were obtained with glass-calomel electrodes; however, the sharpness of the inflections was markedly increased with the substitution of the methanol-modified calomel electrode. The fiber-type calomel electrode may be used with or without methanol modification, but occasionally causes erratic readings due to precipitation of potassium chloride on the fiber. The antimony-calomel electrode system was also usable in pyridine and dimethyl formamide solutions, as well as the antimony–methanol-modified calomel electrodes. These antimony-calomel electrode pairs were not nearly so satisfactory as the glass–methanol-modified calomel electrodes.

Results. The titration curves of phenol in Figure 1 indicate the comparative strength of tetrabutylammonium hydroxide when dissolved in water, isopropyl alcohol–water (9 to 1 by volume), or benzene-methanol (10 to 1 by volume). Curves 2, 4, and 6 illustrate the difference in inflection when phenol is titrated in benzene–isopropyl alcohol, acetonitrile, and pyridine with the same titrant. Comparison of curves 5 and 6 indicates the improvement of the inflection with the methanol-modified electrode.

In addition to a sharper inflection, much steadier potentials were obtained when the modified electrode was used.

Figure 2 shows the titration curves for 2-mercaptobenzothiazole, succinimide, salicyladoxime, acetyl acetone, and α-toluenethiol, all examples of very weak acids with the exception of 2-mercaptobenzothiazole. The samples were dissolved in pyridine and titrated with 0.1*N* tetrabutylammonium hydroxide in benzene-methanol using glass and methanol-modified calomel electrodes.

Figure 3 gives the titration curves for a series of di- and trihydroxybenzenes, in which the samples were titrated with 0.1*N* tetrabutylammonium hydroxide using the glass and methanol-modified calomel electrodes. A single inflection was obtained for each compound. Each of these compounds was found to be monobasic in this titration.

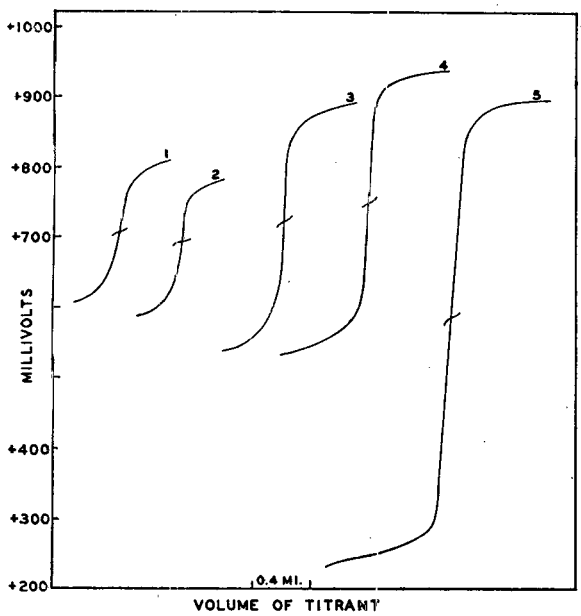

Figure 2. Titration of very weak acids

1. α-Toluenethiol
2. Salicylaldoxime
3. Succinimide
4. Acetyl acetone
5. 2-Mercaptobenzothiazole

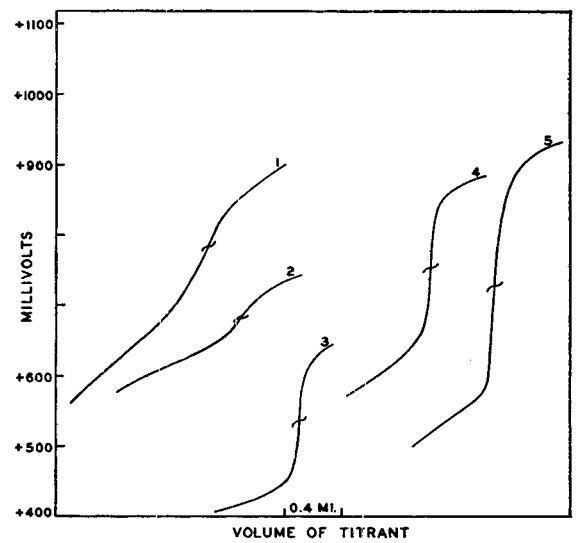

Figure 3. Titration of di- and trihydroxybenzenes

Curve	Solute	Solvent
1	Resorcinol	Dimethyl formamide
2	Hydroquinone	Dimethyl formamide
3	Dihydrodimethylresorcinol	Pyridine
4	Pyrogallic acid	Pyridine
5	Catechol	Pyridine

Titration curves for *o*-, *m*-, and *p*-hydroxybenzoic acids are shown in Figure 4. The solvent-titrant-electrode system was pyridine, 0.1*N* tetrabutylammonium hydroxide in benzene-methanol, and glass–methanol-modified calomel, respectively. Two inflections were obtained for *m*- and *p*-hydroxybenzoic acids, but only a single inflection representing the carboxyl group was obtained for *o*-hydroxybenzoic acid, indicating that the compound is monobasic in this titration.

In Figure 5 the titration curves for several dibasic acids are given. Again, the samples were titrated in pyridine with 0.1*N* tetrabutylammonium hydroxide in benzene-methanol, using the glass and methanol-modified calomel electrodes. The curves for sulfuric acid and succinic acid are of particular interest in that the dissociation constants for sulfuric acid in water are of the order of 10^{-1} and 10^{-2}, and for succinic acid, 10^{-5} and 10^{-6}, yet in pyridine solution sharp inflections are obtained for each equivalent of each acid.

Curves for some of the amino acids are shown in Figure 6. In these titrations the sample was dissolved in a minimum amount of water, and pyridine was added prior to titration with 0.1*N* tetrabutylammonium hydroxide in benzene-methanol. Both the hydrochloride and carboxyl groups show an inflection in the titration of histidine hydrochloride, whereas only the carboxyl group in arginine hydrochloride reacts.

Three inflections were obtained in the titration of tribasic citric acid, of *o*-phosphoric acid and of tetrabasic (ethylenedinitrilo)tetraacetic acid (ethylenediaminetetraacetic acid). This is illustrated in Figure 7. The titrant was 0.1*N* tetrabutylammonium hydroxide in benzene-methanol, and glass–methanol-modified electrodes were used.

Figure 8 shows the titration curves of acid mixtures with three inflections in each curve. Curves 2 and 3 represent the titration of a mixture of a strong acid, a weak acid, and a very weak acid. Curve 1 is for the titration of acetic and malic acids in pyridine solution. Malic acid has dissociation constants in water of the order of 10^{-4} and 10^{-6}, while acetic acid has a dissociation constant of the order of 10^{-5}. In this particular curve, the first and third inflections represent the two acid equivalents of malic

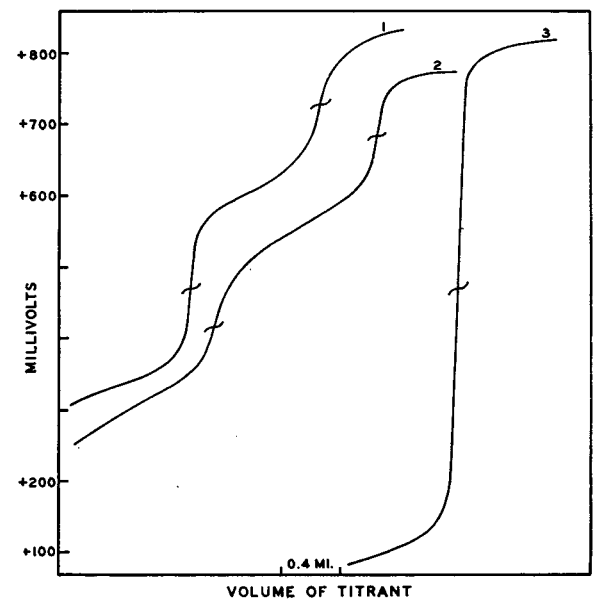

Figure 4. Titration of hydroxybenzoic acids

1. *m*-Hydroxybenzoic acid
2. *p*-Hydroxybenzoic acid
3. *o*-Hydroxybenzoic acid

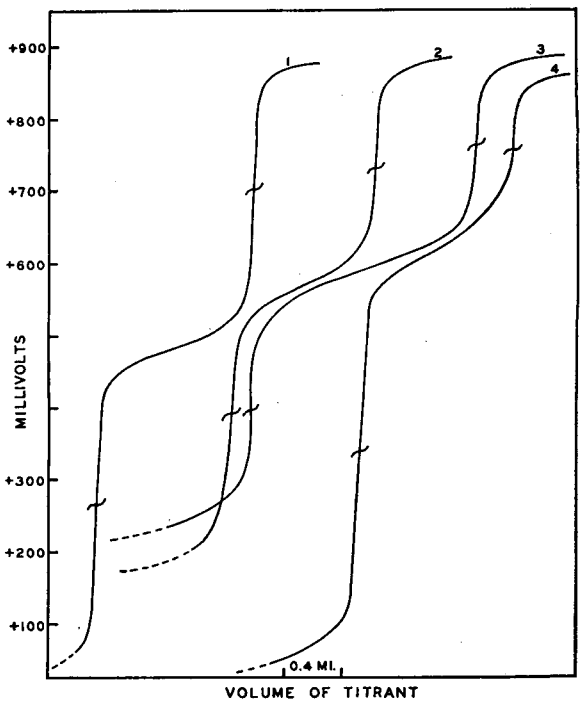

Figure 5. Titration of dibasic acids

1. Sulfuric acid
2. Malic acid
3. Succinic acid
4. *o*-Phthalic acid

acid, while the second inflection represents the acetic acid equivalent.

In Figure 9 two curves with four inflections are shown. The first is a mixture of nitric and malic acids and *p*-cresol; the second, a mixture of sulfuric and succinic acids. In the latter curve the first and third inflections represent the sulfuric acid equivalents, while the second and fourth inflections represent the succinic acid equivalents.

Figure 10 illustrates the specificity possible with this titrant. In this curve five inflections are obtained in the titration of a mixture of nitric acid, malic acid, acetic acid, and phenol. The first inflection represents nitric acid; the second and fourth, malic acid; the third, acetic acid; and the fifth, phenol.

For the curves in Figures 8, 9, and 10, the solvent-titrant-electrode system was pyridine, 0.1*N* tetrabutylammonium hydroxide in benzene-methanol, and glass–methanol-modified calomel electrodes, respectively.

VISUAL TITRATIONS

Accurately weigh a sample which will require 6 to 8 ml. of titrant into a 125-ml. Erlenmeyer flask. Add 25 ml. of solvent and 4 drops of thymol blue or azo violet indicator solution (thymol blue for weak monobasic acids, azo violet for weak dibasic acids and very weak acids). Titrate as rapidly as possible with 0.1*N* tetrabutylammonium hydroxide to the blue end point with thymol blue indicator, or to a violet or in some instances a blue end point with azo violet indicator. It is necessary either to make a blank titration for the solvent or to neutralize the solvent exactly prior to adding it to the sample.

Results. A majority of the compounds titrated in pyridine could be determined by visual titration with either thymol blue or azo violet indicator. The visual change in most instances with thymol blue was from green to blue, and thymol blue is the indicator of choice when titrating weak acids. Azo violet was the preferred indicator when titrating very weak acids. The color at the end point for some compounds was violet; for others, blue. It is necessary to titrate potentiometrically first to ascertain if a compound has an azo violet end point, and if the end point is blue or violet.

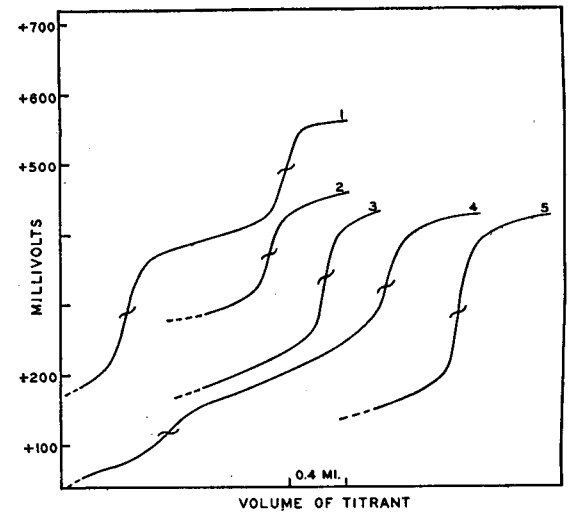

Figure 6. Titration of amino acids

1. Glutamic acid
2. Alanine
3. Arginine hydrochloride
4. Histidine monohydrochloride
5. Glycyl-glycine

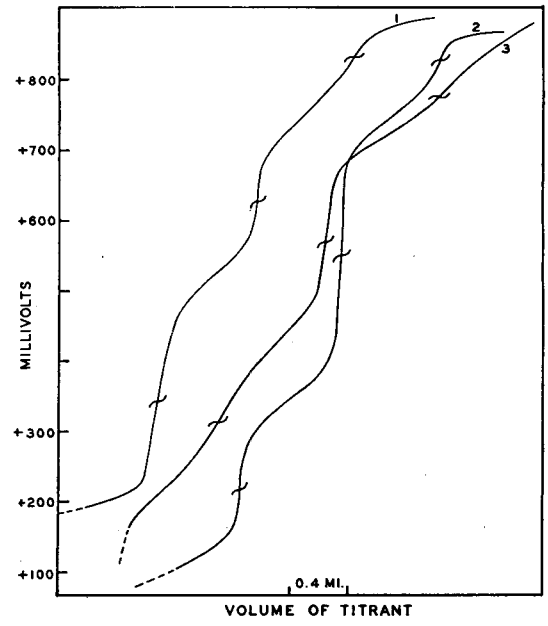

Figure 7. Titration of polybasic acids

Curve	Solute	Solvent
1	(Ethylenedinitrilo)-tetraacetic acid	Dimethyl formamide
2	Citric acid	Pyridine
3	Phosphoric acid	Pyridine

If a compound has a thymol blue end point in pyridine it will also have the corresponding visual end point in the neutral solvents such as acetonitrile, benzene-alcohol, or acetone. A great number of very weak acids will have an azo violet end point in acetonitrile.

Precision. The precision of the indicator titration procedure was tested by titrating 10 samples of 2-mercaptobenzothiazole and 10 samples of citric acid in acetonitrile solution to the thymol blue end point. The mean per cent purity of the 2-mercapto-

benzothiazole was 98.82, with a standard deviation of 0.264. The mean per cent purity of the citric acid was 99.53 with a standard deviation of 0.213.

Similar tests were conducted on 1-naphthol and succinimide compounds which may be titrated to the azo violet end point. The mean for 10 analyses of 1-naphthol in acetonitrile was 99.86% with a standard deviation of 0.166; the mean for 10 analyses of 1-naphthol in pyridine was 99.65% with a standard deviation of 0.271. The mean for 10 analyses of succinimide in pyridine was 99.13% with a standard deviation of 0.348.

COMPOUNDS TITRATED WITH 0.1*N* TETRABUTYLAMMONIUM HYDROXIDE

Listed below are some of the acid compounds which have been successfully titrated in pyridine with 0.1*N* tetrabutylammonium hydroxide in benzene-methanol. Glass and methanol-modified calomel electrodes were used throughout and thymol blue or azo violet indicators were used as indicated.

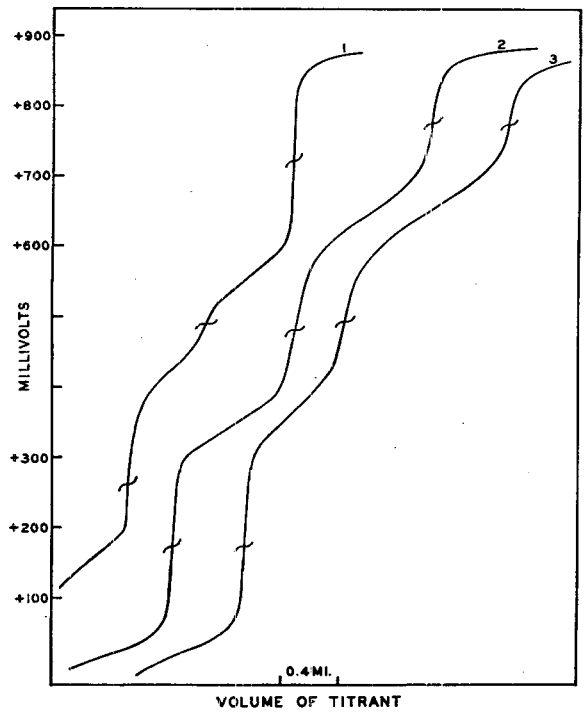

Figure 8. Titration of acid mixtures

1. Acetic and malic acids
2. *p*-Toluenesulfonic acid, benzoic acid, phenol
3. Hydrochloric acid, acetic acid, *p*-cresol

Monobasic Compounds Having Thymol Blue End Point. Acetic acid, benzoic acid, nicotinic acid, salicylic acid, ammonium nitrate, ammonium acetate, *p*-nitrophenol, *o*-nitrophenol, 2,4-dinitrophenol, 2-mercaptobenzothiazole.

Amino Acids Having Thymol Blue End Point. Samples were dissolved in a minimum amount of water and pyridine was added.

Alanine, leucine, threonine, asparagine, methionine, hydroxyproline, glutamine, glycylglycine, glutamic acid, aspartic acid, arginine hydrochloride, histidine hydrochloride.

Monobasic Compounds Having Azo Violet End Point. Phenol, *p*-benzylphenol, *o*-phenylphenol, 1-naphthol, 2-naphthol, 2,5-dimethylphenol, *p*-bromophenol, catechol, pyrogallic acid, dihydrodimethylresorcinol, succinimide, phthalimide, salicylaldoxime, acetyl acetone, *p*-chlorothiophenol, α-toluenethiol, 5-amino-2-benzimidazolethiol.

Dibasic Compounds Having Azo Violet End Point. *m*-Hydroxybenzoic acid, *p*-hydroxybenzoic acid, malic acid, oxalic acid, malonic acid, maleic acid, fumaric acid, succinic acid, *o*-phthalic acid, and sulfuric acid. Two inflections were observed in the titration curves of all these compounds. Citric acid, a tribasic compound, has an azo violet end point coinciding with the potentiometric end point of its second equivalent, and thus would be considered dibasic in a visual titration.

Monobasic Compounds Titrated Potentiometrically but with No Suitable Visual End Point. Thymol, hydroquinone, resorcinol, *p*-toluhydroquinone, *m*-cresol, *p*-cresol.

DISCUSSION

The limitations involved in the titration of polyfunctional acids in aqueous solvents have been discussed by Auerbach and Smolczyk (*1*). These authors have shown theoretically that no inflection occurs at the first equivalence point in the potentiometric titration curve unless the ratio of the first to the second ionization constants (K_1 to K_2) is greater than 16. In fact, it is difficult to locate the break at a ratio of 100. Kolthoff and Stenger (*10*) state that a satisfactory titration can be obtained only when K_1 is at least 10,000 times as large as K_2. Hence, it has been considered impossible to obtain an exact titration to the first equivalence point of such acids as oxalic, tartaric, citric, malonic, succinic, malic, fumaric, phthalic, and sulfuric.

Recently, Deal and Wyld (*3*) obtained two inflections in the titration curves for the three isomers of phthalic acid. Goddu and Hume (*6*) have demonstrated that by a photometric titration method end points can be located without difficulty in $10^{-3}M$ solutions when the ratio of ionization constants is only 100. Higuchi and Rehm (*9*) showed that the two hydrogens of sulfuric acid could be differentiated by means of a conductometric titration in acetic acid. Critchfield and Johnson (*2*) utilized morpholine in acetonitrile to differentiate potentiometrically the two hydrogens of sulfuric acid in their procedure for determination of sulfuric acid–hydrochloric acid and sulfuric acid–nitric acid mixtures. Palit, Das, and Somayajulu (*13*) report the potentiometric titration of the two hydrogens of sulfuric acid and the application of this titration in analysis of acid mixtures. The work of these investigators suggested that, by a proper combination of electrode system, solvent, and titrant, it should be possible to titrate potentiometrically both

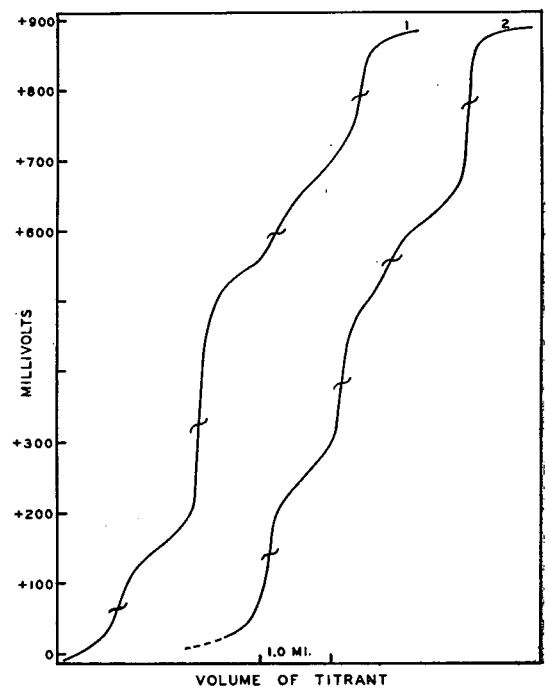

Figure 9. Titration of acid mixtures

1. Nitric acid, malic acid, *p*-cresol
2. Sulfuric and succinic acids

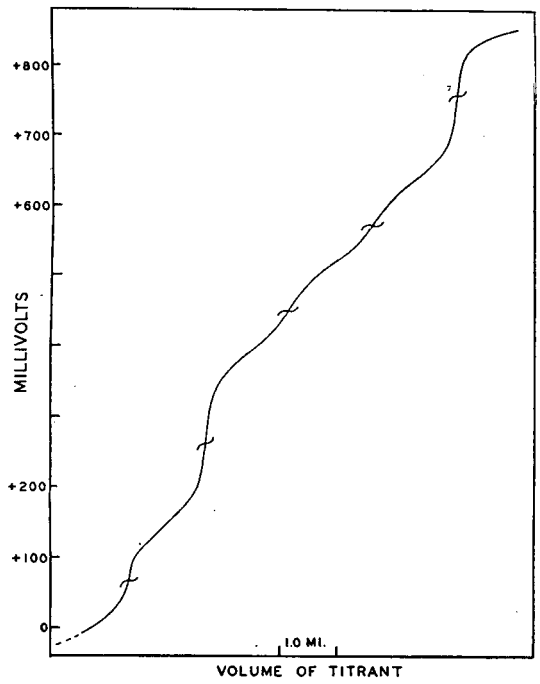

Figure 10. Titration of an acid mixture

Nitric acid, malic acid, acetic acid, and phenol

hydrogens of such acids as citric, malic, tartaric, fumaric, and succinic, whose ratio of ionization constants are not only less than 100, but approach the theoretical limit of 16 proposed by Auerbach and Smolczyk.

An extension of the work already in progress in this laboratory showed that it was possible to titrate these acids potentiometrically and to obtain two sharp inflections in the titration curve as illustrated in Figure 5. Thus, the proposed method afforded a fairly simple and rapid means of exceeding the sensitivity in end point detection of the photometric method and appeared to provide a method for the differentiation of acid mixtures hitherto analyzed by lengthy and cumbersome procedures. For example, preliminary studies indicate that with this titrant it is fairly simple to assay mixtures of sulfuric with all types of mineral, sulfonic, and organic acids; various mixtures of organic acids; mixtures of mineral and organic acids; and a number of inorganic compounds. These particular applications will be reported in the near future.

ACKNOWLEDGMENT

The authors express their appreciation to A. H. Laurene and E. W. Barnhardt for the preparation of the figures.

LITERATURE CITED

(1) Auerbach, Fr., Smolczyk, E., *Z. physik. Chem.* **110A,** 65 (1924).
(2) Critchfield, F. E., Johnson, J. B., ANAL. CHEM. **26,** 1803 (1954).
(3) Deal, V. Z., Wyld, G. E. A., *Ibid.*, **27,** 47 (1955).
(4) Fritz, J. S., "Acid Base Titrations in Nonaqueous Solvents," G. Frederick Smith Chemical Co., Columbus, Ohio, 1952.
(5) Fritz, J. S., Lisicki, N. M., ANAL. CHEM. **23,** 589 (1951).
(6) Goddu, R. F., Hume, D. N., *Ibid.*, **26,** 1679 (1954).
(7) Gran, G., Althin, B., *Acta Chem. Scand.* **4,** 967 (1950).
(8) Harlow, G. A., Noble, C. M., Wyld, G. E. A., ANAL. CHEM. **28,** 787 (1956).
(9) Higuchi, T., Rehm, C. R., *Ibid.*, **27,** 408 (1955).
(10) Kolthoff, I. M., Stenger, V. A., "Volumetric Analysis," 2nd ed., vol. I, Interscience, New York, 1942.
(11) Laitinen, H. A., Wawzonek, S., *J. Am. Chem. Soc.* **64,** 1765 (1942).
(12) Moss, M. L., Elliott, J. H., Hall, R. T., ANAL. CHEM. **20,** 784 (1948).
(13) Palit, S. R., Das, M. N., Somayajulu, G. R., "Nonaqueous Titration," Indian Association for the Cultivation of Science, Calcutta, India, 1954.
(14) Pifer, C. W., Wollish, E. G., Schmall, M., *J. Am. Pharm. Assoc., Sci. Ed.* **42,** 509 (1953).
(15) Riddick, J. A., ANAL. CHEM. **24,** 41 (1952).
(16) *Ibid.*, **26,** 77 (1954).

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Titrations of Halides with Electrolytically Generated Mercurous Ion

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Mercurous ion can be generated with 100% current efficiency at large mercury pool anodes. Accurate and precise titrations of macro quantities (0.2 to 4.0 meq.) of chloride, bromide, and iodide have been carried out with this reagent. The use of mercurous ion in the coulometric titration of halides offers some advantages over the use of silver ion, particularly for the titration of macro quantities.

PRZYBYLOWICZ and Rogers (*2*) have reported the generation of mercurous ion at mercury-coated gold and silver electrodes and at small mercury pool electrodes and have successfully employed this reagent for the coulometric titration of microgram and milligram quantities of halide ions. This paper reports the electrolytic generation of mercurous ion at large mercury pool electrodes and the use of the reagent for the titration of macro quantities of the halide ions.

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EXPERIMENTAL

Reagents. All chemicals used were analytical reagent grade. Standard solutions of the halide ions were prepared from the sodium or potassium salts.

The supporting electrolyte was 0.5*M* perchloric acid for the chloride and bromide titrations. A solution which was 0.4*M* in potassium nitrate and 0.1*M* in perchloric acid was employed in the iodide titrations; the potentials at the end point were more rapidly established and the equivalence point was more sharply defined in this electrolyte than in the 0.5*M* perchloric acid electrolyte.

Apparatus. The constant current power supply was similar to that described by Reilley, Cooke, and Furman (*3*), and was capable of supplying currents of 50, 100, and 200 ma. Electrolysis currents were adjusted and checked frequently by potentiometric measurement of the internal resistance drop across a standard resistance in series with the cell.

The titration cell was a 100-ml. beaker with a sealed-in plati-

num wire to make contact with a mercury pool anode which covered the entire bottom of the beaker to a depth of about 0.5 cm. The generator cathode consisted of a helix of platinum wire dipping into a glass tube 1 cm. in diameter with a fine sintered-glass disk at the bottom. The electrolyte in the cathode compartment was 0.1*M* sulfuric acid; this compartment was always filled to a level above that of the main body of the solution to prevent diffusion of the sample into the cathode compartment. Despite the fact that mercurous sulfate is sparingly soluble, sulfuric acid diffusing from the cathode compartment caused no difficulty. The use of perchloric acid in the cathode compartment was avoided because it was feared that some chloride ion might be produced at the cathode. The results of Przybylowicz and Rogers (*2*) indicate that no reduction of perchlorate occurs, however. During the titration the contents of the beaker were stirred vigorously with a magnetic stirrer; the stirring bar floated on the surface of the mercury pool anode.

Table I. Typical Analytical Results

Amount Taken, Meq.	Current, Ma.	Time, Sec.	No. of Runs	Amount Found, Meq.	Av. Dev., %	Av. Error, %
			Chloride			
0.1949	50.98	369.3	5	0.1951	0.06	+0.10
0.4874	102.1	461.2	5	0.4879	0.14	+0.10
0.9747	102.1	922.2	5	0.9758	0.16	+0.11
0.4891	204.4	230.9	2	0.4889	0.14	−0.09
0.9783	204.4	462.0	6	0.9787	0.09	+0.04
1.4674	204.4	693.5	2	1.4690	0.08	+0.11
1.9566	204.4	924.1	2	1.9573	0.03	+0.04
			Bromide			
0.2696	50.98	510.7	5	0.2698	0.09	+0.07
0.5392	50.98	1021.5	5	0.5397	0.09	+0.09
0.5070	102.1	479.4	5	0.5072	0.08	+0.04
1.0140	102.1	958.3	7	1.0145	0.06	+0.05
2.0280	102.1	1918.7	4	2.0298	0.04	+0.09
1.3480	204.4	636.8	5	1.3488	0.05	+0.06
2.6960	204.4	1272.5	4	2.6957	0.08	−0.01
4.0439	204.4	1910.7	4	4.0471	0.03	+0.08
			Iodide			
0.2482	50.98	470.1	5	0.2485	0.20	+0.12
0.4964	50.98	940.6	5	0.4971	0.12	+0.14
0.4964	102.1	469.9	5	0.4972	0.05	+0.16
0.9927	102.1	940.0	4	0.9945	0.09	+0.18
0.9927	204.4	470.1	5	0.9958	0.12	+0.31
0.9942	204.4	469.9	5	0.9954	0.11	+0.12
1.9854	204.4	940.1	5	1.9912	0.04	+0.29

All titrations were followed potentiometrically with a Beckman Model G pH meter. A saturated calomel electrode in conjunction with a potassium nitrate salt bridge was used as the reference electrode in all titrations. For the titrations of chloride and bromide ions the mercury pool generator anode served also as the indicator electrode. The mercury pool electrode proved to be unsatisfactory as an indicator electrode for the titration of iodide ion, because equilibrium was reached very slowly and the end-point break was very poor. A small silver foil electrode responded more rapidly, and this electrode was used in all iodide titrations reported here. Mercury-coated gold or silver electrodes (*2*) should be suitable as indicator electrodes, but were not employed in any of the present work.

PROCEDURE

A 50-ml. portion of generator electrolyte was placed in the sample cell and a measured volume of sample solution was added. When large sample volumes were used, the sample was added first and then a somewhat more concentrated solution of generator electrolyte was added, so that the final concentration of generator electrolyte was approximately that given in the preceding section. When the sample volume was small, several samples could be titrated in succession without renewing the generator electrolyte.

As the end point was approached the electrolysis current was interrupted and the potential of the indicator electrode measured. Steady potentials were established rapidly in the chloride and bromide titrations, but 30 to 60 seconds were frequently required in the neighborhood of the equivalence point with iodide samples. Generation was then continued in increments of about 0.3 second until the equivalence point potential was reached.

Blanks were found to be negligible for the quantities of sample employed in this work.

RESULTS AND DISCUSSION

The results of typical titrations are shown in Table I. The average deviation from the mean of replicate runs in the chloride titrations is about ±0.1% for all sample sizes between 0.2 and 2.0 meq. Although the average errors seem to indicate a systematic positive error of a few hundredths of 1%, this error does not exceed the uncertainty in the standardization and hence is not significant. The slope of the titration curve at the equivalence point is about 15 mv. per second of generation at a current of 50 ma. Hence, even at the lowest current employed, the equivalence point can be located with an uncertainty not exceeding a few tenths of a second.

The results obtained in the titration of bromide are similar to those obtained in the chloride titrations. Since the slope of the titration curve is greater—about 50 mv. per second at a current of 50 ma.—the precision realized in the titration of bromide is somewhat better than that in the titration of chloride.

The precision realized in the titration of iodide is somewhat poorer than in the titration of bromide, even though the slopes of the titration curves are nearly identical. The slow establishment of steady potentials in the former titrations probably accounts for the poorer precision. Other investigators experienced the same difficulty with a slowly established potential when either silver (*1*) or mercurous (*2*) ion was used for the titration of iodide. A small but significant positive error of 0.1 to 0.3% was observed in all of the iodide titrations attempted. The source of this error was not determined.

Electrolytically generated mercurous ion seems to possess nearly all of the desirable properties of silver ion for the titration of halides, and in some respects mercurous ion is superior to silver ion. Because the mercurous halides are less soluble than the corresponding silver salts, the titration curves are steeper at the equivalence point when mercurous ion is used as the titrant, and equivalence points can be located more precisely. This advantage is particularly pronounced in the titration of chloride ion; the slope of the titration curve at equivalence is nearly twice that for the corresponding titration with silver ion (*1*). Silver wire anodes must be cleaned frequently to remove the coating of silver halide; mercury anodes, on the other hand, never require cleaning, as a fresh surface is constantly exposed by efficient stirring. After one series of titrations has been completed, it is necessary only to drain off the electrolyte, together with most of the suspended precipitate, and replace with fresh generator electrolyte before beginning the next series.

ACKNOWLEDGMENT

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LITERATURE CITED

(1) Lingane, J. J., ANAL. CHEM. 26, 122 (1954).
(2) Przybylowicz, E. P., Rogers, L. B., *Ibid.*, 28, 799 (1956).
(3) Reilley, C. N., Cooke, W. D., Furman, N. H., *Ibid.*, 23, 1030 (1951).

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Coulometric Titrations with Electrolytically Generated Mercury(I and II)

Determinations of Chloride, Bromide, and Iodide

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Mercurous ion was generated at 100% current efficiency from mercury-coated gold and mercury pool electrodes. Potentiometric titrations of halides using this generated reagent were then carried out using similar indicator electrodes. Studies using a mercury-coated silver generator electrode indicated that it could be used equally well, although some silver dissolved. Its endpoint break was nearly the same as that for a mercury-coated gold or a mercury pool electrode and much larger than that obtained with pure silver. Using either an amalgamated gold or silver electrode, the lower limits of an individual halide which were determined with an accuracy of better than 1% in a volume of about 55 ml. were 0.24 mg. of chloride ($1 \times 10^{-4}M$), 0.067 mg. of bromide ($2 \times 10^{-5}M$), and 0.038 mg. of iodine ($6 \times 10^{-6}M$). Smaller amounts of halide down to 0.013 mg. of chloride, 0.0067 mg. of bromide, and 0.0012 mg. of iodide were determined with better than 5% accuracy. The coprecipitation errors encountered in argentimetric titrations of halide mixtures are also present in this method, whereas the usual adsorption errors in the titration of iodide alone were not observed.

INVESTIGATORS (*7, 9, 14*) have reported the use of electrically generated silver ion for the titrimetric determination of halides. Lingane (*9*) obtained greater sensitivity by titrating chloride in 80% ethyl alcohol, thereby rendering the precipitate less soluble. Since mercurous halides are less soluble than their silver analogs, the generation of mercurous ion was studied in the hope of developing a more sensitive titrimetric method for the halides. After the present paper had been submitted for publication, DeFord (*4*) made reference to some unpublished work by Horn (*5*) using generated mercurous ion as a reagent for the determination of the halides.

EXPERIMENTAL

Reagents. All chemicals used were of analytical reagent grade. Standard millimolar halide solutions were made up by weight from their respective potassium salts which had been recrystallized and dried at 110° C. for 2 hours. These solutions were then checked by potentiometric titration with standardized silver nitrate. The stock solutions were diluted, and aliquots of these diluted solutions were then used in the analyses. Iodide solutions were prepared fresh for each day's runs. Others were not kept longer than 2 weeks.

The supporting electrolyte in all of these titrations was $0.5M$ sodium perchlorate plus $0.02M$ perchloric acid. A stock solution of $5M$ sodium perchlorate (that from Fisher Scientific Co. was found to contain only a trace of chloride) and $0.2M$ perchloric acid was prepared and 5 ml. of this solution were added to each sample to be titrated. The total volume of the sample was approximately 55 ml.

Apparatus. The current source was an electronically regulated supply (*12*). The current was determined during each run by potentiometric measurement of the *IR* drop across a standard resistance in series with the titration cell. The latter was similar to the one used by Lingane (*9*).

Three different anodes were used to generate mercurous ion. These consisted of mercury-coated gold and silver electrodes and a mercury pool. The amalgamated silver electrode was prepared by taking a coil of silver wire (0.070 × 10 inches) cleaning it thoroughly in $1N$ nitric acid, and dipping it into metallic mercury. The analogous gold electrode was prepared using a 1.0 × 1.0 inch gold foil. Electrical contact to the gold foil was made with a platinum wire.

These mercury-coated electrodes required only infrequent renewal of the surface after every fourth or fifth titration by putting a drop of mercury on the electrode and removing the excess by shaking. Studies using a mercury-pool generating electrode employed a J-type glass tube 10 mm. in diameter, which held a drop of mercury in its upturned tip. Electrical contact to the external circuit was made through a platinum wire which ran the entire length of the glass tube.

The generator cathode consisted of a platinum foil in a glass tube connected to the main body of the solution through a fine sintered-glass disk. The cathode chamber contained supporting electrolyte of the same concentration as that in the main body of the solution. The level of liquid in this chamber was always kept above that of the liquid in the titration cell.

The indicator electrodes were prepared in exactly the same way as the corresponding generator electrodes, except that the amalgamated electrodes were somewhat smaller in size. Titrations in which the J-type mercury pool electrode was used as a generator electrode employed an identical indicator electrode. These indicator electrodes were used in conjunction with a saturated calomel reference electrode which was connected to the cell through a saturated potassium nitrate liquid salt bridge. The ends of the liquid bridge contained plugs of Corning unfused Vycor glass No. 7930 (*1*). These electrodes were connected to a Leeds & Northrup pH indicator (Model 7664) and the titrations were followed by observing the change in potential with time of generation of mercury ions. Originally the output of the Leeds & Northrup was fed into a recording potentiometer, but the recorder made the accurate determination of the elapsed time more difficult and was therefore abandoned after the preliminary survey work had been completed. In all titrations reported here potential readings were observed periodically on the Leeds & Northrup pH indicator and the potential-time curve was plotted manually with the aid of a stop watch. Replicate titrations were carried out by titrating to a definite potential corresponding to the equivalence point.

PROCEDURE

Samples. The sample was added to the titration cell and diluted to 50 ml. with distilled water, and 5 ml. of the stock solution of supporting electrolyte were added. Mixing the reagents in this order minimized the danger of oxidation by the perchlorate of either iodide or bromide.

The equivalence point potential in these titrations was obtained by determining the potential at which the slope of the curve went through a maximum and from it subtracting 0.018 volt. The 0.018 volt corresponds to the difference in potential between the point of maximum change of slope in the potentiometric curve and the equivalence point potential as calculated from the solubility product (*8*). From the equivalence point potential the elapsed time can be determined. In titrating the smallest amounts of halide reported, the potentiometric curves did not have large breaks and were very flat; hence the titrations were carried out to a definite potential near the point of maximum slope. An appropriate blank was then subtracted. In titration curves of mixtures the end point was determined by inspection and was close to the midpoint of the break.

Blanks. A 5-ml. portion of the stock solution of supporting electrolyte was diluted to 55 ml. with distilled water and the solution was titrated. The resulting potentiometric curve was then superimposed on an actual titration curve. The time required to reach the same potential as that selected for the end point in the titration of the sample provided the blank correction. The method of correction compensated not only for the blank but also for any error in picking the end point potential on the potentiometric curve because the curves for the sample and the

blank were essentially identical beyond the end point. However, a blank of this type can be applied only to the more soluble component in the titration of a binary mixture.

RESULTS

Chloride. Aqueous solutions containing between 0.24 and 13.60 mg. of chloride in 55 ml. of solution were analyzed using generating currents from 5 to 50 ma. The results are summarized in Tables I and II for the amalgamated gold and silver electrodes, respectively. The results show the accuracy to be superior to argentimetric titrations at high dilutions (*9*). When generating currents of less than 5 ma. were used, the potentiometric curve became very flat. Using solutions containing 80% by volume of methanol and the same supporting electrolyte, titrations of amounts as small as 0.014 mg. were possible using generating currents of 0.4 ma. These results are also included in the tables.

Bromide. Aqueous solutions of bromide were titrated under identical conditions in amounts ranging from 0.027 to 14.0 mg. Because of the lower solubility of mercurous bromide it was unnecessary to use alcoholic solutions to obtain curves with sharp breaks when using generating currents as small as 1 ma. By using alcohol it was possible to determine 0.007 mg. of bromide. Tables I and II show results of representative bromide titrations using both amalgamated silver and gold electrodes. It was found that no correction was required for the blank.

Iodide. As anticipated, the titrations of iodide with mercurous ion were the most sensitive of the reactions studied. Of particular interest was the fact that adsorption errors usually encountered in the argentometric titrations of iodide (*9*) were not observed. Hence the limiting factor was, as with the other two halides, the solubility of the precipitate. Table I shows that the accuracy of the method is good down to 1.1 γ of iodide per 55 ml. in aqueous media. Only at the lower limit was a blank correction (34 seconds) found using a current of 10 μa.

Muller and Aarflot (*6, 11*) found that in the volumetric titration of iodide with mercurous ion, negative errors as high as 2% were observed due to the fact that the reaction, $Hg_2^{++} = Hg + Hg^{++}$, was favored by the presence of large concentrations of iodide due to formation of the tetraiodomercurate complex (HgI_4^{--}). As the iodide ion was depleted the above reaction was not quickly and completely reversed, thereby producing a small error in the equivalence point. This error was not observed in the present work, though in titrating iodide it was necessary to wait 15 to 20 seconds before reading the potential near the equivalence point. This may have been due to readjustment of the equilibrium suggested above. In preparing both stock and sample solutions of iodide more dilute than $10^{-5}M$, care was taken to remove oxygen from the water by degassing with prepurified nitrogen.

Mixtures. Adsorption effects were not as great as in the corresponding argentometric titrations, but there appears to be little advantage in using mercurous ion instead of silver ion for analyzing mixtures of halides. First, though the mercurous halides are less soluble than their silver analogs, the difference in solubility between any two halides is approximately the same for both metals. Secondly, coprecipitation and mixed crystal formation, which introduce errors into the titration of halide mixtures with silver (*10*), take place in precipitations of mercurous halides as well. Thus, in equimolar mixtures of $10^{-4}M$ bromide and iodide, the amount of iodide found was high by 5%. When the bromide-to-iodide ratio was increased to 5 to 1, this error increased to +12%. Contrary to expectations, the chloride-iodide system seemed to be a much worse case. When equimolar mixtures of $10^{-4}M$ iodide and chloride were used, the iodide error was +7%; when the chloride content of the mixture was increased to four times that of the iodide, the error for iodide was +23%. Thus, there is no apparent advantage in analyzing mixtures with mercurous ion.

Effect of Acetone. Because acetone is known to form complexes with mercurous and mercuric ions, it was thought that a water-acetone medium could be used to differentiate mixtures of halides by preventing the precipitation of the more soluble of the halides. Actually, however, the mercurous-acetone complex is fairly weak, so that the selectivity of this solvent would depend critically on the concentration of acetone in the solvent. The presence of amounts of acetone up to about 5% by volume gave high results in the titration of chloride or bromide. In solutions

Table I. Titrations of Halides in about 55 Ml. of 0.5*M* Sodium Perchlorate Plus 0.02*M* Perchloric Acid Using Mercury-Coated Silver Electrodes

Taken Mg.	Current, Ma.	Corrected[a] Time, Seconds	Blank	No. of Trials	Found, Mg.	Std. Dev., Mg.	Av. Error, %
			Chloride				
13.60	49.46	747	...	3	13.59	0.007	0.07
3.415	50.00	185	...	3	3.408	0.010	0.20
1.366	20.00	185	...	3	1.363	0.004	0.29
0.683	10.00	185	2.0	3	0.680	0.002	0.29
0.244	5.00	130	4.0	5	0.239	0.002	1.6
0.485[b]	5.00	265	13.0	3	0.487	0.001	0.06
0.244[b]	5.00	132	13.0	5	0.243	0.001	0.12
0.244[b]	3.00	221	24.6	4	0.243	0.001	0.49
0.146[b]	5.00	79	13.0	5	0.145	0.002	0.55
0.146[b]	3.00	132	24.6	6	0.145	0.001	0.60
0.146[b]	1.10	363	57.2	5	0.146	0.0002	0.00
0.0974[b]	3.00	89	24.6	7	0.0979	0.0009	0.53
0.0974[b]	1.10	242	57.2	5	0.0978	0.0012	0.40
0.0974[b]	0.80	331	91.0	6	0.0972	0.0005	0.55
0.0487[b]	1.10	120	24.6	6	0.0483	0.0008	0.79
0.0487[b]	0.80	164	91.0	5	0.0482	0.0002	0.77
0.0487[b]	0.70	188	106.1	6	0.0483	0.0004	0.89
0.0487[b]	0.60	218	124.2	4	0.0481	0.0004	1.3
0.0244[b]	0.70	93	106.1	5	0.0240	0.0003	1.4
0.0244[b]	0.60	109	124.2	6	0.0241	0.0001	1.1
0.0136[b]	0.432	82	150.5	3	0.0131	0.0002	3.7
			Bromide				
13.98	50.00	340	...	3	14.04	0.06	0.43
2.680	20.00	161	...	4	2.673	0.013	0.26
1.340	20.00	81	...	4	1.339	0.004	0.08
1.621	10.00	196	...	3	1.629	0.004	0.49
1.340	5.00	324	..	3	1.345	0.001	0.37
0.670	5.00	161	...	4	0.669	0.002	0.16
0.268	1.10	295	...	4	0.268	0.0003	0.11
0.178	1.10	195	...	3	0.178	0.001	0.00
0.134	1.10	148	...	4	0.134	0.0003	0.10
0.134	0.80	203	...	5	0.135	0.0005	0.45
0.0809	1.10	88	...	4	0.0810	0.0006	0.12
0.0670	0.80	102	...	3	0.0677	0.0004	0.90
0.0670	0.50	160	...	5	0.0662	0.0006	1.2
0.0268	0.50	64	...	4	0.0265	0.0002	1.1
0.0268	0.40	88	...	3	0.0294	0.0002	8.8
0.0268	0.30	123	...	3	0.0304	0.0010	12.0
0.0670[b]	0.40	201	...	4	0.0666	0.0003	0.60
0.0268[b]	0.40	80	...	5	0.0266	0.0002	0.75
0.0134[b]	0.10	167	...	3	0.0136	0.0002	1.5
0.00670[b]	0.10	79	...	3	0.00651	0.00008	4.4
			Iodide				
18.57	49.40	285	...	3	18.47	0.05	0.54
3.496	10.00	265	...	3	3.487	0.01	0.32
1.748	10.00	133	...	3	1.751	0.007	0.17
0.921	5.00	140	...	6	0.919	0.002	0.27
0.350	1.10	240	...	3	0.348	0.004	0.34
0.175	1.10	120	...	3	0.176	0.001	0.51
0.0921	0.50	139	...	5	0.0917	0.0003	0.43
0.0383	0.097	300	...	3	0.0380	0.0002	0.78
0.00897	0.051	130	...	3	0.00878	0.0001	2.1
0.00119	0.010	85	34.0	4	0.00114	0.00002	4.2

[a] Elapsed time was determined to ±0.1 second. Values of corrected time given merely denote approximate time elapsed for replicate titrations.
[b] Titrations run in 80% methanol. No end point was obtained in water alone.

Table II. Titrations of Halides in About 55 Ml. of 0.5*M* Sodium Perchlorate Plus 0.02*M* Perchloric Acid Using a Mercury-Coated Gold Electrode

Taken, Mg.	Current, Ma.	Corrected[a] Time, Seconds	Blank	No. of Trials	Found, Mg.	Std. Dev., Mg.	Av. Error, %
			Chloride				
0.750	10.00	206	...	5	0.752	0.001	+0.25
0.750[b]	10.00	206	7.5	4	0.751	0.001	+0.01
0.0750[b]	1.10	183	77.4	4	0.0741	0.0002	+0.31
0.0372[b]	0.55	181	148.2	4	0.0366	0.0007	−1.61
			Bromide				
0.954	9.80	118	...	3	0.954	0.0004	−0.08
0.0954	1.10	104	...	3	0.0952	0.0002	−0.29
0.0478	0.50	115	...	5	0.0478	0.0002	+0.02
0.0191	0.20	111	...	4	0.0188	0.0003	−1.60
0.0191[b]	0.20	114	...	4	0.0190	0.00004	−0.21
0.00956[b]	0.10	114	...	5	0.00945	0.00007	−1.20
			Iodide				
0.771	5.00	117	...	3	0.769	0.0011	−0.18
0.154	1.10	102	...	4	0.155	0.0004	+0.07
0.0771	0.50	117	...	4	0.0771	0.0002	+0.18
0.00771	0.05	117	10.0	3	0.00767	0.00006	−0.52
0.00154	0.01	120	57.0	6	0.00160	0.00006	+3.70

[a] Elapsed time was determined to 0.1 second. Values of corrected time given merely denote approximate time elapsed for replicate titrations.
[b] Titrations were run in 8% methanol. No end point was obtained in water alone.

containing about 10% acetone no potentiometric curve was obtained for chloride and results for bromide were still high. Potentiometric titration curves of iodide in solutions containing 90% acetone indicate that the ratio of iodide to mercury at the equivalence point is 4 to 1, meaning that in this medium mercuric ion is being preferentially generated with the subsequent formation of the tetraiodomercurate complex. As a result of these experiments, it was concluded that acetone could not be used to advantage to differentiate quantitatively mixtures of halides.

Effect of Alcohol. The effect of increasing percentages of methanol on the sensitivity of halide titrations was studied to determine the cases for which it would be advisable to use alcoholic solutions to increase the sensitivity of the method. It was found that the addition of methanol decreased the solubility of individual mercurous halides to different degrees. Mercurous chloride was most affected and iodide the least. A plot of the logarithm of the apparent solubility products as calculated from the observed potentiometric curves illustrates the relationships of the three halides in various alcohol mixtures. Figure 1 shows that it is most advantageous to use methanol to increase the sensitivity in determining chloride, whereas little is gained in titrating iodide in 80% methanol. It was noticed that in the titrations of iodide in 80% methanol solution, a visible precipitate did not form but the titrated solution was greenish yellow and clear. This could be due either to the formation of the more soluble mercuric iodide or to greater solubility of mercurous iodide in alcohol. In any event, it is apparent that the oxidation state of mercury did not affect the stoichiometry.

Presumably larger amounts of halide than those used in this study can be determined with equal accuracy and convenience using larger generating currents. However, the procedure described above is well suited for the determination of microgram amounts of the halides. Because the coulometric procedure is sensitive, accurate, and essentially independent of changes in temperature, the present procedure should compete favorably with polarography for the determination of anions which produce anodic dissolution waves at the dropping mercury electrode.

DISCUSSION

A comparison of the various electrode systems was made to ascertain the most practical type to use for the generation of mercurous ion. The use of a mercury pool proved impractical because it required large quantities of mercury and because the horizontal electrode surface collected precipitate during the course of a titration, thereby tending to give spurious results. This electrode was therefore abandoned and instead amalgamated electrodes, which could be handled in the same fashion as solid electrodes, were adopted. Both silver and gold can be coated easily with mercury and are convenient to use. According to Sidgwick (*13*), however, the silver electrode will hold three times more mercury than the gold. A freshly coated silver wire behaves like a pure mercury surface. On standing, sufficient silver dissolves in the mercury, so that the activity of the mercury at the surface is significantly less than unity. As a result, when a week-old amalgamated silver electrode was used to generate mercurous ion, some silver ion was also generated. Using this electrode at very high current densities—viz., 2.5 amperes per sq. cm.—it was found that as much as 3% silver ion was produced.

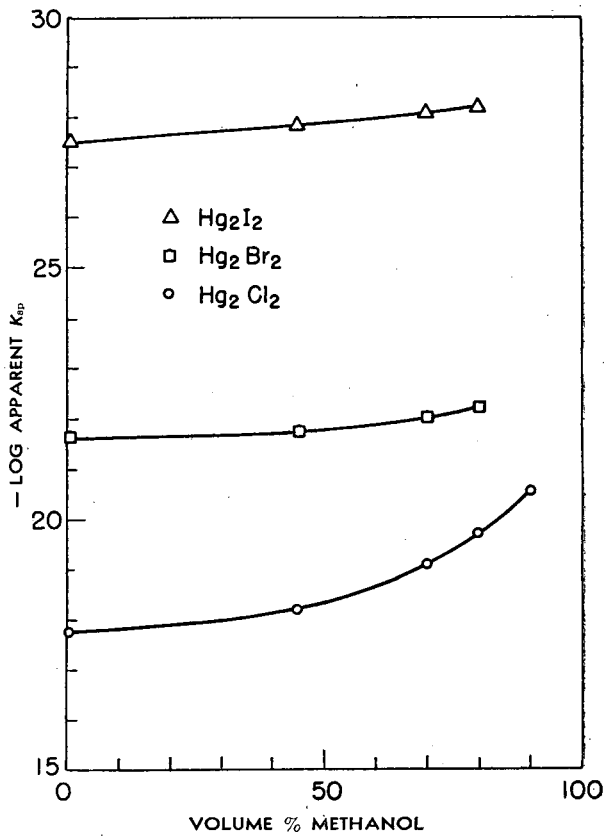

Figure 1. Apparent solubility products of mercurous halides as a function of percentage of methanol

The simultaneous generation of silver did not affect measurably, either the stoichiometry in the titration of halides or the magnitude of the potential break in the vicinity of the equivalence point. On the other hand, some investigators may prefer to use a gold amalgam because it is a "cleaner" system, in the sense that the standard potential for gold is sufficiently more noble than that of mercury to obviate the danger of anodically dissolving significant amounts of gold. In comparing the potentiometric titration curves obtained using a gold amalgam and a mercury pool, it was found that they were almost superimposable. Figure 2 illustrates the sensitivity of the various electrode systems for the determination of chloride. It is evident from these curves that either the mercury pool or the mercury-coated gold electrodes are superior to pure silver and very slightly superior to freshly amalgamated silver for the determination of the halides. Practically speaking,

the mercury-coated electrodes are to be preferred to the mercury pool as a generator electrode because they can be used in a vertical position in which they will collect little of the halide precipitate.

During the preliminary studies for which a recording potentiometer was used, a useful relationship between the generating current and the sharpness of the break at the end point was derived. Several investigators (*2*, *3*) have reported that in using potentiometric indication in coulometric titrations it became apparent that the sharpness of the break at the equivalence point was a function of the magnitude of the generating current. The magnitude of the generating current may then become a limiting factor which will determine the minimum amount of material that can be analyzed in coulometric titrations.

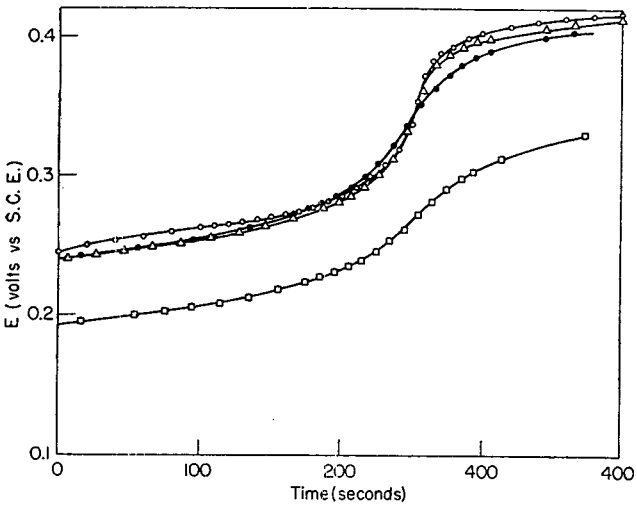

Figure 2. Potentiometric titrations of 0.892 mg. of chloride using four different generator electrodes with a current of 10 ma.

○ Mercury pool
△ Mercury-coated gold
● Mercury-coated silver
□ Silver

This is particularly true if a recording potentiometer is used at a single chart speed. For such case, one can use the Nernst equation to calculate the shape of the potential-time curve for different currents. One can then calculate the current required to produce a given change in potential within a given time interval near the equivalence point—the change being an arbitrary minimum such as that for a "satisfactory" determination of the end point.

Ths simplest function which can be derived for dE/dt as a function of the generating current comes from the first derivative of the Nernst expression. Experimentally, however, the instantaneous dE/dt is difficult to measure accurately. Thus it was decided to select a function which would express the potential E as a function of the generating current over a fixed interval of time. The two potentials chosen for comparison were the equivalence point potential, which is dependent only upon the solubility product of the precipitated salt, and a second potential, t seconds past the equivalence point, which was dependent only upon the rate of generation and the volume of solution. Using the Nernst equation it becomes a simple matter to derive an expression for ΔE.

$$\Delta E = -\frac{0.0591}{n} \log \frac{S \times F_y \times v \times n}{i_g \times t}$$

where

S = solubility of the precipitated compound, moles per liter—i.e., the concentration of mercurous ion at the equivalence point
F_y = faraday, 96,494 coulombs
v = volume of solution, liters
i_g = generating current, amperes
t = time, in seconds, past the equivalence point at which the second potential is measured
n = number of electrons involved in the reaction

After arbitrarily deciding upon the minimum change in potential which, for a given time, will give a fairly sharp break at the equivalence point, a corresponding minimum generating current can be calculated. However, this method is only approximate in that factors, such as ionic strength, which affect the solubility, have not been incorporated for the sake of simplicity.

This relationship was tested using the titrations of halides. Using values of 60 mv. for ΔE and 60 seconds for t, it was found that calculated and observed values of ΔE in the region of the minimum acceptable generating current differed only by ±5 mv. For higher generating currents the agreement was closer. The same relationship should hold for titrations involving complex formation, and only minor changes would need to be made before application to redox systems. One can see that when a recorder is used, another variable, the chart speed, must be considered. As progressively smaller quantities of a substance are determined, one is faced with the dilemma of using smaller generating currents together with slower chart speeds to sharpen the end point breaks, or of using the same generating current with faster speeds to produce longer distances which could be measured more precisely. This dilemma plus the fact that a finite time was required to reach an equilibrium potential were the factors which made continuous automatic recording of the data less desirable than discontinuous generation and measurement.

At present a study is under way to extend the application of generated mercurous and mercuric ions to other systems.

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LITERATURE CITED

(1) Carson, W. N., Jr., Michelson, C. E., Koyama, K., ANAL. CHEM. 27, 472 (1955).
(2) Cooke, W. D., Reilley, C. N., Furman, N. H., *Ibid.*, 23, 1662 (1951).
(3) *Ibid.*, 24, 205 (1952).
(4) DeFord, D. D., *Record Chem. Progr.* 16, 165 (1955).
(5) Horn, H., Ph.D. thesis, Northwestern University, Evanston, Ill., November 1954.
(6) Kolthoff, I. M., Furman, N. H., "Potentiometric Titrations," 2nd ed., pp. 144–89, Wiley, New York, 1931.
(7) Kowalkowski, R. L., Kennedy, J. H., Farringon, P. S., ANAL. CHEM. 26, 626 (1954).
(8) Latimer, W. M., "Oxidation Potentials," 2nd ed., Prentice-Hall, New York, 1952.
(9) Lingane, J. J., ANAL. CHEM. 26, 622 (1954).
(10) Lingane, J. J., "Electroanalytical Chemistry," pp. 100–4, Interscience Publishers, New York, 1953.
(11) Muller, E., Aarflot, H., *Rec. trav. chim.* 43, 874 (1924).
(12) Reilley, C. N., Cooke, W. D., Furman, N. H., ANAL. CHEM. 23, 1030 (1951).
(13) Sidgwick, N. V., "Chemical Elements and Their Compounds," Clarendon Press, Oxford, 1950.
(14) Tutundzic, P. S., Doroslovacki, I., Tatic, O., *Anal. Chim. Acta* 12, 481 (1955).

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Determination of Fluoride in Chromium Plating Solutions

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Fluoride is usually determined by distillation as fluosilicic acid, followed by titration of the distillate with thorium nitrate solution at a controlled pH, a method which requires more time and skill than is desirable for control purposes. The method described here separates fluoride by precipitating the interfering metal ions. Chromium is precipitated as silver chromate and the other metals as hydroxides. After the precipitates are removed the filtrate containing the fluoride is titrated with thorium nitrate. A buffer is used to control the pH. Although the method was devised for chromium plating solutions containing fluorides, on which it has been carried out easily and successfully, it may be adaptable to other analytical problems.

THE chromium plating bath which is used most extensively for industrial applications contains chromic acid and a sulfate catalyst, usually added as sulfuric acid. Other chromium plating baths are catalyzed by compounds containing the fluoride or fluosilicate ions, which may be used separately or with the sulfate catalyst. These other chromium plating baths have had little industrial use, with the exception of a self-regulating type bath that contains the fluosilicate or sulfate ions (*5*, *6*). One reason for their limited use is the problem of maintenance of these baths. This is caused by the difficult and often inaccurate procedures which are available for the determination of the fluoride or fluosilicate content of the bath.

A commonly used procedure for the separation of fluoride, originally suggested by Willard and Winter (*7*), is distillation from a perchloric or sulfuric acid solution. A high degree of accuracy is obtained by this method, but considerable time and skill are required. The method of Berzelius (*2*, *3*) recommends the precipitation of fluoride as calcium fluoride. This procedure is not only time-consuming but also inaccurate. Low results are usually obtained due to the solubility of the calcium fluoride. The method reported herein is considered very good for control purposes. It is easily performed and gives greater accuracy than is usually necessary for controlling chromium plating baths.

THEORY

Sodium hydroxide is added to the bath sample in excess of the amount required to convert the chromic acid to sodium chromate. At this point, the metal contaminants precipitate as hydroxides. Silver nitrate is added in excess to precipitate the chromate as silver chromate. After filtration, a colorless solution is obtained which can be titrated with thorium nitrate.

Fluoride reacts with thorium in an acid solution according to the following equation.

$$Th^{+4} + 4HF \longrightarrow ThF_4\downarrow + 4H^+$$

A buffer must be added to the solution to eliminate interference from the hydrogen ions liberated by the reaction. Hoskins and Ferris (*4*) recommended the use of monochloroacetic acid plus sodium hydroxide.

An aqueous sodium alizarin sulfonate solution was recommended by Armstrong (*1*) as an indicator for the titration. After the fluoride has been precipitated as thorium fluoride, excess thorium reacts with the indicator to give red thorium alizarin sulfonate. The greatest difficulty in performing this determination lies in the recognition of the pink end point of the thorium nitrate titration. The point at which no yellow remained in the solution was the easiest for the authors to duplicate.

The thorium nitrate solution is standardized against a known weight of sodium fluoride in a chromic acid solution. The same procedure is employed in the standardization as in the analysis of a sample. This helps to reduce the error in the method.

EXPERIMENTAL

Reagents. Sodium hydroxide, 1.0*N* (40.0 grams per liter).
Silver nitrate, 0.5*N* (85.0 grams per liter).
Sodium alizarin sulfonate indicator, 0.1% aqueous solution.
Buffer solution. Dissolve 7.56 grams of monochloroacetic acid in 160 ml. of distilled water and add 40 ml. of 1*N* sodium hydroxide. Mix well.
Thorium nitrate, 0.04*N* (5.52 grams per liter).
Nitric acid, 1 to 50.

Procedure. Pipet a 4-ml. sample of the bath into a 250-ml. beaker. From the graph (discussed later) determine the volume of 1.0*N* sodium hydroxide and 0.5*N* silver nitrate necessary for the chromic acid content of the sample. Add these quantities to the sample in the order just mentioned. Agitate the sample while adding silver nitrate, then allow the precipitate to settle. Filter through a No. 40 Whatman paper into a 250-ml. Erlenmeyer flask. Rinse the beaker three times with water and wash the precipitate five times with approximately 10 ml. of water each time.

Add 10 drops of indicator to the filtrate. The indicator should be pink at this point. Add 1 to 50 nitric acid dropwise until the color changes to yellow, then add 2.5 ml. of buffer solution. Titrate with thorium nitrate solution to a pink end point which shows no tinge of yellow.

Standardization of Thorium Nitrate Solution. Standard chromic acid solution, containing 250 grams per liter, and sodium fluoride solution, containing 2.215 grams per liter of reagent grade sodium fluoride (0.001 gram of fluoride ion per milliliter), are needed.

Pipet 4 ml. of standard chromic acid solution and 10 ml. of the sodium fluoride solution into a 250-ml. beaker. Follow the same procedure as for the sample.

Calculations.

$$N \text{ of thorium nitrate} = \frac{0.010}{(\text{ml. of thorium nitrate})\,(0.019)}$$

$$\text{Grams of fluoride ion per liter} = \frac{(\text{ml. of thorium nitrate})(N \text{ of thorium nitrate})\,(0.019)(1000)}{4}$$

RESULTS

Table I shows the accuracy and reproducibility of this method. Standard solutions were prepared containing 250 grams of chromic acid per liter, 2 grams of sulfate as sulfuric acid per liter, and varying amounts of fluoride added as sodium fluoride. Samples of 4-ml. volume were taken in all cases. The best accuracy was obtained where a titration of 10 ml. or more of thorium nitrate was required.

Table II shows the results obtained from chromic acid solutions which contained various concentrations of chromic acid

Table I. Effect of Fluoride Concentration on Accuracy

Fluoride, Grams/Liter		Av. Error, %
Added	Found	
1.00	1.09, 1.11, 1.07, 1.08	+8.75
2.00	2.00, 2.00, 2.00, 2.02, 2.06, 2.06	+1.17
3.00	2.94, 2.91, 2.94, 2.94, 3.02, 3.02	−1.28
4.00	3.92, 3.90, 3.92, 3.92, 3.95, 3.96	−1.79

Table II. Effect of Chromic Acid Concentration on Accuracy

(Each solution contains 2.50 grams of fluoride per liter.)

Chromic Acid Concn., Grams/Liter	Fluoride Found, Grams/Liter	Av. Error, %
200	2.49	−0.4
250	2.50	0.0
300	2.50	0.0
350	2.50	0.0
400	2.48	−0.8

and a constant quantity of fluoride. There was a slight error in the amount of fluoride found in the solutions at the extremes of the chromic acid concentration range.

Standards were prepared which contained constant amounts of chromic acid and fluoride ion per liter, and 2.5 grams of contaminating metals per liter. The addition of these metals had little effect on the accuracy of the method, as shown in Table III. Insufficient washing of the heavy hydroxide and silver chromate precipitates might explain the slightly lower result obtained on the one standard containing all the contaminants.

Table III. Effect of Metallic Contaminants on Accuracy

	Metallic Contaminants, Grams/Liter[1]				
	Cr^{++}, 2.5	Fe^{+++}, 2.5	Ni^{++}, 2.5	Cu^{++}, 2.5	Cr^{+++}, Fe^{+++}, Ni^{++}, Cu^{++}, 2.5 of each
Fluoride added, grams/liter	2.50	2.50	2.50	2.50	2.50
Fluoride found, grams/liter	2.46	2.50	2.50	2.50	2.44
Av. error, %	2.50	2.50	2.50	2.49	2.49
	−0.8	0.0	0.0	−0.2	−1.4

[1] Each bath contained 250 g./liter chromic acid.

A graph was prepared showing the volumes of 1.0*N* sodium hydroxide and 0.5*N* silver nitrate which are required to remove interfering metals from samples of varying chromic acid content (Figure 1). An excess of 2 ml. of sodium hydroxide and 5 ml. of silver nitrate over the stoichiometric amount required by the chromic acid content was added. This excess is sufficient to precipitate the interfering metals as hydroxides because chromium constitutes such a large portion of the interfering ions.

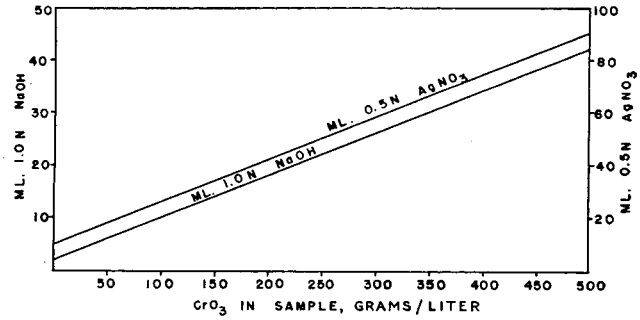

Figure 1. Amount of reagents required for 4-ml. samples of varying chromic acid contents

CONCLUSIONS

The results indicate that this method for determining fluoride provides more than sufficient accuracy for control of chromium plating baths. The method is being used very successfully to control industrial chromium plating baths. After the analyst has become familiar with the method, the determination can be completed in approximately 20 minutes. The usual quantities of contaminating metals do not interfere.

LITERATURE CITED

(1) Armstrong, W. D., *J. Am. Chem. Soc.* **55**, 1741 (1933).
(2) Berzelius, J. J., *Schweigg J.* **16**, 426 (1816); Rose, H., *Ann.* **72**, 343 (1849).
(3) Hillebrand, W. F., Lundell, G. E. F., Bright, M. S., Hoffman, J. I., "Applied Inorganic Analysis," 2nd ed., 742, Wiley, New York, 1953.
(4) Hoskins, W. M., Ferris, C. A., IND. ENG. CHEM., ANAL. ED. **8**, 6 (1936).
(5) Passel, F. (to United Chromium, Inc.), U. S. Patent **2,640,021** (May 26, 1953).
(6) Starech, J. E. (to United Chromium, Inc.), *Ibid.*, **2,640,022** (May 26, 1953).
(7) Willard, H. H., Winter, O. B., IND. ENG. CHEM., ANAL. ED. **5**, 7 (1933).

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Equipment for High Pressure Optical and Spectroscopic Studies

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Equipment has been developed for making optical and spectroscopic studies in liquids at pressures up to 12,000 atm. A combination intensifier and bomb for spectroscopic measurements is described, as well as a source assembly particularly suited for use with a Perkin-Elmer single beam spectrometer. The use of synthetic sapphire windows permits measurements to be made in the wave-length range from 0.2 to 5.0 microns. Experiments are now being undertaken to develop windows which will permit extension of the wave-length range. With this equipment it is possible to study the effect of pressure in all phases of molecular spectroscopy. By suitable modification, it is possible to measure the effects of pressure on the refractive index and on light scattering from polymer solutions, as well as related optical effects.

TO UNDERSTAND, correlate, and predict the physical and chemical effects of pressure, its effect on intermolecular and intramolecular forces must be determined. Optical and spectroscopic investigations provide important evidence as to the nature of these effects.

This work was confined to the study of equipment which was used successfully for optical and spectroscopic work in liquids at pressures up to 12,000 atm.

When infrared spectroscopy is applied to liquids, the vibrational frequencies between atoms in a polyatomic molecule are observed. In the pressure range mentioned, the effect most generally observed is that of crowding solvent molecules around a given bond. Because of the mutual polarizability of bonds, there is an attractive interaction which depends strongly on intermolecular distance. Solution properties, transport phenomena, and other macroscopic properties depend on these interactions,

and many pressure effects can be generalized from these measurements.

Ultraviolet spectra are primarily dependent on the electronic structure of molecules, which can also be affected by interaction with the solvent, and thus by pressure. In particular, interesting pressure effects are anticipated in complexes such as the benzene-iodine system.

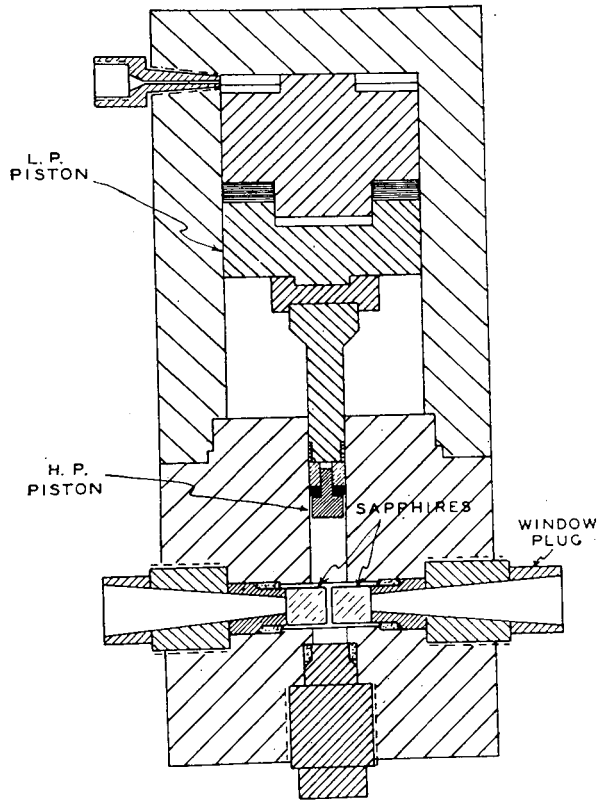

Figure 1. Spectroscopic bomb

Many other high pressure optical experiments, such as light-scattering studies of polymer molecules in solution, are also possible with the equipment described.

INTENSIFIER

Figure 1 shows a cross section of a reasonably small, portable high pressure apparatus which may be introduced into various spectrometers and other optical setups. The device consists of an intensifier with a 3-inch diameter low pressure piston and a $^1/_2$-inch diameter high pressure piston, both utilizing conventional Bridgman unsupported area packing (*1*). Any desired stroke can be incorporated, but for most experiments 1 to 2 inches are ample.

The two ends of the intensifier are conveniently made from SAE 4140, 4340, or 6150 steel hardened to 48–50 Rockwell C. The over-all dimensions of the apparatus are $4^1/_2$ to 5 inches in diameter and 10 to 15 inches high. The ram and high pressure piston are Superdie tool steel (Columbia Tool Steel Co., Chicago Heights, Ill.) hardened to 58–60 Rockwell C.

The low pressure end can be attached to a portable pump and a free piston gage or a good Bourdon gage, such as a Heise gage. In a special run before making optical measurements, the low pressure gage reading is calibrated against the reading of a manganin gage on the high pressure side, but for many purposes it is satisfactory to calculate the high pressure, making a 10% allowance for friction.

The liquid to be studied is introduced directly on the high pressure side. This eliminates the necessity for separating the liquid from a pressure transmitting fluid, and considerably simplifies the operation. Thorough cleaning is, of course, necessary between runs, and corrosive liquids must be handled with care.

WINDOW PLUGS AND WINDOWS

The principle of the window plug (Figure 2) is essentially the same as that established by Poulter (*3*). The plug is made from Columbia Superdie tool steel. The hole is $^1/_4$ inch in diameter at

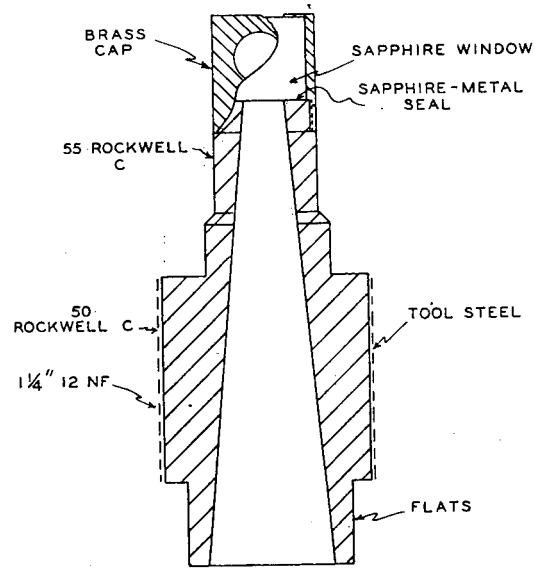

Figure 2. Window plug

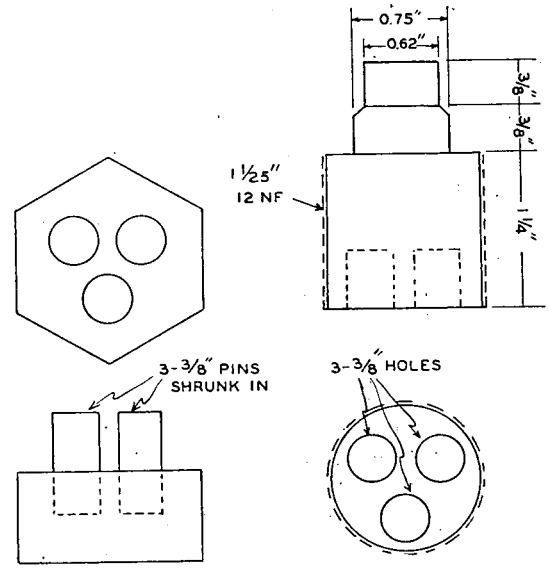

Figure 3. Plug with removable nut

the surface of the window and is tapered out at the angle of the light beam. The main problem in the plug design is to make the surface hard enough to seat the window and still make the plug tough enough to withstand cracks, particularly along the threads. The best compromise found in this laboratory is to make the face 55–56 Rockwell C and to draw the threads to about

50 Rockwell C. Occasionally the face deforms at this hardness, but plugs which have been used for 50 or more runs at 10,000 to 12,000 atm. are still in service. The face of the plug is surface ground and lapped on 0000 emery paper. It is convenient to screw the plug into a lapping block $1^1/_2$ to 2 inches in diameter, which prevents curvature of the surface. When the window seats on the plug and cannot be blown off orally from underneath, or when the plug can be picked up by lifting the window, without separating window and plug, the seal is satisfactory for even the least viscous liquids. The brass cap is useful for holding the window in place during assembling and dissembling, and while applying pressure. The plugs can be sealed in the bomb using steel unsupported area rings as described by Bridgman (*1*). The window spacing can be varied from less than 1 mm. to 1 cm. or more by varying the ring thickness.

Linde synthetic sapphires make convenient windows. The standard window used for this work was $^1/_2$ inch in diameter and $^1/_2$ inch thick. It is important that the *C* axis of the crystal be perpendicular to the window faces. If a flatness of at least 0.0001 inch is specified, the sapphires will usually seal as purchased; otherwise, they must be lapped with diamond paste.

The major limitation on the use of sapphire windows is that they cut off radiation of wave lengths longer than 5 microns. Preliminary information (*2*) on sodium chloride windows indicates that they are satisfactory at least to 1000 atm., and calcium fluoride windows may be useful at still higher pressures.

MISCELLANEOUS FEATURES

It is frequently convenient to have a plug (particularly the bottom plug) tighten flush with the surface of the bomb. A convenient device for this purpose is shown in Figure 3. The square head of the plug is replaced by a removable hexagonal nut which pins to the plug with three $^3/_8$-inch diameter pins of SAE 4340 steel hardened to 50 Rockwell C. The nut can be made easily removable and lasts indefinitely.

A modified design of apparatus involving three window plugs, one perpendicular to the other two, can be used for light-scattering and light-absorption studies.

ACKNOWLEDGMENT

The authors wish to acknowledge the skillful machine work and useful suggestions of W. W. Demlow.

LITERATURE CITED

(1) Bridgman, P. W., "Physics of High Pressure," pp. 34, 37, 39, G. Bell and Sons, London, 1947.
(2) Parsons, R. W., private communication, U. of Illinois, Urbana, Ill.
(3) Poulter, T. C., *Phys. Rev.* 35, 297 (1930).

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Spectrophotometric Determination of Zirconium in Thorium

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At a controlled high acidity, zirconium, in the amount of 0.005 to 0.350%, can be determined colorimetrically using Alizarin Red S. As much as 200 mg. of thorium can be tolerated in the presence of 10 to 700 γ of zirconium. Acetone and heat accelerate the rate of color development and increase the stability of the color. Small amounts of iron and other metals normally present in thorium do not interfere.

AN INVESTIGATION of new techniques for the purification of thorium presented a need for a method for the determination of small amounts of zirconium in thorium.

A number of organic reagents have been studied and recommended for the direct colorimetric determination of zirconium. These include *p*-dimethylaminophenylazobenzenearsonic acid (*5*), alizarin (*2, 8*), Alizarin Red S (*3, 4, 9-11, 14*), purpurin, (*2, 8*) quinalizarin (*2, 8*), thoron (*6*), and chloranilic acid (*10, 13*). In their present form, these methods are time-consuming or require the removal of thorium if present in large amounts.

Alizarin Red S (sodium salt of 3-alizarinsulfonic acid) showed the most promise, because the colors ordinarily produced by interfering metals (other than hafnium) are vitiated in strong mineral acid solution. The various contributory factors were studied to obtain the maximum absorbance due to the zirconium–Alizarin Red S lake under conditions that result in minimum interferences from other sources.

REAGENTS

Standard Zirconium. Dissolve 35.33 grams of C.P. zirconyl chloride octahydrate ($ZrOCl_2 \cdot 8H_2O$) in an aqueous hydrochloric acid solution (pH 1.2) and dilute to 1 liter with the same acid solution. Standardize using the *p*-bromomandelic acid method (*7*). This solution contains 10 mg. of zirconium per milliliter. Prepare solutions containing 0.100 and 0.010 mg. of zirconium per milliliter by properly diluting the stock solution with the aqueous hydrochloric acid solution.

Standard Thorium. Dissolve and fume 29.74 grams of thorium nitrate tetrahydrate [$Th(NO_3)_4 \cdot 4H_2O$] with 150 ml. of concentrated perchloric acid. Dilute to 1 liter with water, making certain that the pH is approximately 1. This solution contains 25 mg. of thorium per milliliter. Analyze by precipitating the thorium as oxalate and weighing the oxide (*12*).

Colorimetric Reagent. Dissolve 500 mg. of Alizarin Red S (National Aniline Division, Allied Chemical and Dye Corp., New York) in a 2 to 3 hydrochloric acid solution and dilute to 1 liter with the same solution. Let stand for 2 days and filter through Whatman No. 40 paper. The solution is stable for at least 1 month.

Sample-Diluting Solution. Prepare a hydrochloric acid–water solution having a pH of 0.70.

PROCEDURE

Sample Preparation. Weigh a sample (metal or compound) containing 0.800 gram of thorium into a platinum dish. Add 10 ml. of concentrated nitric acid and a few drops of 2% hydrofluoric acid. Warm to initiate the reaction, then remove from the heat. If the reaction becomes too vigorous, it may be moderated by the addition of water. When the reaction subsides and solution is complete, add 5 ml. of concentrated perchloric acid. Evaporate to near dryness and cool. Add 2 ml. of nitric acid and 5 ml. of perchloric acid and again evaporate almost to dryness. Dissolve the residue in 5 ml. of 1 to 1 hydrochloric acid with heat. Transfer to a 100-ml. volumetric flask and adjust the volume with water.

Transfer a 25-ml. aliquot (200-mg. sample) to a 50-ml. beaker. Add 4 ml. of acetone and adjust the volume to 32 ± 2 ml. with water. Determine the pH of the solution and adjust the sample to pH 0.70 using 1 to 1 hydrochloric acid solution and water.

Pipet 10 ml. of Alizarin Red S reagent into a 50-ml. volumetric flask and then transfer the sample to the flask. Use the hydrochloric acid–water solution, pH 0.70, for all rinsings and any

volume adjustments. Heat in a water bath (70° to 90° C.) for 10 to 30 minutes to develop the zirconium color, then cool to room temperature.

Measure the absorbance at 540 mμ within 3 hours after the start of heating. Absorbance is measured using 1-cm. cuvettes in a Beckman Model DU spectrophotometer, with water as a reference. Obtain the zirconium concentration from a standard curve showing absorbance *vs.* zirconium content. The curve is prepared by using the same reagents and the standard solutions of zirconium and thorium.

If the zirconium content is high, use a smaller aliquot and add the amount of thorium necessary to provide a total of 200 mg. per sample.

RESULTS

When the recommended procedure is used on 200-mg. samples of thorium metal, it is possible to determine 0.005 to 0.350% zirconium in a 50-ml. volume solution. The standard curve is linear from 0.050 to 0.700 mg. of zirconium, but curves slightly at lower zirconium contents. In the linear portion of the curve, the change in absorbance per milligram of zirconium was 1.14 for the conditions recommended and for the solutions actually used in the authors' laboratory. The daily standard curves originated at 0.7 absorbance unit for 0.0 mg. of zirconium and 200 mg. of thorium; the absorbance for solutions containing 0.11 mg. of zirconium and 200 mg. of thorium (a daily point) was 0.15. The entire curve is reproducible. Larger percentages of zirconium may be determined by using smaller samples and adding standard thorium to provide the necessary 200-mg. total.

Compounds and solutions may be analyzed if the thorium content is known within 10%, if the thorium and zirconium content can be made soluble in water, and if the interfering complex-forming ions are removable.

Table I illustrates the reproducibility of the method as shown by a series of thorium-zirconium melts obtained from a zone-melting experiment. Similar reproducibility has been obtained from several other experiments. A thorium sample reported to be 0.03% in zirconium by spectrographic analysis was found to contain 0.031, 0.031, and 0.033% zirconium. Two other thorium samples contained 0.021, 0.024, 0.025% and 0.027, 0.028, 0.028, and 0.028% zirconium, respectively.

Table I. Zirconium in Thorium

(Analysis of a zone-melting run)

Sample Number	Zirconium, %	Standard Deviation (*1*), s_w
End	0.092, 0.093, 0.093	0.001
1	0.106, 0.106	
2	0.150, 0.155, 0.155	0.001
3	0.062, 0.066	
4	0.079, 0.079, 0.080	0.001
5	0.068, 0.069	
6	0.074, 0.075, 0.077	0.002
7	0.081, 0.082, 0.083	0.001
8	0.089, 0.089, 0.090	0.001
9	0.079, 0.079, 0.079, 0.081	0.002
10	0.005, 0.005	
Thorium (unalloyed)	0.000 0.000	

DISCUSSION

Preliminary investigations showed that the absorbance of a solution containing Alizarin Red S, zirconium, and large concentrations of thorium is dependent on the wave length, conditions of color development, concentration of Alizarin Red S, acidity, and thorium content as well as the zirconium concentration. The effect of each variable was investigated in order to decrease the sensitivity of the method to all factors except zirconium content.

Wave Length. Green (*3*) showed that the effect of varying the necessary excess of Alizarin Red S on the absorbance of the zirconium–Alizarin Red S lake was least at 540 mμ, under the conditions of his experiments. At 540 mμ and at low pH, the absorbance caused by the excess Alizarin Red S is negligible and that resulting from the thorium–Alizarin Red S compound decreases appreciably. The absorbance ascribed entirely to the zirconium compound is at a maximum in the region from 520 to 530 mμ and is only slightly lower at 540 mμ (Figure 1).

Conditions of Color Development. It has been shown (*3*) that high ion content of the system decreases both the rate of color development and the stability of the zirconium–Alizarin Red S lake. Color development by preliminary (temporary) decrease in acidity (*3, 4, 14*) is negated by the presence of thorium, for under the usual conditions for the determination of zirconium in aqueous solution, the high thorium content causes clouding of the mixture before the zirconium–Alizarin Red S color has fully developed.

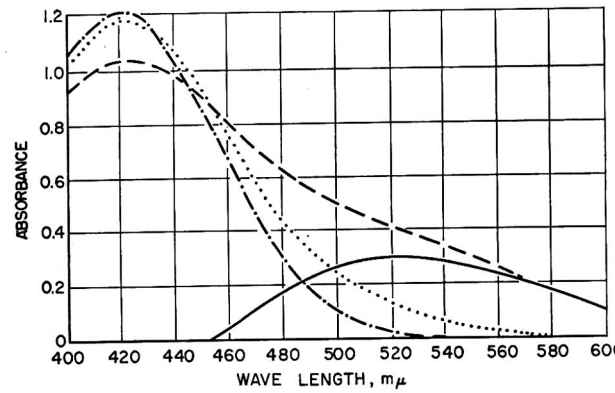

Figure 1. Absorbance spectra of Alizarin Red S complexes

0 mg. of zirconium, 0 mg. of thorium, water as reference
0 mg. of zirconium, 200 mg. of thorium, water as reference
0.293 mg. of zirconium, 200 mg. of thorium, water as reference
0.293 mg. of zirconium, 200 mg. of thorium, reference containing 200 mg. of thorium and no zirconium

Of the stabilizing agents investigated, Carbitol (monoethyl ether of diethylene glycol) prolonged the period of stability but decreased the rate of color development and the over-all sensitivity of the method. An Alizarin Red S–Carbitol reagent was usable only during the second and third day after preparation.

Acetone and heat increase the rate of color development; they have little or no adverse effect on the sensitivity of the system and produce a color stable for several hours. Variation of the acetone content causes no significant change in the color intensity. Four to 10% of acetone by volume is recommended to prevent precipitation.

Reagent Concentration. Because the reaction of zirconium and Alizarin Red S attains equilibrium (*8*), a large excess of the reagent is desirable. Some of the excess is used by the thorium. Too high a concentration of the reagent, particularly in the presence of much thorium, favors the precipitation of the system as manifested by a general cloudiness. Five milligrams of Alizarin Red S per 50 ml. of final solution is a convenient reagent concentration.

Acidity Control. At measurable pH, the absorbance of the Alizarin Red S–zirconium-thorium system is so sensitive to acid that reproducible results are not easily obtained. Simple additions of acid to systems of varying original acid content are unreliable, even though the sensitivity decreases at the higher acidities. Consequently the sample solution should be adjusted to some reproducible pH at less volume and then the final acidity should be obtained by the addition of a prescribed amount of acid. Reproducible acid conditions are readily obtained by the addition of 10 ml. of Alizarin Red S (which is prepared in 2 to 3 hydrochloric acid) to 40 ml. of prepared test samples, which orig-

inally were adjusted to pH 0.70. All rinsings and volume adjustments are made with a solution of pH 0.70. A variation of ±0.20 unit in the original pH adjustment results in an absorbance variation of ±0.005 unit.

Thorium Content. Previously reported methods for zirconium using Alizarin Red S showed that the effects of the presence of large amounts of thorium include the tendency to cause precipitation at previously recommended pH values, delay in color development, and high absorbance caused by thorium–Alizarin Red S combination. The conditions recommended herein eliminate the first two effects and minimize the third. The absorbance attributed to the thorium–Alizarin Red S is accounted for in the standard curve obtained by using 200 mg. of thorium with each synthetic standard. A variation in thorium content of as much as 10% (20 mg.) causes less than 2% error in results in the region from 0.3 to 0.4 mg. of zirconium. The error is only slightly higher for lower zirconium contents.

Table II. Effect of Diverse Ions on Determination of Zirconium in Presence of 200 Mg. of Thorium

Diverse Ion	Mg.	Zirconium, γ Added	Found
Al(III)	5	200	200
Be(II)	1	176	175, 177
U as UO_2(II)	10	176	175, 176
Ni(II)	1	176	176, 176
Fe(III)	5	176	174, 180
Fe(III)	10	176	177, 183
Ce(IV)	1	280	280, 280
Ce(IV)	5	200	182
Mo as MoO_4^{--}	0.04	222	222
Mo as MoO_4^{--}	0.08	222	222
Mo as MoO_4^{--}	0.20	222	225
Mo as MoO_4^{--}	0.50	222	227
H_2SO_4[a]		176	0
$HClO_4$[a]		176	174

[a] 1/2 ml. of acid included before pH adjustment.

Diverse Ions. α-Hydroxycarboxylic acids, inorganic fluorides, and sulfates which form stable complexes with zirconium interfere in the determination. α-Hydroxycarboxylic acids and inorganic fluorides may be removed by repeated treatment with nitric and perchloric acids. The effect of sulfate may be eliminated by the addition of calcium, but not barium.

Data in Table II indicate the effect of a number of diverse ions which might be present and interfere in the zirconium determination. Other ions, specifically reported to cause no interference at lower acid concentrations, do not interfere at the acidity used here, except in concentrations great enough to increase the ion concentration to the point of precipitation or delay of color development. Nitrates, equivalent to the thorium present, decrease the rate of color development. Under the present condition, no reduction of iron is necessary for the quantities investigated. No attempt was made to account for the presence of hafnium in any samples or standards, although this element undoubtedly causes an increase in absorbance when present (*8*).

SUMMARY

The colorimetric determination of zirconium using Alizarin Red S has been adapted to tolerate at least 200 mg. of thorium. The acidity should be controlled to that of a 40-ml. solution with a pH of 0.70 plus 10 ml. of 2 to 3 hydrochloric acid. Acetone and heat are used to develop and stabilize the color.

The thorium content of the final samples should be 200 ± 20 mg. per 50 ml. of final solution, corresponding to the content of the solutions used for the standard curve.

The reproducibility of the determination of zirconium (0.01 to 0.7 mg.) in thorium (200 mg.) is in the range of 0.01% zirconium; the standard deviation is 0.002%. Higher percentages of zirconium may be determined with suitable sample and volume adjustment if the acid concentration is kept constant.

ACKNOWLEDGMENT

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LITERATURE CITED

(1) Dixon, W. J., Massey, F. J., "Introduction to Statistical Analysis," McGraw-Hill, New York, 1951.
(2) Flagg, J. F., Liebhafsky, H. A., Winslow, E. H., *J. Am. Chem. Soc.* **71**, 3630–2 (1949).
(3) Green, D. E., ANAL. CHEM. **20**, 370–4 (1948).
(4) Guenther, R., Gale, R. H., U. S. Department of Commerce, Office of Technical Services, Washington, D. C., KAPL-305, (March 10, 1950).
(5) Hayes, W. G., Jones, E. W., IND. ENG. CHEM., ANAL. ED. **13**, 603 (1941).
(6) Horton, A. D., ANAL. CHEM. **25**, 1331–3 (1953).
(7) Klingenberg, J. J., Papucci, R. A., *Ibid.*, **24**, 1861–2 (1952).
(8) Liebhafsky, H. A., Winslow, E. H., *J. Am. Chem. Soc.* **60**, 1776–84 (1938).
(9) Mayer, A., Bradshaw, G., *Analyst* **77**, 476–83 (1952).
(10) Menis, O., U. S. Department of Commerce, Office of Technical Services, Washington, D. C., **ORNL-1626** (April 7, 1954).
(11) Mills, E. C., Hermon, S. E., *Metallurgia* **51**, 157–8 (1955).
(12) Moeller, T., Sweitzer, G. K., Starr, D. D., *Chem. Revs.* **42**, 85 (1948).
(13) Thamer, B. J., Voigt, A. F., *J. Am. Chem. Soc.* **73**, 3197 (1951).
(14) Wengert, G. B., ANAL. CHEM. **24**, 1449–51 (1952).

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Spectrophotometric Determination of Iron in Strong Alkali Media

4,7-Dihydroxy-1,10-phenanthroline as Iron(II) Organic Chelation Reagent

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4,7-Dihydroxy-1,10-phenanthroline (Snyder's reagent) is used as organic ligand in the ferroine chelation with iron(II), a new procedure for the spectrophotometric determination of iron as a trace element. This procedure is characterized by applicability in concentrated alkali solutions, a property which is not shared by any previously known ferroine-reacting organic reagent. Snyder's reagent may be employed for the rapid and specific determination of iron impurities of alkali hydroxides, carbonates, and reagent ammonium hydroxide, alkaline earth metal oxides and hydroxides, alkaline phosphates, and other reagent chemicals. The spectrophotometric constants have been determined. The use of Snyder's reagent for the determination of iron in glass sand is described, and the method is compared with that employing 4,7-diphenyl-1,10-phenanthroline (bathophenanthroline). Variable factors such as anion and cation interferences, reagent stability, and the selection of the most suitable reducing agent for iron have been investigated. Snyder's reagent is commercially available.

THE 1,10-phenanthrolines and their derivatives have proved to be widely applicable in the spectrophotometric determination of trace elements including iron(II), copper(I), cobalt(II), and other elements. Although there are favored pH magnitudes governing instantaneous metal chelation, the complex cations formed are, in general, stable over the pH range from 2 to 10. The interferences from anions and cations which form colored complexes are very limited, and, by the use of suitably substituted modifications of the parent reagent, they may be eliminated. The metal complex cations involved have a large area of variegated analytical applications.

The newly described 4,7-dihydroxy-1,10-phenanthroline (Snyder's reagent) is unique in its type for its stability in strong alkali media. Its applicability extends into the pH range represented by a saturated sodium hydroxide solution (about 18*M*), with full color chelation for iron(II).

The determination of iron in glass sand is described, illustrating one practical application of this new 1,10-phenanthroline.

SNYDER'S REAGENT

Preparation. The new 4,7-dihydroxy-1,10-phenanthroline was prepared according to the synthesis by Snyder and Freier (*3*). Their yield of impure material was approximately 90%; however, they did not describe a method for purification of the reagent. Their attempts to purify the reagent by recrystallization failed because no suitable organic solvent was found. The presence of two hydroxyl groups suggested the solution of the impure product in strong alkali, followed by filtration to remove insoluble impurities. The filtrate was then neutralized and the pure product was obtained as a flocculent gelatinous precipitate. Two such recrystallizations were required and the process was not entirely satisfactory. A second method involved the formation of the reagent as the hydrochloride. The impure sample was dissolved in hydrochloric acid, filtered free of impurities after addition of decolorizing carbon, and crystallized by concentration of the filtrate, followed by cooling and final filtration. The finished product is light canary yellow in color, nicely crystalline, and dissolves to give a clear solution in caustic soda, in which form it may be used. The yield of pure product was found to be 25% based upon the Snyder and Freier (*3*) synthesis.

Determination of Molecular Ratio in Snyder's Reagent–Iron(II) Complex. On application of the Vosburgh and Cooper method of continuous variations (*4*), it was established that a tris complex of this organic chelate is formed with ferrous iron in alkaline media. This would indicate that the instability constant of the ferrous complex is beyond the value of that for ferrous hydroxide. Other ferrous complex ions of the 1,10-phenanthroline are, in general, readily dissociated, and ferrous hydroxide is precipitated when the complexes are made alkaline by high concentrations of caustic soda. This procedure has been employed (*2*) for the recovery of the organic ligand in accumulated waste solutions of these ferroine complexed species.

General Properties of Reagent and Its Ferrous Complex. These are classified as follows.

The reagent is insoluble between pH 1 and 8, and also in any common organic solvent. It is soluble in hydrochloric acid and in alkalies, including the hydroxides of ammonia, potassium, and sodium. It is most conveniently prepared and is commercially available as the hydrochloride.

Snyder's reagent forms a highly colored iron(II) complex at a pH no lower than 8, with no upper limit. Solutions of the ferrous complex in this pH range are stable at the boiling temperature. The ferrous complex is not extracted by any commonly employed immiscible organic solvent.

Solutions of the ferrous complex in stoppered containers are stable in alkaline media in the presence of a slight excess of sodium hydrosulfite for over 24-hour intervals of storage. The maximum color intensity of air-oxidized solutions in alkali can be restored by the addition of the same reducing agent.

EXPERIMENTAL

Preparation of Reagents. SODIUM HYDROSULFITE. A fresh solution is prepared containing 200 mg. per milliliter of water. The iron may be removed by treating the solution of sodium hydrosulfite at pH 4 with bathophenanthroline (4,7-diphenyl-1,10-phenanthroline), then extracting the iron(II) complex thus formed, as well as excess ligand, with *n*-hexyl alcohol as an immiscible solvent. Because commercial grades of sodium hydroxide invariably contain appreciable amounts of iron, it is preferable to use all reagents in solution form in controlled amounts and to correct for their iron content by a blank determination. Ammonium hydroxide and sodium carbonate also contain, to a lesser degree, interfering amounts of iron as impurities.

SNYDER'S REAGENT. A 0.01*M* solution of 4,7-dihydroxy-1,10-phenanthroline hydrochloride was prepared containing sufficient slight excess of ammonium hydroxide to neutralize the acid of the reagent hydrochloride and to adjust the pH to a suitable value to dissolve the substituted phenanthroline.

STANDARD IRON SOLUTION. A standard was prepared from reference purity iron dissolved in excess hydrochloric acid and diluted to give a 0.1*N* concentration of free acid. It contained 0.0001163 gram of iron per gram of solution. Weight burets were employed to deliver samples of this solution.

OTHER REAGENTS. In all cases C.P. grade chemicals from various commercially available sources were used.

Procedure for Preparation of Solutions. SODIUM HYDROXIDE. Portions of standard iron solution were weighed into 25-ml. volumetric flasks. By pipet, 10 ml. of 10*M* sodium hydroxide was added, followed by 2 ml. of sodium hydrosulfite and 2 ml. of Snyder's reagent. The solution was then diluted to volume with

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water. Blank determinations of the same mixed reagents, without addition of iron, were simultaneously prepared.

AMMONIUM HYDROXIDE. Portions of standard iron solution were weighed into 50-ml. breakers, then 10 ml. of concentrated ammonium hydroxide, 1 ml. of sodium hydrosulfite, and 2 ml. of Snyder's reagent were added. The solution was heated to boiling, then cooled and transferred quantitatively into a 25-ml. volumetric flask. A 1-ml. portion of sodium hydrosulfite was then added, and the solution was diluted to volume. If the heating is omitted, a 2-hour period of aging is required for the maximum development of color.

SODIUM CARBONATE. The procedure involved is the same as for ammonium hydroxide, except that 4 grams of sodium carbonate is substituted for ammonium hydroxide. If the heating is omitted in this case, a 24-hour period of time is required for full color development.

Determination of Spectrophotometric Constants. The various alkaline solutions of the iron(II) complex, prepared as described above, were measured spectrophotometrically employing either a Cary recording spectrophotometer, Model 11, or a Beckman DU spectrophotometer. The wave length of maximum absorbance of the solutions was found to be 520 mμ. A typical absorption spectrum of the complex from 400 to 600 mμ is shown in Figure 1, and the experimental data and results are tabulated in Table I.

From the results listed in Table I (excluding No. 6), the average value for the molecular extinction coefficient is found to be 14,800. This value is believed to be accurate within 100 units. Beer's law is thus found to be valid at least for iron(II) concentrations up to 6 p.p.m. at absorbances less than 1.5.

Table I. Determination of Molecular Extinction Coefficient of Iron(II) Complex in Alkaline Solutions

Determination No.	Iron Taken, Mg.	Alkaline Solution	Molecular Extinction Coefficient	Absorbance
1	0.03546	4*M* NaOH	15,160	0.385
2	0.05757	4*M* NaOH	14,950	0.617
3	0.08189	4*M* NaOH	15,100	0.886
4	0.10275	4*M* NaOH	14,800	1.089
5	0.12744	4*M* NaOH	14,720	1.344
6	0.04741	.6*M* NH_4OH	15,460	0.525
7	0.09458	6*M* NH_4OH	14,920	1.011
8	0.04818	1.5*M* Na_2CO_3	14,810	0.505
9	0.09808	1.5*M* Na_2CO_3	14,730	1.023
10	0.04842	4.0*M* NaOH + 0.7*M* Na_2CO_3	14,590	0.506
11	0.07033	4.0*M* NaOH + 0.7*M* Na_2CO_3	14,690	0.740
12	0.08961	4.0*M* NaOH + 0.7*M* Na_2CO_3	14,630	0.939
13	0.11348	4.0*M* NaOH + 0.7*M* Na_2CO_3	14,730	1.197
14	0.12522	4.0*M* NaOH + 0.7*M* Na_2CO_3	14,870	1.344
15	0.14046	4.0*M* NaOH + 0.7*M* Na_2CO_3	14,750	1.484

REDUCING AGENTS FOR IRON IN ALKALINE MEDIA

The preferred reducing agents, based upon established applications of the various 1,10-phenanthrolines in the spectrophotometric determination of iron(II) and copper(I), are hydroxylamine hydrochloride and hydrazine hydrochloride. Other reductants occasionally recommended are sulfur dioxide and hydroquinone.

Hydroxylamine was found to be unsuited for use in concentrated caustic solutions. Reduction is slow, and gaseous evolution effects further complicate the reaction. Hydrazine hydrochloride proved to be slow in reaction under the same conditions, although no inconvenience was encountered from gaseous evolution. Sulfurous acid and hydroquinone were not subjected to test.

Sodium hydrosulfite was found best suited for the reduction of iron(III) to iron(II) in strong caustic solution. It was rapid in its reduction of iron for all alkaline concentrations, and its decomposition products introduced no interferences. Because of decomposition during short periods of storage, solutions of sodium hydrosulfite must be freshly prepared. The best grades of commercially available sodium hydrosulfite contain iron as an impurity. These two drawbacks constitute the only complications in the use of this reducing agent, and both are readily provided for in the procedure.

Table II. Time for Complete Chelation of Iron(II) by Snyder's Reagent in Various Alkaline Media at Ordinary Temperatures

Solutions of Snyder's Reagent	Approximate Required Time
0.1*M* NaOH	17 hours
1.0*M* NaOH	2 hours
3.0*M* NaOH	15 minutes
4.0*M* NaOH	2 minutes
6.0 to 10*M* NaOH	Immediate
6.0*M* NH_4OH	2 hours
4.0*M* NaOH + 0.7*M* Na_2CO_3	20 hours

INTERFERENCES

A solution 4*M* in sodium hydroxide, $10^{-3}M$ in Snyder's reagent, $8 \times 10^{-5}M$ in iron, and $5 \times 10^{-2}M$ in sodium hydrosulfite was prepared. This solution was added to various amounts of the anions and cations to be tested for interference. After 30 minutes the resulting solutions were examined spectrophotometrically for any diminution in color intensity as compared with the test solution itself. Of a large number of ions tested, only cyanide, thiosulfate, and tartrate were found to interfere, and these differed considerably in magnitude of effect. Concentrations of cyanide in excess of 2 p.p.m., of tartrate in excess of 1000 p.p.m., or of thiosulfate greater than 10,000 p.p.m. interfere significantly. (A reduction in absorbance greater than 3% is considered significant.)

The marked interference of the cyanide ion indicates that it forms a much more stable complex with iron(II) than does Snyder's reagent. On addition of sodium cyanide to solutions of the Snyder's reagent–iron(II) complex in 4*M* sodium hydroxide, the characteristic red complex can be converted to an intensely yellow complex. Although this yellow complex has not been identified, it seems unlikely that it is ordinary ferrocyanide because of its intense color.

The following anions were not found to interfere significantly, even at 1*F* concentrations: CNS^-, S^{--}, SO_3^{--}, SO_4^{--}, SiO_3^{--}, Cl^-, Br^-, I^-, ClO_3^-, IO_3^-, ClO_4^-, F^-, CO_3^{--}, $C_2O_4^{--}$, $HCOO^-$,

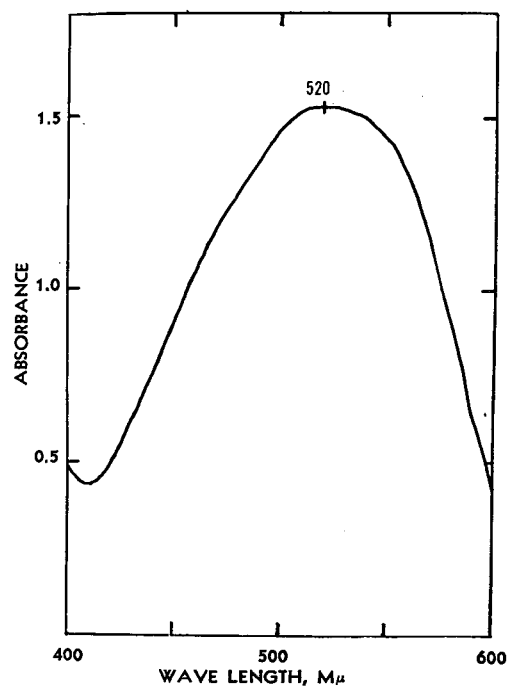

Figure 1. Absorption spectrum of tris(4,7-dihydroxy-1, 10-phenanthroline) iron(II) complex

NO_3^-; NO_2^-, PO_4^{--},$B_4O_7^{--}$, citrate, Versenate, polymetaphosphate, and pyrophosphate. Oxalates and fluorides may be insoluble at higher caustic concentrations than are required for iron(II) chelation with Snyder's reagent.

Copper(I) and cobalt(II) gave light-yellow colored solutions in caustic soda when treated with Snyder's reagent in the presence of sodium hydrosulfite. Their maximum absorbance does not occur in the visible portion of the spectrum but is limited to the near ultraviolet. Alkaline solutions of Snyder's reagent also absorb strongly in the near ultraviolet range. Under the conditions outlined previously for the formation of the ferrous chelate with Snyder's reagent, nickel, molybdenum, titanium, and zirconium form no colored complexes in the visible spectrum range. Alkaline earth metals, when present in sufficient concentration, precipitate but do not constitute an interference.

INFLUENCE OF ALKALI CONCENTRATION ON TIME OF REACTION

At room temperature alkaline solutions of Snyder's reagent require various time intervals for complete formation of iron(II) complexes. The required time interval is a function of the alkali concentration and type; these data are given in Table II.

The time intervals indicated in Table II were all shortened to instantaneous chelation if the test solutions were heated to near boiling for brief intervals (1 to 2 minutes), followed by cooling before spectrophotometric examination.

Table III. Determination of Iron in Glass Sand Using Snyder's Reagent

(National Bureau of Standards sample 81, 0.051% iron)

Sample, Gram	Absorbance, 520 Mμ		Iron, %
0.1485	0.764		0.0483
0.1122	0.569		0.0477
0.1079	0.564		0.0491
0.1087	0.564		0.0488
0.0894	0.455		0.0478
0.1542	0.792		0.0483
0.1375	0.714		0.0488
0.1662	0.845		0.0478
0.1240	0.645		0.0489
0.1264	0.662		0.0492
0.0794	0.417		0.0485
		Av.	0.0485
		Av. dev.	0.0005
		Std. dev.	0.0002

DETERMINATION OF IRON IN GLASS SAND

Snyder's Reagent Method. As a practical analytical application of Snyder's reagent, the determination of iron in glass sand was selected. This determination, following decomposition with fused ammonium bifluoride to evolve silica, has been described by Shead and Smith (*1*). Their accurate and rapid procedure is even more conveniently and accurately carried out by dissolving the sample with a carbonate fusion and using Snyder's reagent for spectrophotometric determination of iron.

A 100-mg. sample of glass sand is dried for 1 hour at 120° C., accurately weighed, and transferred to a 30-ml. iron-purged platinum crucible. A 2-gram sample of an equimolar mixture of sodium and potassium carbonates is added and the crucible contents are thoroughly blended. The sample is fused in the usual manner with the cover in place. When a clear fused melt is obtained, the temperature is lowered and the cover removed. The melt is rotated to cause solidification with increased surface area. When the melt is cool, 5 ml. of water, 1 ml. of sodium hydrosulfite solution, and 2 ml. of Snyder's reagent (0.01*M* in 1*M* sodium hydroxide) are added. The crucible is covered and continuously heated to dissolve the contents at or near the boiling point. The solution is cooled, transferred quantitatively to a 25-ml. volumetric flask, and diluted to the mark. The absorbance at 520 mμ is measured, using a 1-cm. cell in a Beckman DU spectrophotometer. A blank determination for iron in the reagents used must be applied. The iron present is then calculated employing the value 14,800 for the molecular extinction coefficient (absorbance = ϵlc and l = unity).

The use of either nickel or silver crucibles is ill-advised. Some nickel and silver are always taken into solution. The presence of nickel is objectionable, because more Snyder's reagent must be employed to complex with the nickel present. Any silver in solution is reduced by the sodium hydrosulfite (black colloidal silver sols), and interferes.

It was observed that the heating of the carbonate fusion melt to dissolve the sample gave a more rapid production of the maximum color intensity than if the melt was dissolved out of the crucible and transferred to a beaker before treatment with the chelation reagents. This effect was postulated to be due to the formation of a silicate in the melt, which to a very minor degree inhibits complete and rapid chelation by Snyder's reagent. Digestion of the sample together with sodium hydrosulfite and complexing reagent in contact with platinum eliminates this slight tendency to give incomplete color formation without aging the samples before determination of their optical densities.

Platinum crucibles containing surface-alloyed iron stains give unnecessarily large and irregular blank corrections. Some tendency for precipitation of silica was observed. To avoid this, Snyder's reagent is added as a solution in 1*M* sodium hydroxide; this is sufficient to maintain a favorable pH and prevent silica precipitation.

The results from a series of glass sand analyses are given in Table III.

Bathophenanthroline Method. This method was applied to the determination of iron in the same sample of glass sand for the purpose of comparing results with those obtained using Snyder's reagent.

Accurately weighed 100-mg. samples of glass sand are transferred to 30-ml. platinum crucibles. Five milliliters of 48% hydrofluoric acid are then added from a polyethylene graduated cylinder, and the crucible contents gently heated to evolve silicon tetrafluoride. A small volume of sulfuric acid (2 ml. of concentrated acid, diluted 1 to 1 with water) is added and heating is continued until dense fumes of sulfur trioxide are evolved to eliminate excess hydrofluoric acid.

When the solution is cool, 4 to 5 ml. of water are cautiously added and the crucible contents neutralized with ammonia using but a slight excess. The solution is reacidified by the addition of a slight excess of 1 to 6 hydrochloric acid, and the contents of the crucible are transferred quantitatively to a 25-ml. volumetric flask. The following reagents are then added from a pipet: 5 ml. of 2*M* ammonium acetate, 2 ml. of 10% hydroxylamine hydrochloride solution, and 5 ml. of 0.002*M* bathophenanthroline (4,7-diphenyl-1,10-phenanthroline) dissolved in ethyl alcohol. The contents of the 25-ml. flask are diluted to the mark with ethyl alcohol and thoroughly mixed. The absorbance of this solution is determined at 533 mμ, employing 22,400 as the molecular extinction coefficient and calculating iron values from the data thus obtained.

Table IV. Determination of Iron in Glass Sand Using Bathophenanthroline

(National Bureau of Standards sample 81, 0.051% iron)

Sample, Gram	Absorbance, 533 Mμ	Iron, %
0.1261	0.969	0.0480
0.1229	0.940	0.0476
0.0891	0.695	0.0488
0.1199	0.940	0.0490
0.1270	1.000	0.0492
0.0945	0.745	0.0492
	Av.	0.0486

The presence of hydrofluoric acid does not constitute an interference, but its removal is recommended because fluoride retards attainment of maximum absorbance. A blank correction for iron impurities in the reagents employed is chiefly due to the hydrofluoric acid. Consequently the volume of this reagent should be a definite measured amount.

Results from a series of these determinations are given in Table IV.

Examination of the data of Tables III and IV shows that the values for iron in glass sand determined by use of Snyder's reagent or bathophenanthroline agree. These values are 4% lower than those reported for U. S. Bureau of Standards Sample No. 81.

PROPOSED APPLICATIONS

The use of Snyder's reagent is helpful in estimating the purity of commercially available alkaline type reagents with respect to iron contamination. This application to sodium hydroxide, sodium carbonate, and aqueous ammonia has been discussed in the quantitative investigations of the present work.

Trisodium phosphate, sodium silicate, sodium tetraborate, and sodium sulfide in aqueous solution require no addition of sodium hydroxide before reduction of their iron content and addition of Snyder's reagent. Rapid and full color production in these cases requires that the test mixture be heated to boiling for a brief period.

Sodium sulfite, sodium pyrophosphate, and ammonium fluoride require the addition of sodium hydroxide to provide a pH high enough to promote chelation of iron(II) with Snyder's reagent. Only in the case of ammonium fluoride may heating be omitted to obtain prompt color formation for these three reagents.

Sodium metaphosphate and solutions of the tetrasodium salt of (ethylenedinitrilo)tetracetic acid give color development at ordinary temperatures. The color is dissipated upon heating in these two cases but returns in short periods of time after cooling.

DISCUSSION

Snyder's reagent greatly facilitates the determination of iron impurities in strongly basic aqueous solutions of reagent chemicals. Quality control and label statements of trace iron contamination may thus be stated with a high degree of accuracy.

The use of the Snyder's reagent–ferrous complex may prove of value as a redox indicator in strong alkali media. Experimentation in substantiation of this premise has not been attempted. The redox potential in such applications has not been investigated.

Other hydroxy substituted derivatives of 1,10-phenanthroline are being investigated and will be reported in the near future.

LITERATURE CITED

(1) Shead, A. C., Smith, G. F., IND. ENG. CHEM., ANAL. ED. 3, 61 (1931).
(2) Smith, G. F., Cagle, F. W., ANAL. CHEM. 20, 574 (1948).
(3) Snyder, H. R., Freier, H. E., *J. Am. Chem. Soc.* 68, 1320 (1946).
(4) Vosburgh, W. C., Cooper, G. R., *Ibid.*, 63, 437 (1941).

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Thoron–Tartaric Acid Systems for Spectrophotometric Determination of Thorium

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Thoron is commonly used for the spectrophotometric determination of thorium. An undesirable feature of its use is its high sensitivity to zirconium. This study describes the use of tartaric acid as a masking reagent for zirconium. Three tartaric acid–thoron systems, developed for the determination of thorium, differ with respect to the concentrations of thoron and tartaric acid. Mesotartaric acid, used in one of the systems, is most effective in masking zirconium. The behavior of rarer elements, usually associated with thorium ores, is determined in two systems, and a dilution method is described for the direct determination of thorium in monazite concentrates.

THORON—the disodium salt of 2-(2-hydroxy-3,6-disulfo-1-naphthylazo)-benzenearsonic acid—is probably the most common reagent for the spectrophotometric determination of thorium. Originally introduced by Kuznetsov (*5*) for the detection of thorium, the reaction was developed into a quantitative spectrophotometric procedure by Thomason, Perry, and Byerly (*7*). Several important studies and applications of this reaction have been made by Ingles (*4*), Banks, Klingman, and Byrd (*1–3*), and Taylor and Dillon (*6*). The report by Byrd and Banks (*3*) is comprehensive.

An undesirable feature limiting the use of thoron in the spectrophotometric method for thorium is that the reagent is highly sensitive to zirconium. This is shown in Figure 1, where the net absorbance due to zirconium alone and in the presence of 150 γ of thorium dioxide is plotted. Zirconium gives increased absorbance in the presence of thorium. It can be shown that 1 γ of thorium dioxide in 25 ml. of solution gives a net absorbance of 0.012 for a 5-cm. light path. Thus 0.6 γ of zirconium dioxide is equivalent to 1 γ of thorium dioxide when 150 γ of thorium dioxide is present. Several organic hydroxy acids were studied as possible masking reagents for zirconium; this report deals with the use of tartaric acid.

The thoron-thorium–tartaric acid system is very complex. At least five equilibria are involved: thorium-thoron, thorium-tartrate, zirconium-thoron, zirconium-tartrate, and thoron-tartrate. The last equilibrium must be considered because thoron and tartaric acid also react. An additional variation is introduced when mesotartaric acid is substituted for the common *d*-tartaric acid.

EXPERIMENTAL DATA

In the studies of the tartaric acid–thoron systems a high acidity (pH 0.65) was chosen to minimize the effect of interfering elements. Three systems were studied. In System I, 2 mg. of thoron per 25 ml. of final solution was used. This amount is just sufficient to complex 150 γ of thorium dioxide at low tartaric acid concentrations. By limiting the thoron concentration it was thought that interference from elements, such as rare earths, that react weakly with thoron would be decreased. In System II the concentration of thoron was twice that in System I. System II would be expected to have less anion interference. In System III the thoron concentration was the same as in System I, but mesotartaric acid was substituted for the common *d*-tartaric acid used in the other two systems.

All experiments were made from a total volume of 25 ml.

The order of addition of the reagents was always the same: acid first, zirconium in (1+1) hydrochloric acid solution second, thorium third, hydroxylamine hydrochloride (1 ml. of a 10% solution) fourth, 30% tartaric acid solution fifth, and thoron last. Two milliliters of 0.1% thoron was used in Systems I and III, and 4 ml. was used in System II. The total amount of acid in all solutions amounted to 1 ml. of (1+1) hydrochloric acid (including that supplied by the zirconium solutions). The hydroxylamine hydrochloride is included because it is used in the final procedure to decrease the interference of ferric iron. Absorbances were measured with a Beckman spectrophotometer, using 5-cm. cells at 545 mμ, the wave length which gives maximum absorbance difference between the thorium-thoron complex and the blank.

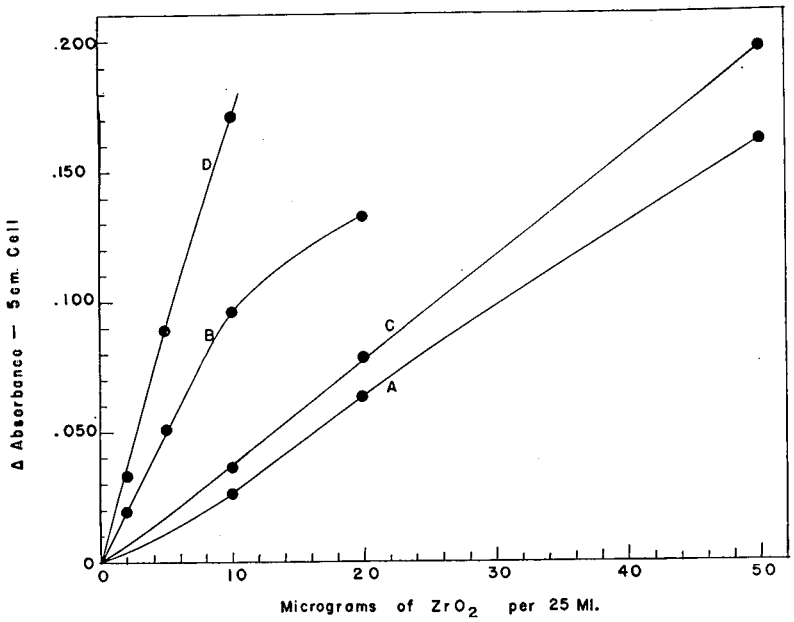

Figure 1. Zirconium interference in absence of tartaric acid

A. ZrO_2 *vs.* reagent blank, 2 mg. of thoron
B. 150 γ ThO_2 plus varying ZrO_2 *vs.* 150 γ ThO_2, 2 mg. thoron
C. ZrO_2 *vs.* reagent blank, 4 mg. thoron
D. 150 γ ThO_2 plus varying ZrO_2 *vs.* 150 γ ThO_2, 4 mg. thoron

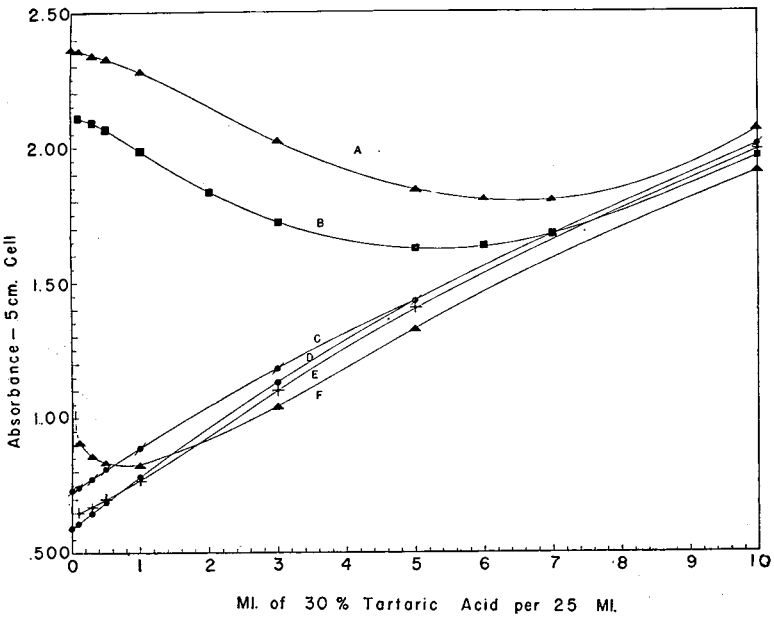

Figure 2. Effect of tartaric acid, System I

A. 150 γ ThO_2
B. 150 γ ThO_2 + 1 mg. ZrO_2
C. 10 γ ThO_2
D. 2 mg. thoron
E. 200 γ ZrO_2
F. 1 mg. ZrO_2

System I. The effect of tartaric acid in System I is shown in Figure 2. Absorbances were obtained differentially but are plotted with water as reference. Thoron is seen to react with tartaric acid, progressively increasing absorbances being obtained with increase in tartaric acid (curve *D*). The thoron–tartaric acid complex shows a maximum absorbance difference from the thoron alone at 533 mμ. The nature of this interaction is not known. The curves for 10 γ of thorium dioxide (curve *C*) and 150 γ of thorium dioxide (curve *A*) show that as the tartaric acid is increased more of the thorium enters into a tartrate complex. The 10-γ thorium dioxide curve (curve *C*) intersects the thoron curve (curve *D*) at about 5 ml. of tartaric acid and then follows the thoron curve. This is apparently due to full release of thorium from its complex with thoron and the complete formation of the thorium-tartrate complex. However, 10 ml. of tartaric acid is not enough to complex 150 γ of thorium dioxide completely (curve *A*). Small amounts of tartaric acid exert a strong complexing action on zirconium as indicated by a sharp drop in the absorbance curve, *F*, at small concentrations of tartaric acid. However, the zirconium reaction is peculiar.

If full zirconium-tartrate complex were obtained, the zirconium curves, *E* and *F* (200 γ and 1 mg. of zirconium dioxide), would be expected to intersect the thoron curve, *D*, and then coincide with it as the tartaric acid is increased, as was true for 10 γ of thorium dioxide in curve *C*. Actually, for these amounts of tartrate the zirconium solutions absorb less than the thoron and this decrease cannot be accounted for simply by the amount of tartaric acid combined with the zirconium. Solutions containing both thorium and zirconium (curve *B*) show less absorbance than solutions containing only thorium (curve *A*). No completely satisfying explanation was found.

It is clear from the nature of the curves plotted in Figure 2 that the thorium-thoron reaction would be highly sensitive only for media containing small amounts of tartaric acid. Accordingly, the region up to 2 ml. of tartaric acid was examined in greater detail. The thorium in the test solutions was varied from 0 to 150 γ of thorium dioxide, and the zirconium was varied from 100 to 1000 γ of zirconium dioxide. The absorbance of each test solution was determined relative to a comparable reference solution containing the same amounts of all reagents but without

Table I. Milligrams of Various Elements (as Oxides) Equivalent to 1 γ of Thorium Dioxide

Elements Tested	System I (5 Ml. of 0.04% Thoron, 1 Ml. of 9% Tartaric Acid) 0 ThO_2	10 γ ThO_2	100 γ ThO_2	150 γ ThO_2	System II (5 Ml. of 0.08% Thoron, 1 Ml. of 30% Tartaric Acid) 0 ThO_2	50 γ ThO_2	150 γ ThO_2
Sc_2O_3	0.52	0.18	0.070	0.070	0.63	0.11	0.10
Y_2O_3	3.5	3.5	7.5	>7.5	2.0	2.5	2.7
La_2O_3	0.72	0.72	1.0	...	0.35	0.77	2.5
Ce_2O_3	0.46	0.70	1.2	1.8	0.34	0.50	0.72
Pr_2O_3	0.74	0.76	0.96	...	0.49	0.50	0.85
Nd_2O_3	0.88	0.96	1.6	1.7	0.48	0.63	0.71
Sm_2O_3	0.68	0.72	0.95	0.95	0.58	0.58	0.82
Eu_2O_3	1.9	1.9	1.9	...	0.81	1.2	1.6
Gd_2O_3	2.8	2.8	2.8	...	1.2	1.7	1.7
Tb_2O_3	...	...	> 5.0	...	1.4	...	...
Dy_2O_3	...	...	...	...	...	...	...
Ho_2O_3	...	...	> 5.0	...	...	...	...
Er_2O_3	...	...	> 5.0	...	3.6	...	...
Tm_2O_3	...	...	> 6.0	...	...	...	...
Yb_2O_3	> 5.0	> 5.0	> 5.0	...	3.1	...	4.8
Lu_2O_3	...	...	> 5.0	...	4.0	> 5.0	...
TiO_2	1.0	0.040	0.016	0.013	0.50	0.021	0.014
ZrO_2	0.13	0.13	0.18	0.045	0.34	0.36	> 1.0
Nb_2O_5	0.20	0.40	0.060	0.043	0.83	0.060	0.050
Ta_2O_5	0.10	0.25	0.13	0.04	0.59	0.090	0.077
WO_3	> 5.0	2.8	1.0	0.50	> 5.0	3.0	4.7
UO_2	0.54	0.54	1.3	2.4	0.28	0.38	0.47
Fe_2O_3	0.35	0.18	0.15	0.14	0.13	0.045	0.028
Au_2O_3	> 5.0	> 5.0	> 5.0	> 5.0	> 5.0	> 5.0	> 5.0
HgO	>20	>20	>20	>20	>20	>20	>20
Al_2O_3	44	82	50	23	2.3	3.0	8.5
SnO_2	0.060	0.060	0.079	0.098	0.056	0.094	0.078
PbO	>10	>10	>10	>10	5.0	10	10
Bi_2O_3	...	...	...	...	> 5.0	> 5.0	> 5.0
P_2O_5	> 5.0	2.0	0.16	0.065	> 5.0	0.70	0.60
SO_3	> 5.0	4.3	0.60	0.32	> 5.0	2.2	2.2
F	> 5.0	0.0064	0.001	0.0006	> 2.0	0.005	0.0027

zirconium. In Figure 3 the data for solutions containing 0 and 150 γ of thorium dioxide and 0.1 to 0.5 ml. of tartaric acid are shown. Curves for amounts of tartaric acid greater than 0.5 ml. are not shown, but above 0.5 ml. of tartaric acid slightly greater interference resulted from low concentrations of zirconium. Other curves for intermediate amounts of thorium dioxide fall in proper sequence between the limits shown in Figure 3. The zirconium interference is least at a level of tartaric acid between 0.3 and 0.5 ml. Differences here are small and the 0.3-ml. level of tartaric acid was selected for the operating system. The operating conditions adopted for System I follow: In a total volume of 25 ml. are contained 1 ml. of (1+1) hydrochloric acid, 1 ml. of 10% hydroxylamine hydrochloride, 0.3 ml. of 30% tartaric acid, and 2 ml. of 0.1% thoron. In the final procedure 1 ml. of 9% tartaric acid is substituted for the 30% acid for ease in pipetting.

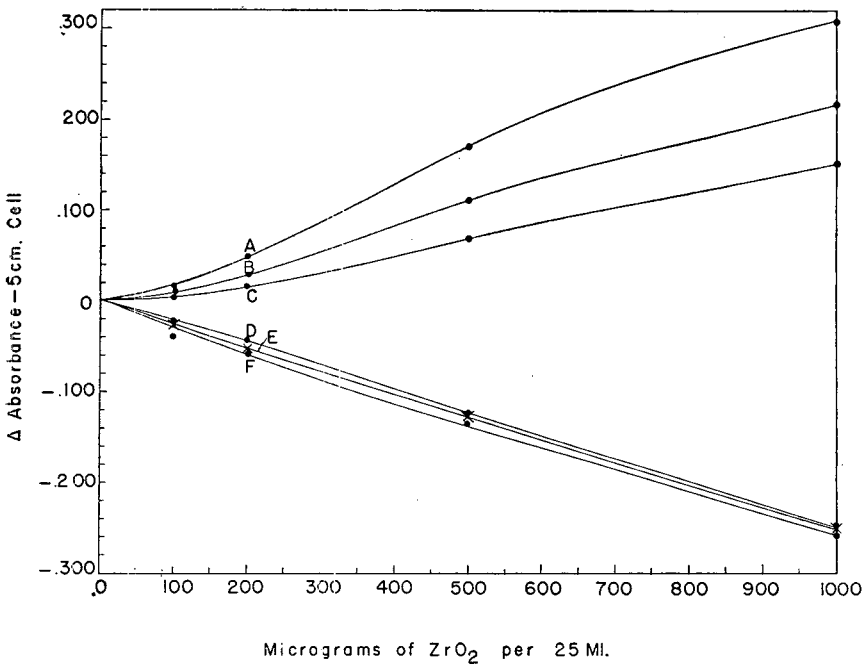

Figure 3. Effect of Zirconium at various concentrations of thorium and tartaric acid

	ThO_2, γ	30% Tartaric Acid, Ml.
A	0	0.1
B	0	0.3
C	0	0.5
D	150	0.1
E	150	0.3
F	150	0.5

Under the operating conditions of System I, the effects of various elements usually found in thorium ores, calculated as oxides, are given in Table I. Absorbances were determined differentially in 5-cm. cells. The amount of each element is equivalent to an error of 1 γ of thorium dioxide at the various levels of thorium. The cations were introduced as chlorides and the anions as the sodium salts. Niobium and tantalum solutions were prepared by fusing each oxide (purity >99.5%) with potassium carbonate and leaching the melts with water to give very dilute solutions. The titanium solution was prepared by fusing the oxide with potassium carbonate and dissolving the melt in (1 + 9) hydrochloric acid. The rare earths were spectrographically pure and determined to be free from thorium and zirconium by chemical tests. Some elements give both positive and negative errors. Such

elements probably form strong thoron complexes and upset the thorium-thoron equilibrium.

System II. In System II the amount of thoron was increased to 4 mg. The effect of tartaric acid on solutions containing 200 to 1000 γ of zirconium dioxide alone and in the presence of 150 γ of thorium dioxide is shown in Figure 4. The absorbance differences from solutions free from zirconium but containing the same amounts of other reagents are plotted. One milliliter of tartaric acid was chosen as optimum. The operating conditions in System II are thus the same as in System I, except for the substitution of 1 ml. for 0.3 ml. of 30% tartaric acid and of 4 mg. for 2 mg. of thoron.

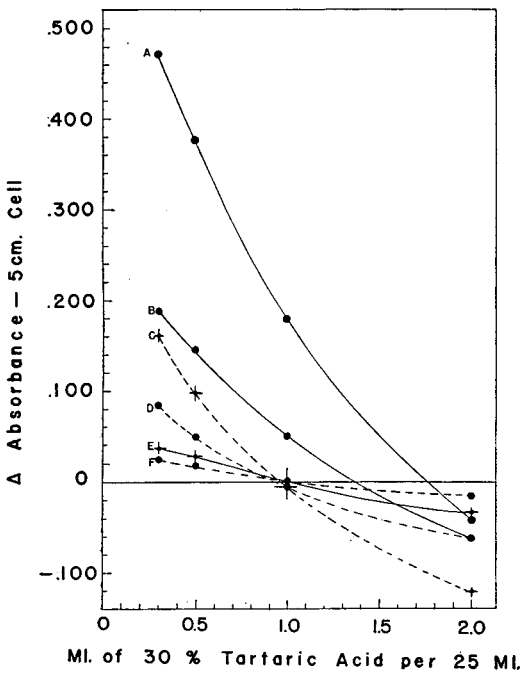

Figure 4. Effect of tartaric acid, System II

A. 1 mg. ZrO_2
B. 500 γ ZrO_2
C. 150 γ ThO_2 + 1 mg. ZrO_2
D. 150 γ ThO_2 + 500 γ ZrO_2
E. 200 γ ZrO_2
F. 150 γ ThO_2 + 200 γ ZrO_2

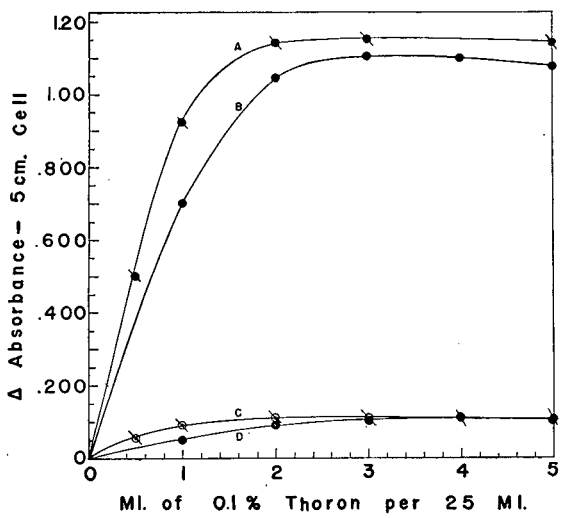

Figure 5. Effect of thoron concentration

A, C. System I
B, D. System II
A, B. 100 γ ThO_2
C, D. 10 γ ThO_2

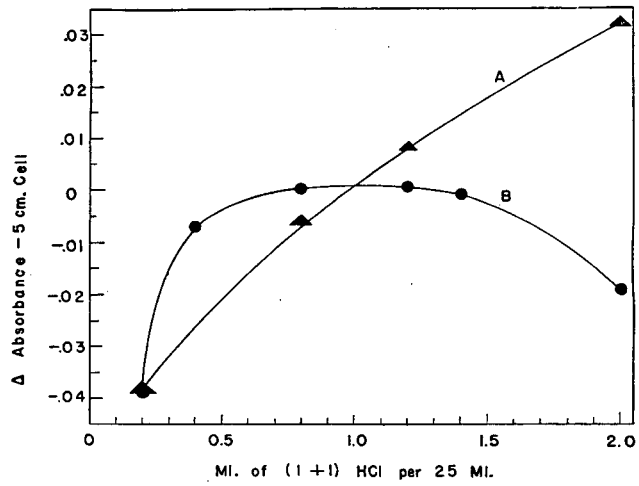

Figure 6. Effect of acidity, System II

A. 15 γ ThO_2
B. 100 γ ThO_2

The effects of thoron concentration at the 0.3-ml. and 1.0-ml. levels of tartaric acid, other conditions being the same, are illustrated in Figure 5. A calculated absorbance–thorium concentration curve, based on the assumption that a 1 to 2, thorium-thoron complex is formed, was found to be within 5% of the experimental curve for System I. The slope ratio method also indicated a 1 to 2 complex. The effect of pH under the operating conditions of System II is illustrated in Figure 6. One milliliter of (1 + 1) hydrochloric acid was used in the reference solution. The slightness of the changes in absorbance with increasing acidity shows that the pH dependency is not too important. Similar considerations hold for System I.

The effects of various elements (System II), are given in Table I. Higher concentrations of rare earths, iron, aluminum, and uranium can be tolerated in System I than in System II. System II can tolerate higher concentrations of anions as well as zirconium and tungsten.

System III. Two milligrams of thoron was used in System III, which is similar to System I, except that mesotartaric acid was substituted for the *d*-form used in Systems I and II. The effects of mesotartaric acid on the absorbances of thoron and of various solutions containing thorium or zirconium or both are shown in Figure 7. Absorbances were obtained differentially, but are plotted with water as reference. A simple system is indicated. Mesotartaric acid should be an ideal reagent to mask at least 1 mg. of zirconium dioxide. The system seems promising and will be the subject of a separate study.

SPECTROPHOTOMETRIC DETERMINATION OF THORIUM IN MONAZITE CONCENTRATES

By the use of a dilution technique and measuring absorbances with a 5-cm. light path, it was possible to determine thorium in monazite sands directly after the solution of the sample. An aliquot representing 0.5 mg. of sample is used for the spectrophotometric determination.

Reagents and Apparatus. Thoron solution, System I, 0.04 gram per 100 ml. of aqueous solution.

Thoron solution, System II, 0.08 gram per 100 ml. of aqueous solution.

d-Tartaric acid solution, System I, 9 grams per 100 ml. of aqueous solution.

d-Tartaric acid solution, System II, 30 grams per 100 ml. of aqueous solution.

Hydroxylamine hydrochloride solution, 10 grams per 100 ml. of aqueous solution.

Beckman Model DU spectrophotometer supplied with 5-cm. cells.

Procedure. Mix 0.1000 gram of a representative finely ground monazite sample with 1 gram of sodium peroxide in a platinum crucible.

Sinter the mixture (covered) in a small furnace at 440° ± 20° C. for about 30 minutes At higher temperatures sodium peroxide would seriously attack the platinum vessel. In the range 440° ± 20° C. attack of the platinum crucible is negligible.

Leach with 25 to 50 ml. of water. Digest the solution on the steam bath until the precipitate is filterable.

Filter the solution through a medium-speed filter paper and wash with 1% potassium hydroxide solution. The filtrate obtained should be clear. If not, digest it and refilter.

Dissolve the residue off the paper with 20 ml. of hot (1 + 1) hydrochloric acid (use pipet), adding a drop or two of hydrogen peroxide (30%) to reduce cerium to the trivalent state. Wash thoroughly with water. The filtrate should be about 40 ml.

Remove hydrogen peroxide by heating the solution on the steam bath in a covered beaker for about 30 minutes. Cool.

Transfer the solution to a 100-ml. volumetric flask and adjust to the mark with water. Mix.

Transfer a 10-ml. aliquot to a dry 100-ml. volumetric flask. Add (1 + 9) hydrochloric acid to the mark. Mix.

Transfer a 5-ml. aliquot to a 25-ml. volumetric flask, and add 1 ml. of hydroxylamine hydrochloride and 5 ml. of water. If System I is used, add 1 ml. of 9% tartaric acid solution and 5 ml. of 0.04% thoron solution, adjust to the mark with water, and mix. If System II is used, add 1 ml. of 30% tartaric acid solution and 5 ml. of 0.08% thoron solution, adjust to the mark with water, and mix. Volumetric pipets are used throughout.

Measure the absorbance of the solution in 5-cm. cells against a reagent blank prepared by adding 1 ml. each of (1 + 1) hydrochloric acid, hydroxylamine hydrochloride, the proper tartaric acid, and 5 ml. of the proper thoron solution. Measure absorbances with the Beckman DU spectrophotometer at 545 mμ, using a slit width of 0.05 mm. for System I and 0.15 mm. for System II.

Determine the amount of thorium by reference to a standard curve. The relationship between absorbance and thorium concentration is linear up to 100 γ of thorium dioxide for System I and up to 60 γ of thorium dioxide for System II. Five micrograms of thorium dioxide was the smallest amount tested. For amounts of thorium dioxide greater than these limits, the curvature is very gentle and all points are reproducible. The systems are stable enough so that absorbance measurements can be made at any time within 1 hour.

The suggested dilution procedure is peculiarly suited for the determinination of thorium in monazite concentrates, because the ratios of titanium and other interfering ions to thorium are usually sufficiently low in these ores. The dilution technique should be useful on other samples, provided that the final aliquot contains smaller amounts of interfering elements than those given in Table I. Ordinarily titanium and iron determine the limits of applicability. For an aliquot containing 0.5 mg. of sample, no more than 4% of titanium dioxide and at least 1% of thorium dioxide should be present. Iron need not be a serious interference if it is reduced to the bivalent state, in which form at least 10 mg. can be tolerated. For the reduction of iron, the final aliquot (which is acid) is evaporated to dryness, 1 ml. of hydroxylamine hydrochloride solution is added, and the mixture is warmed briefly. At the low acidity of such solutions the reduction of iron is instantaneous. After the reduction, water, hydrochloric acid, tartaric acid, and thoron are added for the color development.

Test of Procedure. A standard monazite sand, No. 2601, obtained from the New Brunswick Laboratory of the U. S. Atomic Energy Commission, was analyzed according to the proposed procedure. Three aliquots of the solution were analyzed by the method of System I, and three aliquots of the same solution were analyzed by the method of System II. The results are given in Table II.

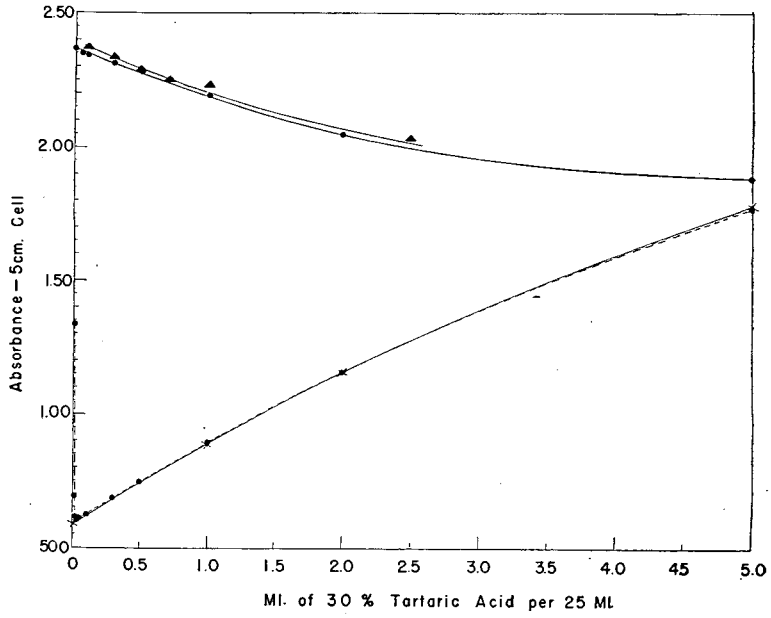

Figure 7. Effect of mesotartaric acid, System III

Upper. 150 γ ThO_2 + 1 mg. ZrO_2
Next. 150 γ ThO_2
Dashed. 1 mg. ZrO_2
Lowest. 4 mg. thoron

Table II. Test of Procedure on Monazite Sand

Sample No.	ThO_2, % System I	ThO_2, % System II	Certificate Value
NBS 2601	9.88	9.80	9.65
	9.88	9.78	
	9.88	9.82	

ACKNOWLEDGMENT

The authors thank Hewitt G. Fletcher of the National Institutes of Health for his generosity in supplying the mesotartaric acid. This work is part of a program being conducted by the U. S. Geological Survey on behalf of the Division of Raw Materials of the U. S. Atomic Energy Commission.

LITERATURE CITED

(1) Banks, C. V., Byrd, C. H., ANAL. CHEM. **25**, 416 (1953).
(2) Banks, C. V., Klingman, D. W., Byrd, C. H., *Ibid.*, **25**, 992 (1953).
(3) Byrd, C. H., Banks, C. V., "Spectrophotometric Determination of Thorium with the Trisodium Salt of 2-(2-Hydroxy-3,6-disulfo-1-naphthylazo)-benzenearsonic Acid and Some Properties of Complexes Involved," Iowa State College, **ISC-456**, 1953.
(4) Ingles, J. C., Canada Dept. Mines Tech. Surveys, Mines Branch, **NP-3069**, 1951.
(5) Kuznetsov, V. I., *J. Gen. Chem.* (*U.S.S.R.*) **14**, 914 (1944).
(6) Taylor, A. E., Dillon, R. T., ANAL. CHEM. **24**, 1624 (1952).
(7) Thomason, P. F., Perry, M. A., Byerly, W. M., *Ibid.*, **21**, 1239 (1949).

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Quantitative Estimation of Vitamins D_2 and D_3 in Pure Solution

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A method which has been developed for the estimation of vitamin D_2, vitamin D_3, or a mixture of the two forms in pure solution is based upon the formation of a colored reaction product when the vitamins are treated with furfural and sulfuric acid under carefully controlled conditions. Distinctive absorption curves are obtained for the two forms of the vitamin, and spectrophotometric techniques can be applied in the analysis of binary mixtures. The method is applicable to samples containing as little as 15 γ of total vitamin D, regardless of the relative abundance of each form.

MANY color reactions have been proposed for the estimation of vitamin D, and an excellent review of the methodology is given by Ewing, Kingsley, Brown, and Emmett (*4*). The antimony trichloride reaction, introduced by Carr and Price (*3*) for the estimation of vitamin A, was modified for use with vitamin D by Brockman and Chen (*1*) and by Nield, Russel, and Zimmerli (*5*). Sobel, Mayer, and Kramer (*7*) proposed the use of glycerol dichlorohydrin, but the reaction was found by Campbell (*2*) to be unsuitable for the estimation of vitamin D_3 in low potency oils. The formation of colored complexes between vitamin D and various aldehydes in the presence of sulfuric acid has been studied by Schaltegger (*6*). This paper describes a rapid chemical assay procedure for the identification and quantitative estimation of vitamins D_2 and D_3 in pure solution.

REAGENTS AND APPARATUS

Absolute Ethyl Alcohol. Reflux for 8 hours with 20 grams of potassium hydroxide and 10 grams of silver nitrate per liter, then distill.

Furfural Reagent. A 0.0046% (w/v) solution of furfural in 95% ethyl alcohol.

Sulfuric Acid. Reagent grade; specific gravity, 1.84.

Crystalline Vitamins D_2 and D_3. These were obtained from Nutritional Biochemicals Corp., 21010 Miles Ave., Cleveland, Ohio. A portion of each of these preparations was converted to the 3,5-dinitrobenzoyl ester and recrystallized five times from acetone. The absorption characteristics of the furfural reaction products obtained with the vitamins isolated after saponification of the esters were the same as those found for the original preparations. The purity of the vitamin D_3 preparation was further demonstrated by comparison with a sample of pure vitamin D_3. The original preparations were therefore used without further purification.

Spectrophotometer. A recording spectrophotometer fitted with a repetitive scanning accessory was used to obtain the data. The instrument used in this work was manufactured by Warren Electronics, Bound Brook, N. J.

PROCEDURE

Preliminary experiments suggested that colored complexes which formed between furfural and sulfuric acid might be used as a basis for analysis. It was found, however, that the nature of the reaction was greatly influenced by acid concentration, temperature, and the length of time employed for color development. The conditions cited below should be followed carefully in order to obtain reproducible results.

Place a 2.0-ml. aliquot (not less than 15 γ total vitamins) of an ethyl alcohol solution of the vitamins in a 50.0-ml. centrifuge tube. Add 1.0 ml. of the furfural reagent and cool the tube by immersion in a mixture of ethyl alcohol and dry ice in a Dewar flask. Stir the solution with a fine stream of nitrogen and maintain the temperature at 13° ± 5° C. by raising or lowering the tube. Add 7.0 ml. of concentrated sulfuric acid dropwise at a rate of approximately 1.0 ml. per minute. After the addition of acid is complete remove the tube from the bath and allow it to come to room temperature. Determine the absorption curve 20 minutes after commencing to add the acid.

RESULTS

The influence of reaction temperature on the wave length of maximum absorption of the vitamin D_2 complex is shown in Figure 1. In the case of the vitamin D_3 complex (Figure 2), temperature does not alter the wave length of maximum absorption but there is a marked change in the value of the extinction coefficient. In view of the negligible contribution of the vitamin D_3 complex to the absorbance at 565 mμ, the ratio 490 mμ/565 mμ has been used as a criterion of the relative abundance of the two forms of the vitamin.

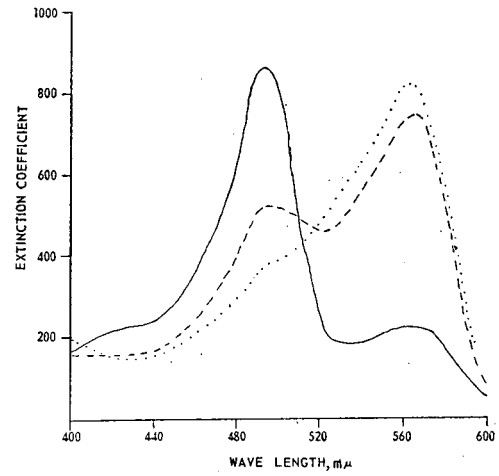

Figure 1. Effect of reaction temperature on vitamin D_2 complex formation

———— −20° C.
– – – – 0° C.
..... 20° C.

The influence of temperature on this ratio was studied and it was found that the values remained constant over the range from 10° to 30° C. in the reaction with vitamin D_2. Constant values were found over the range from 10° to 20° C. for vitamin D_3 when the absorbance at 490 mμ was plotted against temperature. These results indicated that the influence of temperature on the reaction was minimal in the range from 10° to 20° C., and in subsequent work samples were maintained at 13° ± 5° C. during the addition of acid.

The distinctive absorption curves of the furfural reaction products of vitamins D_2 and D_3 are shown in Figure 3. The negligible contribution of vitamin D_3 to the absorption at 565 mμ permits the accurate estimation of vitamin D_2 by using a value of 950 for the extinction coefficient at this wave length. It is

possible to construct a curve of the type shown in Figure 4 by using a value of 1060 for the extinction coefficient of vitamin D_3 at 490 mμ and 0.465 for the absorption ratio (490 mμ/565 mμ) of vitamin D_2. The weight percentage composition of mixtures of vitamins D_2 and D_3 can be determined by the use of this curve.

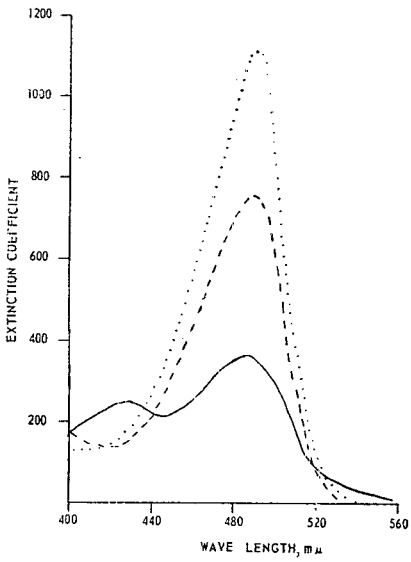

Figure 2. Effect of reaction temperature on vitamin D_3 complex formation

——— 20° C.
– – – – 0° C.
..... 20° C.

The method outlined above has been tested by analyzing a series of stock solutions which were prepared to contain approximately the same total amount of the vitamins D but which differed in the relative proportion of D_2 and D_3. The weight percentage of vitamin D_2 in these mixtures was calculated by means of Figure 4, and the appropriate extinction coefficients at 565 and 490 mμ were used to determine the absolute amounts of the two forms. The calculated and theoretical values for the composition of these mixtures are given in Table I.

Table I. Results Obtained with Mixtures of Vitamins D_2 and D_3

Stock Solution	Vitamin D_2, Wt. %		γ of Vitamin			
			D_3		D_2	
	Calcd.	Theor.	Calcd.	Theor.	Calcd.	Theor.
1	13.0	14.4	39.3	39.6	6.0	6.8
2	30.5	28.8	33.6	33.0	15.0	13.1
3	45.0	43.2	26.4	26.4	21.0	20.2
4	56.0	57.4	21.3	19.8	27.4	26.8
5	72 0	71.7	15.3	13.2	34.5	33.9
6	87.0	85.9	6.9	6.6	37.0	40.4
7	100.0	100.0	0.0	0.0	47.2	47.1
8	1.0	0.0	44.8	46.2	0.0	0:0

The specificity of the reaction has been tested by applying the method to a series of compounds which resemble vitamins D_2 and D_3 in structure. The results of these tests are given in Table II, in which the extinction coefficients for the compounds are listed at three wave lengths. In comparison with the value of 950 for the extinction coefficient of vitamin D_2 at 565 mμ and 1060 for vitamin D_3 at 490 mμ, the extinction coefficients listed in Table II are relatively small.

DISCUSSION

The method which has been described permits the rapid estimation of vitamins D_2 and D_3. An alternative method of calculation may be of value under certain circumstances in that the total amount of the D vitamins present in the sample may be calculated by using a value of 465 for the extinction coefficient at the isosbestic point at 509 mμ. Among the compounds which were tested, 7-dehydrocholesterol and ergosterol were found to interfere to the greatest extent. The absorption curves of these compounds in the reaction with furfural are quite different from those obtained with vitamins D_2 and D_3, and corrections can easily be applied to simple mixtures.

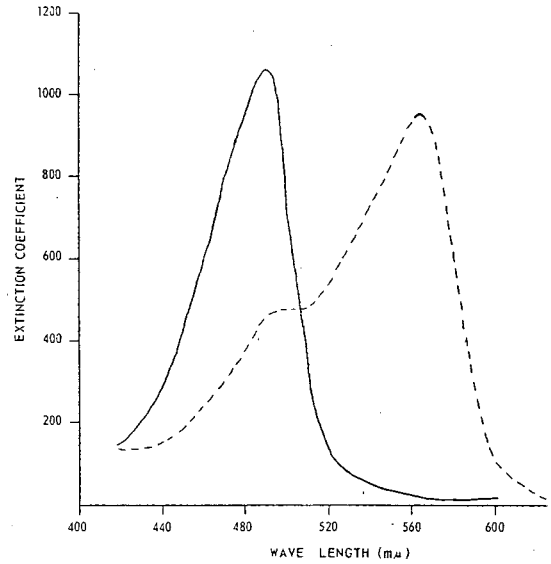

Figure 3. Absorption curves of complexes of vitamins D_2 and D_3 with furfural

– – – – – Vitamin D_2
——— Vitamin D_3

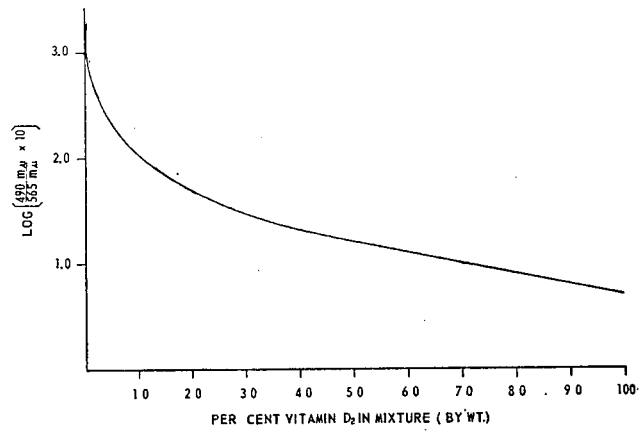

Figure 4. Relationship between mixture composition and absorbance

Table II. Extinction Coefficients of Compounds Structurally Related to Vitamins D_2 and D_3

Compound	Extinction Coefficient 490 mμ	565 mμ	400 mμ
Progesterone	16.4	2.7	120.6
Desoxycorticosterone acetate	16.6	6.6	25.0
Testosterone	16.2	5.4	18.9
Dehydroisoandrosterone	33.8	11.1	542
Δ-5-Androstene-3,17-diol diacetate	27.7	8.2	337
Δ-5-Androstene-3β,17β-diol-3-acetate-17-benzoate	19.0	0	283
Pregnenolone	11.0	0	413
Ergosterol	70.1	46.9	220
Sitosterol	14.5	2.4	41.2
Stigmasterol	37.1	11.8	141
Cholesterol	23.4	13.1	62.7
7-Dehydrocholesterol	118.5	50.3	112.5
Squalene	24.8	15.4	28.8

The sensitivity of the method is good because of the magnitude of the extinction coefficients at the wave lengths of maximum absorption. The method appears adequate for the estimation of as little as 15 γ of vitamin D, but conditions for the reaction must be carefully controlled if good reproducibility is to be obtained.

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LITERATURE CITED

(1) Brockman, H., Chen, V. H., *Z. physiol. Chem.* **241**, 129 (1936).
(2) Campbell, J. A., ANAL. CHEM. **20**, 766 (1948).
(3) Carr, F. H., Price, E. A., *Biochem. J.* **20**, 497 (1926).
(4) Ewing, D. T., Kingsley, G. V., Brown, R. A., Emmett, A. D., IND. ENG. CHEM., ANAL. ED. **15**, 301 (1943).
(5) Nield, C. H., Russel, W. C., Zimmerli, A., *J. Biol. Chem.* **136**, 73 (1940).
(6) Schaltegger, H., *Helv. Chim. Acta* **29**, 285 (1946).
(7) Sobel, A. E., Mayer, A. M., Kramer, B., IND. ENG. CHEM., ANAL. ED. **17**, 160 (1945).

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Identification of Ozone in the Los Angeles Atmosphere

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Although the oxidant occurring in the Los Angeles atmosphere resembles ozone in its properties, its true identity has been the subject of much discussion. Because the analytical methods commonly used are not entirely specific for ozone, a technique was employed which utilized silica gel at the temperature of liquid oxygen for the concentration of ozone. The adsorbed gases were flushed into an optical cell and their ultraviolet spectrum was recorded. The resulting spectrum was compared with spectra of synthetic ozone-oxygen mixtures. The results indicate that the material retained on and desorbed from silica gel was ozone. The quantities recovered account for 30 to 45% of the total oxidant. The remainder may well be handling losses, or another oxidant, or both.

THE presence of a powerful oxidant in the Los Angeles atmosphere has long been recognized; its concentration has been recorded continuously since May 1951 in the Pasadena Laboratories of Stanford Research Institute, using an automatic recorder based on iodine release from potassium iodide. The concentration has been shown to vary from 0 to 60 parts per hundred million (p.p.h.m.) calculated as ozone (by volume) and to follow a characteristic diurnal pattern. The occurrence of oxidant appeared to coincide with the incidence of "smog" and to constitute an objective measure of this disagreeable condition: high oxidant values—about 25 p.p.h.m.—were usually accompanied by plant damage and irritation of the eye.

As the oxidant resembled ozone in some of its properties, an attempt was made to identify the ozone fraction of the oxidant. The nature of the oxidant in the Los Angeles area has been the subject of previous discussions. Haagen-Smit (*7*) expressed the opinion that no more than a few parts per hundred million of ozone are present and that the bulk of the oxidant is made up of organic peroxides. Nitrogen dioxide, known to be present in the air in amounts up to perhaps 20 p.p.h.m., was also suspected as the cause of the high oxidant values. Bartel (*2*) and others suspected that ozone is present, largely on the basis of the severe rubber cracking encountered in this area. In a later paper, Haagen-Smit and Bradley (*3*) reported up to 22 p.p.h.m. of ozone (based on rubber cracking) in the Los Angeles atmosphere—about 10 times the "normal" amount reported in other parts of the world.

Since the liberation of iodine from neutral potassium iodide solutions, which is the basis of the recorder used by Stanford Research Institute (*8*), is not specific for ozone (nitrogen dioxide and sulfur dioxide are the principal interfering agents), more specific methods were sought. Three different techniques were eventually employed for the identification of ozone: rubber cracking, direct spectroscopic measurements, and isolation and identification by spectroscopic and chemical tests.

DETERMINATION BY RUBBER CRACKING AND LIBERATION OF IODINE

The tools used in these tests were a continuous oxidant recorder and a modification of the rubber-cracking technique described by Haagen-Smit and Bradley (*3*).

The oxidant recorder has been described in detail (*8*); a schematic diagram is shown in Figure 1. The setup used for the rubber-cracking test is shown in Figure 2. The test strips were made up according to Haagen-Smit's formulation and were extracted with boiling carbon tetrachloride for 24 hours in a Soxhlet extractor, after which they were dried under vacuum for a week. A calibration curve was obtained using ozonized oxygen (Figure 3), showing that the time required for initial rubber cracking was a function of the ozone concentration.

Table I and Figure 4 show a typical result obtained by these methods. It appears from these and many similar runs that, within the accuracy of the methods, identical values can be obtained.

The specificity of the rubber-cracking technique has recently been challenged by Crabtree and Biggs (*4*), who reported that identical cracking can be obtained with free radicals, even in the

Table I. Ozone Concentration in the Los Angeles Atmosphere by Iodine-Release and Rubber-Cracking Methods

Time (P.S.T.)[a]	Rubber-Cracking Time, Min.	Ozone Concn., P.P.H.M.	Recorder Ozone, P.P.H.M.
	6/10/52		
9:30 A.M.	14	8	8
10:30 A.M.	15	7.5	9
11:30 A.M.	9	12.5	13.5
11:50 A.M.	5	22	20
12:30 P.M.	7.5	15	14
1:30 P.M.	10.5	11	12.5
2:30 P.M.	10	11.5	11
3:30 P.M.	14.5	8	9
	6/12/52		
10:00 A.M.	10	11.5	12
11:00 A.M.	11	11	15
11:10 A.M.	6.5	18	17
12:15 P.M.	6	19	18
1:15 P.M.	7	16	14
2:30 P.M.	14	8	9

[a] Pacific standard time.

absence of oxygen. Thus, the above tests do not provide an unequivocal identification of ozone.

DIRECT SPECTROSCOPIC DETERMINATION OF OZONE

Ozone has an intense absorption spectrum in the ultraviolet, centered around 2550 A. It has long been determined in the atmosphere by its absorption in this region, usually by "shooting the sun"—that is, in a vertical column between the earth's surface and the sun.

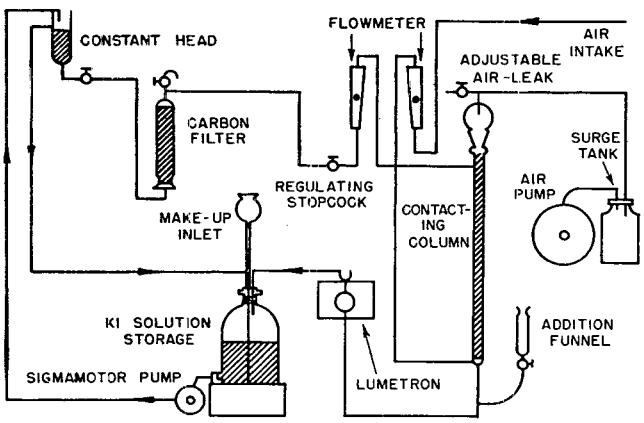

Figure 1. Schematic flow diagram of continuous oxidant recorder

A few measurements of ozone concentration near the earth's surface were carried out by the same method, notably by Regener (*9*) in New Mexico. The values thus obtained were generally below 5 p.p.h.m. Because of the much higher values reported in Los Angeles, Regener and his coworkers set up their instrument at Pasadena in July 1952. The equipment consisted of a hydrogen discharge lamp and a special spectrograph, using a light path of 1000 feet. In general, their results agreed within 10% with those obtained by the potassium iodide method. [Since this manuscript was prepared a similar method for the measurement of ozone concentrations has been described (*1*).]

At this laboratory a similar technique was employed, using an instrument developed by H. Kruger Associates (P. O. Box 164, San Gabriel, Calif.). This device, which is essentially a double-beam ultraviolet filter photometer, utilizes a light path of only 10 inches, yet has a full-scale sensitivity of less than 100 p.p.h.m of ozone. A comparison of the readings of this instrument with those of the potassium iodide recorder showed values ranging from 65 to 100%. The shapes of the curves were essentially identical.

While the good correspondence of the three methods—liberation of iodine, rubber cracking, and ultraviolet absorption—makes it highly probable that the oxidant is ozone, it does not constitute an absolute proof. Therefore, another approach was taken.

Table II. Ozone Decomposition on Silica Gel at Room Temperature

Run No.	Ignition Temp., ° C.	Average Oxidant Concn. as O_3, P.P.H.M. by Vol. Inlet	Exit	Undecomposed, %	Remarks
1	400	300	206	69	
2	500	313	218	70	
3	600	294	236	80	
4	700	294	229	78	
5	800	288	236	82	
6	800	306	282	92	
7	800	0	0	..	Gel ozonized 1 hr.
8	800	257	236	92	

SPECTROSCOPIC DETERMINATIONS AFTER CONCENTRATION ON SILICA GEL

Development of Method. Edgar and Paneth (*6*) described a technique which they have used in London for estimating the concentration of ozone in the ambient air. It consists of passing the air stream through an absorption train made up of a calcium chloride drying tower, followed by silica gel traps immersed in liquid oxygen. The purity of the silica gel appeared to be of considerable importance. The authors prepared their own, then ozonized it to destroy all oxidizable matter. The ozone was retained by the silica gel at −180° C., then desorbed at about −100° C., and quantitative estimation was achieved by ultraviolet spectroscopy, using a set of standards of known ozone concentrations.

An attempt was made to apply the silica gel method to the Los Angeles atmosphere. For the experiments, commercial

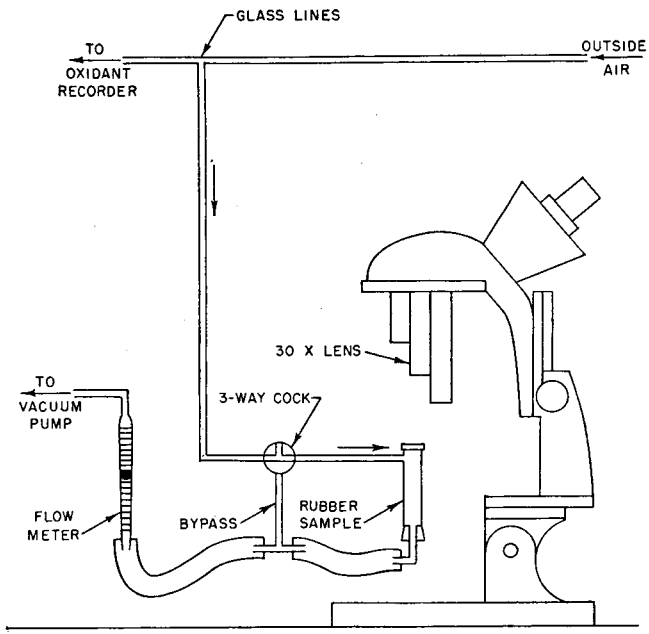

Figure 2. Experimental setup for rubber-cracking tests

silica gel was used (Davison Chemical Co., refrigeration grade, 14–20 mesh). The gel was first ignited, then treated by exposure to relatively high ozone concentrations; these measures were taken to remove oxidizable substances, which would tend to destroy ozone on contact. The effect of treating the gel by heating to various temperatures for 1 hour is shown in Table II. The tests were performed by passing ozonized oxygen at room temperature through a U-tube containing silica gel, or through a bypass line into a fritted-glass bubbler containing neutral 20% potassium iodide solution, and determining the amount of liberated iodine. The silica gel in runs 6 to 8 was ozonized after ignition. Run 7, a blank run with ozonized silica gel, shows that no residual ozone remained on the silica gel after this treatment.

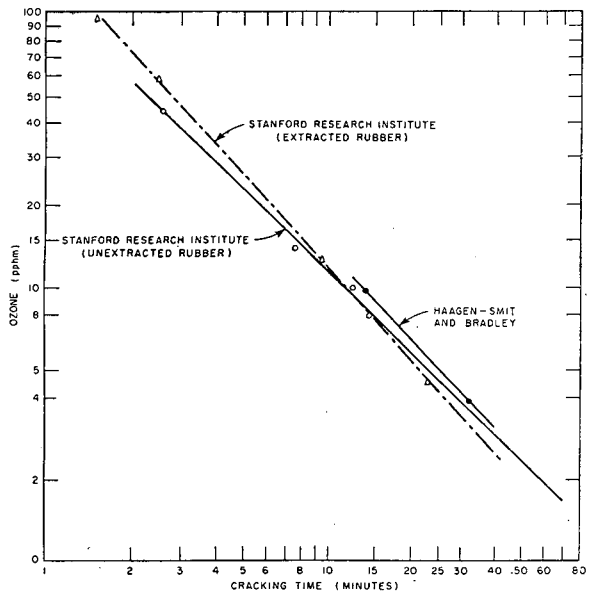

Figure 3. Comparison of calibration curves for rubber-cracking tests

Several runs were made to establish the absorption efficiency of silica gel at liquid oxygen temperatures, using gel ignited to 800° C. and treated with ozone. The amount of gel was varied from 100 to 25 ml. at throughput rates of 5 liters per minute. In no case did any ozone pass through the silica gel trap at ozone concentrations from 50 to 120 p.p.h.m. (by volume). A spray contactor, similar to the device used by Crabtree and Kemp (*5*), was used as an ozone-detecting device.

Because the interference of nitrogen dioxide with these tests was anticipated, the behavior of nitrogen dioxide in this system was explored. Prior to tests on the adsorption and desorption of nitrogen dioxide with silica gel, a test was made of the extent of the reaction of this gas with neutral buffered potassium iodide in the spray contactor. A 250-ml. glass bulb, equipped with a stopcock on one side and an extremely fine capillary leak on the other side, was evacuated and then filled with nitrogen dioxide to a pressure of 6 pounds per square inch. The magnitude of the leak was measured by a displacement of a slug of mercury in a 1-ml. graduated pipet. The discharge rate remained constant for a considerable period of time. This stream of nitrogen dioxide was then bled into a stream of oxygen (5 liters per minute), and the mixed gases were conducted to the spray contactor. On the basis of the measured discharge rate of the capillary leak, the concentration of nitrogen dioxide produced was 784 p.p.h.m., and the spray contactor titrations indicated that approximately 80% of the nitrogen dioxide reacted, assuming that 1 mole of nitrogen dioxide is equivalent to 1 mole of ozone for the reaction with potassium iodide.

A stream of oxygen containing 1140 p.p.h.m. of nitrogen dioxide was passed for 5 minutes through 100 cc. of Davison silica gel cooled in liquid oxygen. No trace of nitrogen dioxide was detected in the spray contactor to which the gases were conducted after coming in contact with the silica gel. Following removal of the liquid oxygen bath and the nitrogen dioxide source, the system was flushed into the spray contactor with cylinder oxygen. No desorption of nitrogen dioxide occurred up to a temperature of +20° C. and only a trace between +20° and +25° C. This was in complete accord with the results of Edgar and Paneth, who found that a boiling water bath was required for desorption of nitrogen dioxide from silica gel.

The silica gel from the test described above was used for the adsorption of ozone at liquid oxygen temperature from a stream of ozonized cylinder oxygen. Adsorption was carried on for 15 minutes in the manner described above. Five liters per minute of oxygen containing 131 p.p.h.m. of ozone were passed through the silica gel during the adsorption.

Subsequent removal of the liquid oxygen bath and flushing of the stream with cylinder oxygen resulted in the desorption of an amount of ozone equivalent to only 8 p.p.h.m. on the volume of gases used during adsorption. It is perhaps not unreasonable to expect that ozone and nitrogen dioxide would react at the high local concentrations on silica gel, although the experiments of Edgar and Paneth indicated that this reaction was not appreciable in extent.

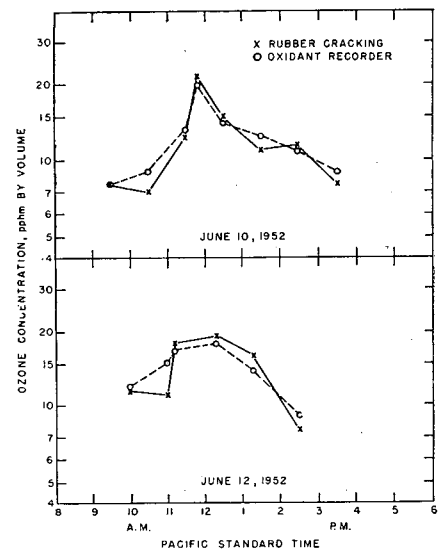

Figure 4. Comparison of atmospheric ozone concentrations at Pasadena

In the tests described above, adsorption of ozone was carried out for approximately 0.5 hour in each case. At the end of this period the liquid oxygen bath was removed and cylinder oxygen was passed through the silica gel to the spray contactor to flush out the desorbed ozone as the temperature of the silica gel rose. Previous work had shown that cylinder oxygen contained no detectable amount of oxidant.

Titration of the iodine liberated in the spray contactor during the desorption step in each case was then translated into an equivalent concentration of ozone in the gas passed through the silica gel during the adsorption. Comparison of these concentrations with those obtained for the gas bypassing the silica

Table III. Recovery of Adsorbed Ozone

Test No.	Silica Gel, Cc. (Approx.)	Ozonized Gas	Ozone, P.P.H.M.	Recovery on Desorption, %
1	100	Oxygen	58	95
2	100	Oxygen	51	73
3	100	Air	118	75
4	25	Air	65	50

Table IV. Effect of Freeze-Out Drying Traps on Oxidant Concentration

Trap Packing	Cooling Bath	Oxidant Concn. as O_3, P.P.H.M. by Vol. Inlet	Oxidant Concn. as O_3, P.P.H.M. by Vol. Exit	Exit Concn., % of Inlet
Glass Raschig rings	Liquid oxygen	16	9	56
Stainless steel helices	Acetone–dry ice	11	6	55
		11	7	64
		9	7	77
		11	10	91
	Liquid oxygen	12	9	75
$CaCl_2$	....	17	4	23

gel during the adsorption step served to establish the efficiency of recovery of ozone on desorption (Table III).

A pentane thermometer placed in the gas stream on the exit-gas side of the silica gel indicated that, at the flushing and heating rates employed, desorption began at about −90° C. and was essentially complete at −50° C. The above four tests are not believed to be extensive enough to allow much significance to be read into the differences in recovery efficiency obtained, beyond the fact that the percentage recovery appears to decrease at lower ozone concentrations and is slightly higher for ozonized oxygen than for ozonized smoggy air. Test 4, performed with only one fourth the quantity of silica gel used in the other tests, did not result in an improvement in recovery efficiency.

Thus, this method apparently did provide a means for concentrating ozone, at least from simple mixtures of ozone and oxygen. The next step was the application of this technique to the atmosphere.

Apparatus and Procedure. The adsorption train used for the initial experiments consisted of a series of traps. Air was drawn through a train consisting of a drying tube containing anhydrous calcium chloride, an unpacked tube immersed in liquid oxygen, a tube containing activated silica gel (also immersed in liquid oxygen), and finally into the spray contactor. At intervals during the adsorption cycle a bypass line was used to check the oxidant concentration in the incoming air.

Approximately 100 cc. of silica gel (Davison Chemical Co., 6–16 mesh, ignited to 800° C.) was used as adsorbent. Five liters of air per minute were drawn through the train during adsorption, which was carried out for a total of 45 minutes.

At the end of this period the Dewar flasks were removed, and cylinder oxygen was passed into the air inlet at a rate of 5 liters per minute to flush out the oxidant as it was desorbed. No detectable amount of oxidant was desorbed up to a temperature of 20° C., as indicated by the pentane thermometer on the exit side of the silica gel. The oxidant concentration measured during adsorption (by means of the bypass line) was equivalent to 25 p.p.h.m. by volume of ozone.

In attempting to locate the reason for this poor result, the elements of the adsorption train were isolated and tested separately. It was found that most of the loss occurred in the calcium chloride drying trap: Of 17 p.p.h.m. of oxidant, the calcium chloride trap passed only 4 p.p.h.m.

To improve the results obtained above, three types of freeze-out traps were tested. The first type of trap tested was packed with glass Raschig rings and immersed in liquid oxygen. When air containing 16 p.p.h.m. of oxidant (expressed as ozone) was drawn through this trap, about 56% of the oxidant passed through. No attempts were made to improve this performance by special cleaning or treatment of the packing.

The second type of freeze-out trap tested contained stainless steel helices and was immersed in an acetone–dry ice bath. From 55 to 91% of the oxidant initially in the air drawn through this trap passed through, at an initial oxidant level between 9 and 11 p.p.h.m.

In the third test of predrying by freeze-out, the same trap was utilized as for test 2, but with a liquid oxygen cooling bath in place of the acetone–dry ice bath. Results were substantially identical with those obtained with the acetone–dry ice bath, as 75% of the oxidant passed through the trap without decomposition. These tests showed, incidentally, that the oxidant must be a very low boiling gas, as it was not retained by condensation at liquid oxygen temperature.

The results of the above tests are summarized in Table IV. For comparison the data obtained with anhydrous calcium chloride are also included.

As a result of these tests, the adsorption train was modified. The calcium chloride trap was discarded; in its place a freeze-out trap containing stainless steel helices (and in later experiments, borosilicate glass helices) was used. This was followed by a Millipore filter in an aluminum holder (to retain the ice fog formed in the freeze-out traps), and a trap containing about 100 cc. of silica gel, immersed in liquid oxygen.

Preliminary Collections of Atmospheric Oxidant. Using the above freeze-out train, several preliminary runs were made to ascertain that the oxidant could be absorbed on silica gel at liquid oxygen temperatures and recovered on warming to dry ice temperatures.

In each of these runs the air was dried by means of the freeze-out train, which consisted of three traps packed with glass helices and cooled in liquid oxygen. The experimental procedure was the same in all cases; the weather conditions were different on each of these occasions, and it is likely that the observed differences in recovery efficiency must be somehow related to variations in atmospheric conditions.

Table V. Atmospheric Oxidant Collections in November 1951

Coll. No.	Date	Weather	Atmospheric Oxidant as O_3, P.P.H.M. by Vol. Total	Through freeze-out train	Desorbed from silica	Retained in freeze-out train
3	11/5/51	Severe smog	37	21	16.6	Not detd.
4	11/8/51	Slight smog	14	11	5.0	Not detd.
..	11/9/51	Slight smog	17	5	Not detd.	5
5	11/15/51	Clear	11	3.6	0.7	2.1

Following the fourth atmospheric collection, it was decided to check whether any oxidant was retained in the drying freeze-out train. For this test contaminated air was drawn through the train for 42 minutes, after which the liquid oxygen baths were removed and the silica gel trap was flushed into the spray contactor with cylinder oxygen. In addition, the traps were afterwards rinsed with neutral buffered potassium iodide solution, and the liberated iodine was determined by titration. The oxidant found by these two means amounted to 29% of the total atmospheric oxidant on November 9, 1951, the date of this test, and 20% on November 15, the date of the fifth atmospheric collection. In each case approximately half of the oxidant recovered from the traps was capable of being flushed out with oxygen as the traps were warmed. Data for these experiments are shown in Table V.

The material responsible for the liberation of iodine was not identified; it may have been present in the atmosphere, or it may have been formed in the trap by the action of the oxidant on oxidizable substances, thus accounting for the relatively low recoveries on desorption.

A similar run was made at a later date at Palmdale, in the desert about 30 air miles north of Pasadena. The recoveries of ozone upon desorption from the silica gel were much higher, ranging from 85 to 92% at total oxidant levels of 4 to 9 p.p.h.m. This may be due to much lower levels of pollution at Palmdale compared to Pasadena.

Stability of Adsorbed Ozone. Because it was important to

know whether ozone adsorbed on silica gel could be stored for extended periods without loss, successive adsorptions and desorptions were performed with the same batch of gel, with the desorption taking place immediately in one case, and being delayed until the following morning in the other. A duplicate run showed excellent agreement between recoveries on immediate and delayed desorption of the ozone from silica gel maintained at liquid oxygen temperature until the time of desorption. These data are tabulated in Table VI.

Table VI. Stability of Ozone Adsorbed on Silica Gel Maintained at Liquid Oxygen Temperature

Average Inlet Ozone, P.P.H.M. by Vol.	Recovery on Desorption, %	Remarks
276	89	Immediate desorption
241	90	Desorption after 15 hours. Good agreement with recovery on immediate desorption

Table VII. Calibration Data for Ozone Ultraviolet Absorption Spectra

Film No.	Film Position	Nominal Ozone Dilution	Exposure, Minutes	Titration, Ml. 0.001N $Na_2S_2O_3$	Ozone per Sq. Cm. Optical Path, γ
1616	4	Initial	2		
	9		20		
	14		2	4.40	41.4
1617	4	0.5	2		
	9		20		
	14		2	2.38	22.5
1618	4	0.25	2		
	9		20		
	14		2	1.30	12.2
1619	4	0.125	2		
	9		20		
	14		2	0.67	6.3
1620	4	0.0625	2		
	9		20		
	14		2	0.38	3.6
1621	4	Oxygen	2	..	..
	9		20		

Identification of Ozone by Its Ultraviolet Spectrum. After a method for the collection and concentration of a material which behaved like ozone had thus been established, the positive identification by means of its ultraviolet absorption spectrum was undertaken next.

EQUIPMENT AND PROCEDURE FOR CALIBRATION. The equipment consisted of a 1.5-meter ARL spectrograph which was modified to accommodate an optical tube 50 cm. long. A Beckman hydrogen discharge lamp was used as the source of ultraviolet light and was operated at the second highest filament temperature setting.

Spectra were obtained for five ozone dilutions and for pure oxygen. In order to define all portions of the spectra with adequate precision and to evaluate the extent of ozone decomposition during the photography, three exposures were made of each dilution. A 2-minute exposure was followed by a 20-minute exposure and then by another 2-minute exposure. The 2-minute exposures were intended to serve as a measure of decomposition during the time interval between, which averaged about 23 minutes. Maximum precision at the lower wave lengths required longer exposures, however, and for this purpose the 20-minute exposures were more suitable.

CALIBRATION RESULTS. Table VII lists the schedule of exposures, together with the results of the titration of the ozone for each dilution. Each titration was performed at the end of the corresponding series of exposures.

Evaluation of the films by means of the photoelectric densitometer involved several simplifying assumptions regarding the constancy of the film contrast at all wave lengths and the validity of the film calibration under conditions of light intensity and exposure time differing widely from those employed for its calibration. As a result, the absorption coefficients calculated from the data cannot be compared quantitatively with those published in the literature, since there are several sources of appreciable systematic errors. Because the absorption due to ozone is obtained as a difference between the total absorption and that due to oxygen alone, such systematic errors may not only result in a shift in the magnitude of the apparent ozone coefficients but may also cause a displacement in the apparent wave length of maximum absorption by ozone.

Table VIII. Absorption Data for Ozone-Oxygen Mixtures

	Transmittance Referred to Oxygen, %														
	Nominal Ozone Dilution														
	O_2			$1/2$ O_3			$1/4$ O_3			$1/8$ O_3			$1/16$ O_3		
Wave Length,	Exposure, Minutes														
A.	2	20	2	2	20	2	2	20	2	2	20	2	2	20	2
2150	..	86	..	..	94	..	..	88	..	..	99	..	..	96	..
2175	..	80	..	..	90	..	..	87	..	..	96	..	..	94	..
2200	..	75	..	..	86	..	..	86	..	..	95	..	..	94	..
2225	..	65	..	..	82	..	..	84	..	..	93	..	..	96	..
2250	..	60	..	..	79	..	..	85	..	..	92	..	..	99	..
2300	..	43	..	..	63	..	..	79	..	..	89	..	..	98	..
2325	..	36	..	..	54	..	..	70	..	..	85	..	..	96	..
2350	..	28	..	..	45	..	..	62	..	..	80	..	..	94	..
2375	..	27	..	..	41	..	..	61	..	..	82	..	..	100+	..
2400	..	22	..	..	33	..	..	54	..	..	75	..	..	95	..
2425	..	20	..	..	29	..	..	49	..	..	72	..	..	94	..
2450	..	16	..	..	24	..	..	43	..	..	66	..	..	89	..
2475	..	15	..	..	21	..	..	40	..	..	63	..	..	88	..
2500	50	13	51	54	19	62	56	37	73	69	62	74	89	86	100+
2525	46	12	48	49	17	59	51	34	66	67	61	70	88	88	100+
2550	42	11	43	44	16	55	48	35	60	64	61	67	86	90	98
2575	39	9.5	41	42	16	52	47	34	57	65	61	68	88	87	98
2600	34	8.5	36	36	15	47	44	35	51	63	65	63	84	87	92
2625	31	8.0	33	33	16	44	42	36	49	66	66	66	82	86	88
2650	28	7.5	32	32	18	41	44	38	49	67	71	67	82	88	88
2675	26	7.7	31	31	21	39	46	45	51	69	74	69	86	94	91
2700	25	8.0	30	31	26	38	50	49	53	70	74	70	89	98	91
2725	25	8.8	30	35	30	40	54	53	57	72	80	72	91	100+	94
2750	23	11	30	39	37	44	61	62	64	76	83	76	93	100+	96
2775	24	15	30	46	46	49	67	69	70	80	84	80	98	100+	98
2800	26	20	32	56	57	59	74	77	76	86	86	86	100+	100−	100+
2825	32	28	37	64	69	66	77	82	80	100+	91	100+	100	100+	100
2850	41	36	44	73	77	74	84	87	84	90	100	90	100+	100	100+
2875	50	50	50	80	100	80	88	100	88	90	100+	90	100+	100+	100+
2900	58	66	62	88	100+	88	92	100+	92	92	100+	92	100+	100+	100+
2925	68	87	68	92	100+	92	94	100+	94	96	100+	96	100+	100+	100+
2950	76	92	76	100+	100+	100+	100+	100+	100+	94	100+	94	100+	100+	100+
2975	84	100	84	100+	100+	100+	99	100+	99	94	100+	94	100+	100+	100+
3000	100+	100+	100+	100+	100+	100+	100+	100+	100+	94	100+	94	100+	100+	100+

Because of these considerations, the calculated absorption coefficients for ozone cannot be represented as being quite correct quantitatively. Nevertheless, if the same method of film evaluation is employed for the spectra of atmospheric oxidant, it should be possible to make use of the calibration data both for qualitative and quantitative confirmation of the presence of ozone, as the same systematic errors would be present in both cases.

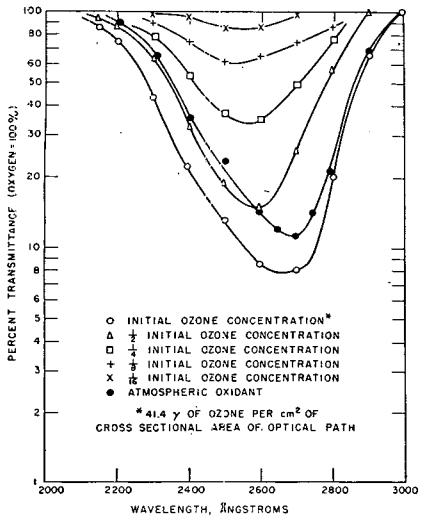

Figure 5. Ultraviolet absorption spectra of ozone and of atmospheric oxidant

Table VIII lists the calculated transmittance data, with the transmittance of oxygen taken as 100%. Figure 5 is a plot of transmittance *vs.* wave length, for the data calculated from the 20-minute exposures. The shift from curve to curve of the wave length at which the transmittance is at a minimum illustrates one of the effects of the systematic errors discussed above. The transmittance minimum appears to be near 2600 A. for all the 20-minute exposures; Figure 6 is a plot of the transmittance at 2600 A. (referred to oxygen as 100%) *vs.* the concentration of ozone. Figure 6 may be used for quantitative estimation of the ozone in atmospheric samples, provided that the absorption data for atmospheric oxidant are found to be qualitatively like those of Figure 5.

Collections for atmospheric oxidant were made during the peaks of the moderate smogs of April 2, 1952; 144 liters of air were sampled, and desorption in this case produced an amount of oxidant (by titration) comparable with results obtained on previous occasions during the development of the collection technique. Of the 22 p.p.h.m. present in the air during collection, an amount of oxidant equivalent to 6.9 p.p.h.m., or 31%, was desorbed from the silica gel and photographed. As previously indicated, the percentage recovery could be expected to be still higher at higher atmospheric oxidant levels.

Test Procedure. The optical setup used for the atmospheric sample was identical with that employed for the calibration spectra. Transfer of the oxidant to the optical tube was accomplished in several steps, because of the large amount of oxygen which is desorbed prior to and together with the oxidant.

To minimize the dilution of the oxidant with desorbed oxygen, the oxidant was first transferred from the silica gel on which it was collected to a much smaller tube containing about 5 grams of gel. From this tube desorption was allowed to take place into the evacuated optical tube in several portions, as sufficient gas was desorbed to fill the optical tube several times. Desorption was stopped for each portion when the pressure in the optical tube reached 1 atm. The last portion was brought to 1 atm. by sweeping cylinder oxygen through the silica gel into the optical tube.

The first portion of gas desorbed from the silica gel was discarded; past experience indicated that it would be essentially pure oxygen.

A 20-minute exposure was made of the next portion of gas, after which its oxidant content was determined by titration and found to be negligible. The third and final portion was found to contain the bulk of the oxidant. It also was exposed 20 minutes and titrated, after which an exposure was made of pure oxygen.

Table IX. Photography of Ultraviolet Absorption Spectra of Atmospheric Oxidant[a]

Film No.	Film Position	Sample Portion	Exposure, Minutes	Titration, Ml. 0.001*N* $Na_2S_2O_3$
1632	..	1	..	Discarded
	4	2	20	0.20
	9	3	20	3.95
	14	Oxygen	20	..

[a] Collected April 2, 1952.

Results. Table IX summarizes the schedule of exposures and titrations for the April 2 sample. The absorption data for this sample are listed in Table VIII; the spectrum is also shown in Figure 5. Although there is a slight shift of the position of the minima, this shift is considered to be within the experimental error for the method of film evaluation employed. Qualitatively, therefore, the ultraviolet absorption of this portion of the atmospheric oxidant and that of ozone are identical.

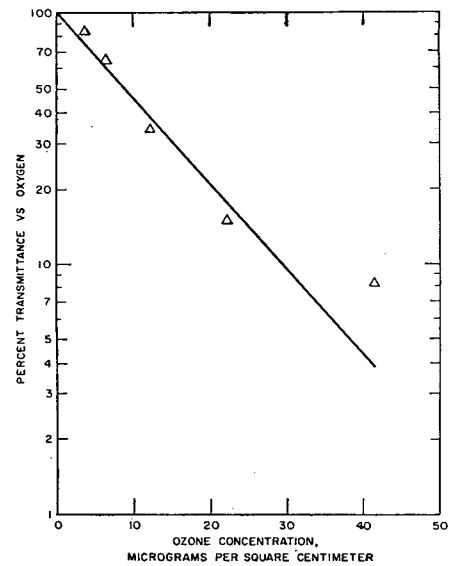

Figure 6. Ultraviolet absorption of ozone at 2600 A. Calculated from 20-minute exposures

Shortcomings inherent in the use of film are emphasized when attempting to use these spectra for quantitative estimation of the ozone concentration. At 2700 A. the ozone concentration in portion 3, estimated from the calibration data of Figure 1, agrees exactly with the chemical titration, while at wave lengths above 2700 A. the spectroscopic value appears to be too high, and at wave lengths below 2700 A. the spectroscopic value appears to be too low. Both the spectroscopic and the chemical deter-

minations for portion 2 are uncertain because of the small quantities of ozone involved.

Within the limits of experimental error, however, the spectroscopic data appear to confirm that all of the atmospheric oxidant desorbed from silica gel is ozone.

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LITERATURE CITED

(1) Air Pollution Foundation, Los Angeles, Calif., Rept. 12 (1955).
(2) Bartel, A. W., Temple, J. W., *Ind. Eng. Chem.* 44, 857 (1952).
(3) Bradley, C. E., Haagen-Smit, A. J., *Rubber Chem. and Technol.* 24, 750 (1951).
(4) Crabtree, J., Biggs, B. S., *J. Polymer Sci.* 11, 280 (1953).
(5) Crabtree, J., Kemp, A. R., *Ind. Eng. Chem.* 38, 278 (1946).
(6) Edgar, J. L., Paneth, F. A., *J. Chem. Soc. (London)* 1941, 511.
(7) Haagen-Smit, A. J., "Chemical Analysis of Air Pollutants," Air Pollutant Proc., U. S. Technical Conference on Air Pollution, p. 193, McGraw-Hill, New York, 1950.
(8) Littman, F. E., Benoliel, R. W., ANAL. CHEM. 25, 1480 (1953).
(9) Regener, V. C., "Atmosphere Ozone in the Los Angeles Region," Univ. New Mexico, Contract AF 19(122)-381, Sci. Rept. 3 (July 22, 1954).

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Polarographic Study of Alkyl Hydroperoxides

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The polarographic behavior of alkyl hydroperoxides was investigated systematically in order to define the most suitable conditions for their determination. Seventeen examples of these compounds varying from four to nine carbon atoms in chain length were investigated. Aqueous alcohol solutions of various compositions were used as solvents in this work. Several supporting electrolytes were investigated, and, of these, sulfuric acid was found to be the most satisfactory. The best polarographic waves were obtained with the compounds of higher molecular weight. These were steeper, better defined, and occurred at a less negative potential. The half-wave potentials for all of the hydroperoxides were so close together that the determination of any individual compound in a mixture was impossible. In general, diffusion currents were directly proportional to concentration. However, the proportionality constants for the various hydroperoxides varied widely, limiting the usefulness of the method for functional group analysis.

IN RECENT years there has been considerable interest in methods for the determination of the various types of organic peroxides. The polarographic procedure is among the several methods which have been suggested for this. However, only a relatively small number of examples of organic peroxides have been investigated because of the difficulty in preparation of pure samples of many of these compounds.

As a result of the recent work of Williams and Mosher (*10*), it is possible to prepare the alkyl hydroperoxides of lower molecular weight in a fairly pure state, and it seemed worth while to study the polarographic behavior of a number of these in order to establish conditions best suited for their determination.

Several references are found in the literature to the polarographic reduction of the first two members of the alkyl series—namely, methyl and ethyl hydroperoxide (*2, 6, 7, 9*). In addition Willits and others (*11*) have reported on the reduction of 3-butyl hydroperoxide as well as several high molecular weight compounds including cumene, menthane, pinene, and cyclohexane hydroperoxide. Their work was done in a nonaqueous solvent of methanol and benzene with lithium chloride as the supporting electrolyte. MacNevin and Urone (*5*), in a paper describing a method for the determination of hydrogen peroxide in the presence of hydroperoxides, show a polarogram for 3-pentyl hydroperoxide from a 0.1*N* solution of potassium chloride. Bruschweiler and Minkoff (*1*) have recently reported on the reduction of methyl, ethyl, and 3-butyl hydroperoxide from lithium sulfate and lithium hydroxide solutions.

This paper describes a systematic study of the reduction of a number of typical alkyl hydroperoxides varying from four to nine carbon atoms in chain length. Water and aqueous ethyl alcohol solutions were used as solvents in this work and several supporting electrolytes were investigated.

APPARATUS AND MATERIALS

All of the polarograms reported herein were recorded with a Sargent Model XXI Polarograph. An H-type cell similar to that described by Lingane and Laitinen (*4*) was used throughout this work. The cell was thermostated at 25° ± 0.1° C. The dropping mercury electrode was of the usual type and had a capillary constant, $m^{2/3}t^{1/6}$, of 2.644.

With the exception of 3-butyl hydroperoxide, which was obtained commercially, all of the hydroperoxides were prepared according to the method of Williams and Mosher (*10*), and some of the compounds studied were furnished by them. Table II lists the 17 alkyl hydroperoxides which were investigated. Based on iodometric titration data, the purity of all of the compounds listed is believed to be at least 95% and in most cases better than 99%.

Solutions. Sulfuric Acid, 0.5*F*.
Potassium Hydroxide, 0.5*F*.
Gelatin Solution, 0.1%.
Methyl Red, 0.4%.

BUFFER SOLUTIONS. Approximately 330 ml. of 0.2*F* acetic acid was mixed with 20 ml. of 0.2*F* sodium acetate; 35 ml. of this was used for each 100 ml. of solution prepared. This solution buffers at approximately pH 4.0. For a buffer at approximately pH 9.0, about 53 ml. of 0.1*F* potassium dihydrogen phosphate was mixed with 347 ml. of 0.05*F* borax; 40 ml. of this was used for each 100 ml. of solution prepared.

STOCK SOLUTIONS OF PEROXIDES. Approximately 0.1*F* stock solutions of the peroxides were prepared by diluting carefully weighed portions of the compounds to exactly 25 or 50 ml. with 95% ethyl alcohol. These solutions were stored in brown bottles sealed with aluminum foil and kept at −10° C. except when used. Such solutions were found to be stable for several months under these conditions.

STANDARD PEROXIDE SOLUTIONS. The stock peroxide solutions were allowed to come to room temperature and measured volumes were then transferred to 100-ml. volumetric flasks. The supporting electrolyte and sufficient 95% ethyl alcohol to give the desired concentration of this reagent were added and the solution was diluted to the mark. The alcohol concentrations referred to in this paper are volume percentages of the 95% ethyl alcohol—i.e., milliliters of 95% ethyl alcohol per 100 ml. of solution.

Table I. Effect of Supporting Electrolyte on Polarograms of Hydroperoxides

Hydroperoxide	Half-Wave Potential, Volt *vs.* S. C. E. H_2SO_4, 0.3*F*	Acetate buffer, pH 4	Phosphate buffer, pH 9	KOH, 0.1*N*
1 Butyl	−0.26	−0.21	−0.20	Drawn out wave between −0.3 and −1.3 volts
3-Butyl	−0.35	−0.27	−0.27	.....
1-Pentyl	−0.20	−0.19	−0.20	Drawn out wave
2-Pentyl	−0.20	−0.15	−0.14	.....

PROCEDURE

The solutions were transferred to the polarographic cell, brought to 25° C., and freed of oxygen by bubbling nitrogen through the cell for 10 to 20 minutes. The nitrogen was first passed through a tower containing a solution similar to that being studied to minimize composition changes due to evaporation.

The solutions were blanketed with nitrogen during the run to prevent absorption of oxygen. All of the polarograms were recorded at a rate of potential increase of 0.148 volt per minute.

In calculating half-wave potentials no correction for *iR* drop was made. The cell resistance throughout this work was 350 to 450 ohms, and generally such corrections would be smaller than the accuracy with which potential measurements were made. Furthermore, the uncertainties introduced by variations in the liquid junction potential with solvent composition were probably of the same order of magnitude as the variations in *iR* drop with composition.

EXPERIMENTAL RESULTS

Solvent. Of the compounds investigated only the butyl hydroperoxides were found to be sufficiently water-soluble for convenient study in this medium. Aqueous solutions of ethyl alcohol, on the other hand, readily dissolved all of the compounds under investigation, and mixtures containing from 5 to 60% ethyl alcohol by volume were used.

The concentration of ethyl alcohol was found to have a profound effect on the polarograms for the hydroperoxides, particularly the lower molecular weight members of the series. The effect is illustrated in Figures 1 and 2. In general, an increase in the alcohol concentration led to less well-defined, and more drawn out waves with smaller slopes. Half-wave potentials were more negative and diffusion currents smaller at higher alcohol concentrations. A similar variation in behavior for the reduction of *p*-nitroaniline in ethyl alcohol–water mixtures has been discussed by Shreve and Markham (*8*). The higher molecular weight peroxides gave satisfactory waves from solutions containing 40% or more alcohol. However, to obtain well-defined waves with the pentyl peroxides, the alcohol concentration had to be kept below 20%, and with the butyl peroxides below 10%. For analytical purposes, the alcohol concentration of the solvent should be kept as low as possible.

Supporting Electrolytes. Several types of supporting electrolytes were investigated. These included 0.1*N* potassium hydroxide, a phosphate buffer of pH approximately 9, an acetate buffer of pH approximately 4, and sulfuric acid varying in concentration from 0.1*F* to 0.5*F*. In general the most satisfactory waves were obtained from the sulfuric acid solutions, although the polarograms from the phosphate and acetate buffer solutions were nearly as satisfactory in most cases. With 0.1*N* potassium hydroxide, extremely drawn out and analytically unsatisfactory waves were obtained.

In Table I are listed data obtained for four of the hydroperoxides in the various supporting electrolytes. It will be noted that the half-wave potentials become slightly less negative with increasing pH; however, this effect is comparatively small. Variation in the supporting electrolyte had a negligible effect on the diffusion currents obtained.

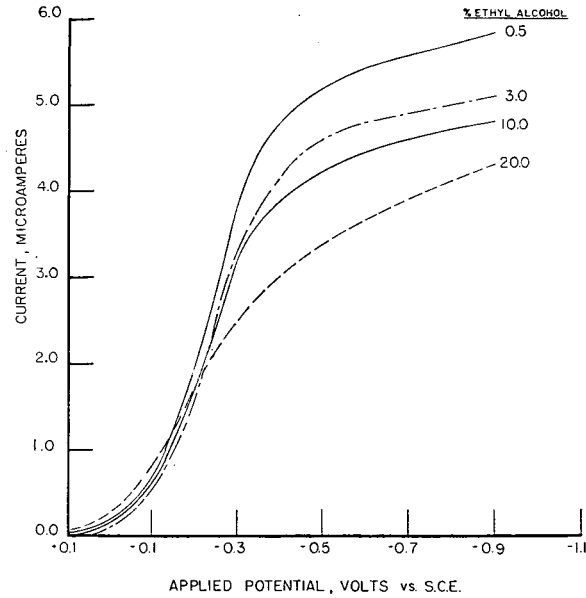

Figure 1. Effect of alcohol concentration on polarograms for 1-butyl hydroperoxide

Solutions 5 × $10^{-4}F$ in hydroperoxide and 0.5*F* in sulfuric acid

For purposes of analysis, sulfuric acid appeared to be well suited as a supporting electrolyte. Less trouble with maxima was encountered with this than with the other electrolytes, and satisfactory waves were obtained for all of the peroxides studied. It was found that the concentration of sulfuric acid could be varied from 0.1 to 0.5*F* without appreciably affecting the polarograms either as to half-wave potential or diffusion current, and most of the data reported in this paper are for solutions of sulfuric acid in this concentration range.

Effect of Molecular Weight and Chain Branching on Polarograms of Hydroperoxides. Table II gives half-wave potentials and diffusion current constants, i_d/C, for all of the hydroperoxides studied. The data obtained were for solutions 0.1*F* in sulfuric acid and, with the exception of the butyl compounds, 20% by volume in ethyl alcohol. The half-wave potentials became less negative with increasing molecular weight. Furthermore, there appears to be a slight negative shift in half-wave potentials in going from the normal isomers to the secondary and tertiary compounds. However, none of the half-wave potentials is sufficiently different to allow analysis of any particular hydroperoxide in a mixture of the compounds.

Table II. Half-Wave Potentials for Various Alkyl Hydroperoxides

Hydroperoxide	Alcohol, Vol. %	$E_{1/2}$, Volt *vs.* S.C.E.	i_d/C
1-Butyl	5	−0.26	8.4
2-Butyl	5	−0.28	8.2
3-Butyl	5	−0.34	8.2
1-Pentyl	20	−0.20	7.3
2-Pentyl	20	−0.24	7.0
3-Pentyl	20	−0.22	6.7
3-Methyl-1-butyl	20	−0.23	7.0
Cyclopentyl	20	−0.25	7.0
1-Hexyl	20	−0.12	6.9
2-Hexyl	20	−0.16	6.7
3-Hexyl	20	−0.16	6.6
Cyclohexyl	20	−0.14	6.7
1-Heptyl	20	−0.03	..
2-Heptyl	20	−0.12	6.3
1-Octyl	20	−0.02	6.0
2-Octyl	20	−0.80	6.0
1-Nonyl	20	−0.01	5.8

The diffusion current constants also decrease appreciably with increasing molecular weight. Probably, this decrease simply reflects the decrease in diffusion rate which would accompany the increase in the size of the molecules.

The shapes of the polarograms vary considerably in going from the lower molecular weight compounds to the higher (Figure 3). With the butyl and pentyl hydroperoxides, the waves are drawn out and rather poorly defined, whereas the octyl and nonyl compounds yield steep, sharp curves which are in every way more satisfactory. It is apparent that with the smaller molecules, the electrode process is a highly irreversible one. With the octyl and nonyl compounds, on the other hand, the slopes of the waves approach those for a two-electron reversible reduction. Plots of $\log i/(i_d - i)$ against potential for these were straight lines with slopes of 0.026 and 0.024, respectively, compared with a theoretical slope of 0.030 for a two-electron reduction. However, the independence of these waves of pH changes indicates that the electrode reaction is not reversible even with the highest molecular weight compounds studied.

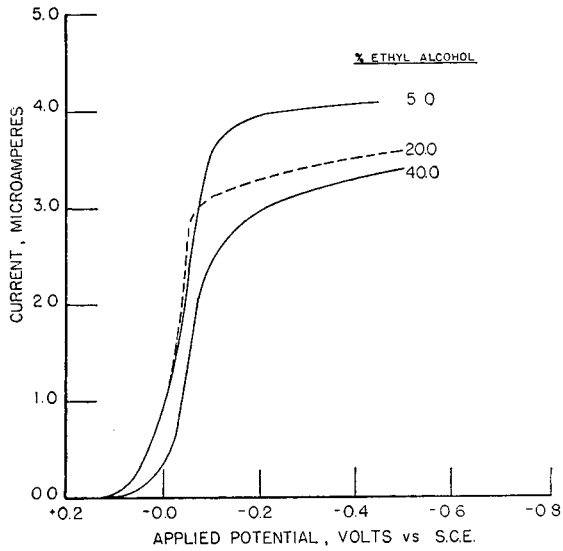

Figure 2. Effect of alcohol concentration on polarograms for 1-octyl hydroperoxide

Solutions 5 × $10^{-4}F$ in hydroperoxide and 0.5F in sulfuric acid

Current Maxima. Current maxima were encountered from time to time in this work. In general, these appeared more frequently when working at lower alcohol concentrations and particularly with the higher molecular weight peroxides. The alcohol appeared to act as a suppressor and maxima were seldom found when alcohol concentrations of 20% or greater were used. Maxima occurred more frequently and were larger in the acetate buffer than in either the sulfuric acid or the phosphate buffer solutions. Generally, the maxima could be suppressed readily by addition of a few drops of 0.1% gelatin solution or 0.4% methyl red to the peroxide solution. Addition of these had little effect on the diffusion current for the peroxides.

Effect of Concentration on Polarograms. Several of the hydroperoxides were investigated extensively for the effect of concentration on the polarographic waves. In every case, the half-wave potential was independent of concentration. Moreover, the diffusion currents were found to increase linearly with concentration. This is illustrated in Table III for three of the hydroperoxides. The markedly smaller diffusion current constant, i_d/C, shown for the 1-octyl hydroperoxide, results in part from the high alcohol concentration used with this compound. This effect was mentioned earlier.

From the data in Table III and other similar data, it was concluded that it is possible to determine any one of the hydroperoxides studied with an accuracy of 1 to 2% by the polarographic method. However, the rather large variation in the diffusion current constants (i_d/C) among the various hydroperoxides seriously limits the accuracy of the method for a functional group analysis, at least for hydroperoxides in the range of 4 to 9 carbon atoms. Thus, in Table II it will be seen that a 20% decrease in i_d/C is found in going from the 1-pentyl (n-amyl) to the 1-nonyl compound.

Table III. Effect of Concentration on Polarograms

Hydroperoxide	Solvent and Supporting Electrolyte	Hydroperoxide Concn., C, Mmoles per Liter	Diffusion Current, i_d, μa.	i_d/C	$E_{1/2}$, Volt vs. S.C.E.
3-Butyl	5% ethyl alcohol, 0.3F H_2SO_4	0.30	2.5	8.3	−0.30
		0.50	4.2	8.4	−0.30
		1.00	8.1	8.1	−0.32
		2.00	16.2	8.1	−0.31
		3.00	24.5	8.2	−0.32
1-Pentyl	5% ethyl alcohol, 0.3F H_2SO_4	0.30	2.5	8.3	−0.18
		0.50	4.2	8.4	−0.18
		1.00	8.5	8.5	−0.18
		2.00	16.5	8.3	−0.18
		3.00	24.5	8.2	−0.19
1-Octyl	40% ethyl alcohol, 0.3F H_2SO_4	0.30	1.7	5.6	−0.06
		0.50	2.7	5.4	−0.07
		1.00	5.5	5.5	−0.06
		2.00	11.2	5.6	−0.05

Stability of Solutions. The stock solutions of the hydroperoxides in 95% ethyl alcohol were found to be stable for several months at −10° C. The more dilute aqueous solutions containing the peroxides and supporting electrolyte were stable for at least a week when refrigerated, but at room temperature and in contact with mercury, slow decomposition of these solutions took place. The rate of decomposition, however, was slow enough to cause no serious difficulties in obtaining reproducible polarograms. For example, with a 0.001F solution of 2-butyl hydroperoxide in sulfuric acid, a decrease in current from 7.95 to 7.6 μa. was observed in 1 hour of standing in contact with mercury.

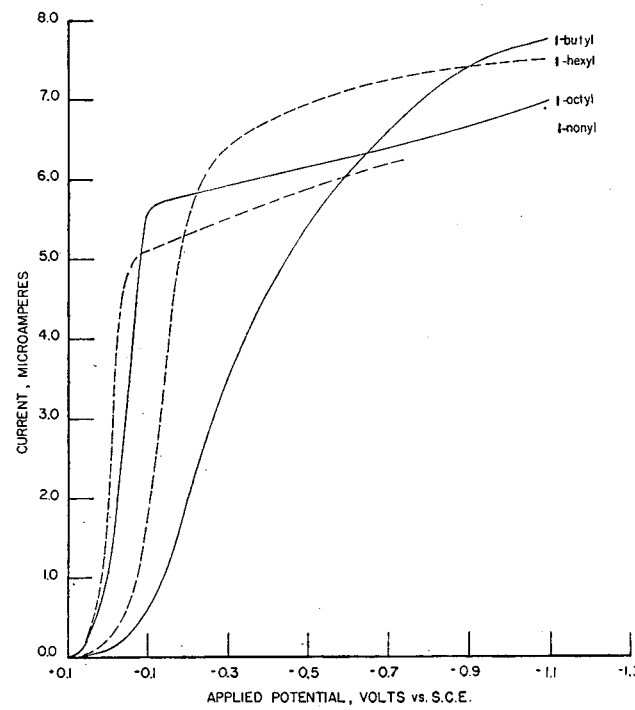

Figure 3. Polarograms for normal hydroperoxides

Solutions approximately 1 × $10^{-3}F$ in hydroperoxides, 0.1F in sulfuric acid, and 20% ethyl alcohol

Electrode Reaction. It seems probable that the electrode reaction involves reduction of the peroxides to the corresponding alcohols as follows:

$$ROOH + 2H^+ + 2e \rightarrow ROH + H_2O$$

A calculation of n, the number of electrons involved in the reduction of 1-butyl hydroperoxide from aqueous solutions was made by means of the Ilkovič equation. This yielded a value of approximately 2. In this calculation a value of 0.77×10^{-5} cm.^{2} sec.$^{-1}$ for the diffusion coefficient of the peroxide was used. This is the value found in the literature for n-butyl alcohol (*3*) and it seems probable that the diffusion coefficient for the corresponding peroxide should be rather close to this. With this approximation n was found to be equal to 1.9.

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LITERATURE CITED

(1) Bruschweiler, H., Minkoff, G. J., *Anal. Chim. Acta* **12**, 186 (1955).
(2) Dobrimskaya, A. A., Nieman, M. B., *Acta Physicochim. U.R.S.S.* **10**, 297 (1939).
(3) "International Critical Tables," vol. 5, p. 70, McGraw-Hill, New York, 1929.
(4) Lingane, J. J., Laitinen, H. A., IND. ENG. CHEM., ANAL. ED. **11**, 504 (1939).
(5) MacNevin, W. M., Urone, P. F., ANAL. CHEM. **25**, 1760 (1953).
(6) Nieman, M. B., Gerber, M. I., *Zhur. Anal. Khim.* **1**, 211 (1946).
(7) Roberts, E. R., Meek, J. S., *Analyst* **77**, 43 (1952).
(8) Shreve, O. D., Markham, E. C., *J. Am. Chem. Soc.* **71**, 2993 (1949).
(9) Shtern, V., Pollyak, S., *Acta Physicochim. U.R.S.S.* **11**, 797 (1939); *J. Gen. Chem., U.S.S.R.* **10**, 21 (1940).
(10) Williams, H. R., Mosher, H. S., *J. Am. Chem. Soc.* **75**, 2984, 2987, 3495 (1954).
(11) Willits, C., Ricciuti, C., Knight, H. B., Swern, D., ANAL. CHEM. **24**, 785 (1952).

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Voltammetry at Solid Electrodes

Anodic Polarography of the Phenylenediamines

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The anodic polarography of the phenylenediamines at a rotating platinum electrode has been examined using a current-scanning technique. Measurements were made of the variation of $E_{1/2}$ with pH, and from these data the pK_a values of reduced and oxidized species were determined. In all cases examined, linear limiting current *vs.* concentration behavior was observed. The quantitative determination of *o*- and *p*-phenylenediamine can be accomplished with a reproducibility of 2 to 3% in the concentration range from 10^{-4} to $10^{-5}M$, but analysis of mixtures of the two cannot be done except in favorable cases. This study was undertaken to illustrate a potentially valuable adjunct of solid electrode polarography—the measurement of formal potentials of labile organic redox systems which cannot be determined by classical potentiometric techniques.

THE solid electrode polarography of a variety of organic compounds has been investigated to date. The majority of these studies have concentrated on quantitative analysis by means of limiting current measurements. The important variation of $E_{1/2}$ with pH that accompanies organic polarographic reactions has received less emphasis. Hedenburg and Freiser examined the mechanism and the dependence of $E_{1/2}$ *vs.* pH for the oxidation of phenol at a stationary platinum electrode (*9*). A comprehensive study of the oxidation of phenolic compounds at graphite electrodes by Gaylor, Elving, and Conrad includes some data on the behavior of $E_{1/2}$ *vs.* pH for phenol, hydroquinone, and 2,4-dimethyl-6-*tert*-butylphenol (*8*).

The work reported herein is mainly concerned with the evaluation of dissociation constants of the electroactive species from $E_{1/2}$ *vs.* pH curves taken at the rotating platinum electrode. Although the dropping mercury electrode has been widely used for this purpose, similar studies with solid electrodes have apparently not been made. All of the present work was done with the rotating platinum electrode using the current-scanning technique previously described (*1*, *6*). Similar results can be secured using conventional voltage-scan polarography, but it is believed that more reproducible results were obtained with less effort using the new technique. *o*- and *p*-phenylenediamine were chosen for study because the diamine-diimine couple represents a highly unstable organic redox system.

Comparatively little work has been reported on the anodic oxidation of phenylenediamines at solid electrodes. In a comparison of gold, graphite, and platinum electrodes, Lord and Rogers briefly investigated the oxidation waves of *o*-, *m*-, and *p*-phenylenediamine (*11*). More attention has recently been given the substituted *p*-phenylenediamines which are useful as antioxidants and photographic developers. A summary of half-wave potentials of some 50 *p*-amino-*N*-dialkylanilines obtained by automatic recording at a stationary platinum electrode has been given (*2*, *10*). Gaylor, Conrad, and Landerl recently described a wax-impregnated graphite electrode for the determination of antioxidants of the *p*-phenylenediamine class (*7*).

EXPERIMENTAL

Reagents. Samples of *o*- and *p*-phenylenediamine were Eastman White Label, used without further purification because it was desired to work with practical samples. Stock solutions ($0.01M$) for polarographic work were prepared by dissolving the required weight of compound in the minimal quantity of either $1M$ hydrochloric or $1M$ acetic acid (for buffer studies) and diluting to volume with air-free distilled water. These solutions were prepared fresh each day to minimize the effect of air oxidation.

Britton and Robinson buffer solutions were used for the pH studies and were prepared according to the directions given by Müller (*15*). The stock buffer solution prepared was $0.04M$ in phosphoric, boric, and acetic acids, respectively. Varying volumes of this solution were mixed with $0.2M$ sodium hydroxide to prepare the desired buffers. The exact pH was measured at the end of each polarographic run using a Leeds & Northrup pH meter, which was standardized prior to each of these pH deter-

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minations by checking against a saturated potassium hydrogen tartrate solution (pH 3.56). Citrate-phosphate buffers were examined but found to be unsatisfactory because the limiting current plateaus of the phenylenediamines were poorly defined in this medium.

Stock solutions of hydrochloric, sulfuric, and perchloric acids were prepared by proper dilution of the concentrated C.P. acids.

Electrodes. An 18-gage platinum wire, sealed in soft glass and extending about 2 cm. vertically downward, was used as the rotating electrode. A Sargent synchronous rotator (600 r.p.m.) was used for all the work. Two other electrodes completed the assembly. One, a lead amalgam–lead sulfate half-cell, functioned as the shielded cathode (*1*); the other, a saturated calomel electrode, was used only as the reference electrode. In all cases, the platinum electrode cleaning procedure consisted merely of rotation for 2 to 5 minutes in 15*M* nitric acid after each polarographic run, followed by thorough rinsing with distilled water. The electrode was stored for overnight periods in distilled water and cleaned with nitric acid before the first run.

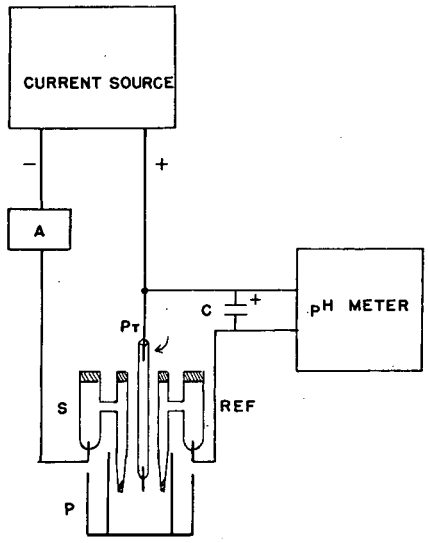

Figure 1. Apparatus for current-scanning polarography

A. Microammeter, Tripplett Model 726, 0 to 50 μa.
C. 20-μf. electrolytic capacitor
P. Polarographic cell
Pt. Rotating platinum electrode
Ref. Saturated calomel electrode half-cell
S. Shielded cathode, Pb, Hg–$PbSO_4$–1*M* H_2SO_4

Polarographic Cell. A 50-ml. open beaker, centered in a 4-inch crystallizing dish, served as the polarographic vessel. Temperature control was achieved by keeping the outer dish filled with water at 25° C. Provision was made to position the electrode and cell in a reproducible fashion for each polarographic run. Figure 1 indicates the complete apparatus.

Current Source. A simple, one-tube current-scanning circuit was used. Such a device is far more convenient than the battery and variable resistance combinations previously described (*1*). A single adjustment gives the required current sweep. The circuit is shown in Figure 2; it is recognized as a modified difference amplifier. The power supply consists of five 45-volt B batteries. The current drain is low enough to enable a single set of batteries to be used for over a year. The 6SL7 heaters are supplied from a small filament transformer. A current output from 0 to about 250 μa. can be supplied to the polarographic cell. The 5-K resistor in the plate circuit serves as a zero adjust and the current scanning is accomplished by means of the 50-K potentiometer which provides grid signal to the left half of the 6SL7. In actual practice, the zero current reading was taken with one electrode lead disconnected, because the inexpensive microammeter used did not indicate true readings close to zero. The cell leads indicated in Figure 2 are connected to the rotating platinum electrode and the shielded cathode ($Pb/PbSO_4$).

Voltage Measurement. A Leeds & Northrup Model 7664 pH meter was used to provide continuous indication of the polarographic voltage. The pH meter leads are attached to the rotating platinum and the saturated calomel electrode. Because the polarographic current does not pass through the saturated calomel electrode, current-potential curves are obtained directly and do not have to be corrected for the usual *iR* drop. A 20-μf. electrolytic capacitor may be connected across the pH meter leads to decrease the voltage fluctuations in the vicinity of the limiting current plateau.

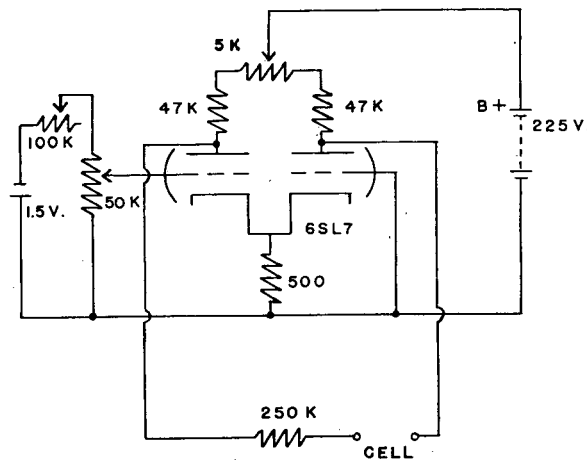

Figure 2. Variable current circuit

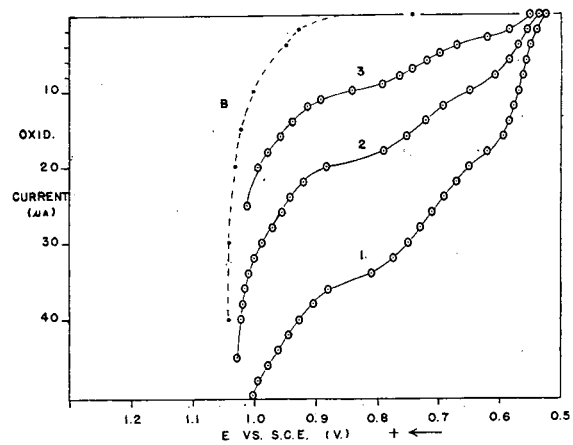

Figure 3. Anodic waves of *p*-phenylenediamine in 1*M* hydrochloric acid

1. $2 \times 10^{-4}M$
2. $1 \times 10^{-4}M$
3. $5 \times 10^{-5}M$

To measure the voltage, the zero current polarographic voltage —i.e., solution redox potential—was recorded, then the current supply leads were connected and the voltage was observed in increasing current increments, usually 1- or 2-μa. steps. The data were plotted directly on standard graph paper. The limiting currents and half-wave potentials were taken from these plots using conventional polarographic practice. In general, the polarographic voltage indicated by the pH meter is steady immediately after each current increase. Only in the vicinity of the limiting current plateau is there any voltage lag (for oxidations, the voltage drifts toward more positive values). In this case it is best to allow 30 to 60 seconds for the equilibrium voltage to be attained. This sluggishness varies with the polarographic system, buffer conditions, and the like. However, the lag is not serious enough to interfere in any way with the reproducibility of results. In practice, the rate of stepwise current increase may be as fast as the operator can read and record the values, except as noted above.

RESULTS

Anodic Waves in Acid Solution. Figure 3 illustrates the oxidation waves obtained for varying concentrations of *p*-phenylene-

Table I. Half-wave Potentials of *p*-Phenylenediamine in 1*M* Hydrochloric Acid

Concentration, *M*	$E_{1/2}$ (volt *vs.* S.C.E.) 1st wave	2nd wave
2×10^{-4}	0.57	0.72
1×10^{-4}	0.58	0.73
5×10^{-5}	0.58	0.74

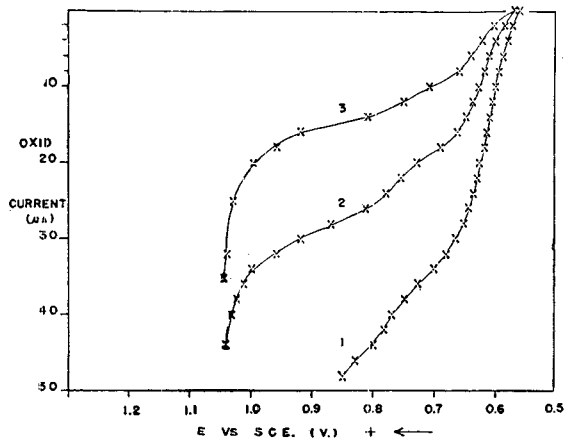

Figure 4. Anodic waves of *o*-phenylenediamine in 1*M* hydrochloric acid

1. $2 \times 10^{-4}M$ 2. $1 \times 10^{-4}M$ 3. $5 \times 10^{-5}M$

diamine in 1*M* hydrochloric acid. The background oxidation wave ($2Cl^- \rightarrow Cl_2 + 2e$) is indicated as wave *B*. The para isomer gives two waves of about equal height, although the separation is not complete. Table I indicates that the values of $E_{1/2}$ for these waves are independent of concentration within the estimated reproducibility of ±0.01 volt. Based on data discussed later in connection with the buffer studies, these split waves can be correlated with single-electron oxidation stages—i.e., semiquinone formation. The relative limiting currents for the individual waves are about one half of that obtained for the two-electron process in buffer media. Such an interpretation is in accord with the stability of the semiquinone of *p*-phenylenediamine in acid solution found by Michaelis, Schubert, and Granick (*14*). Further support of this view is found in the color of the solutions after the polarographic runs. These solutions were pale yellow, stable for several days in some cases. Indeed, oxidized solutions up to about pH 4 showed the same yellow color, while above this pH, the color after the polarographic run was pink. According to Michaelis, Schubert, and Granick, the color of the semiquinone is yellow, while the fully oxidized diimine is pink (*14*). Although only a small fraction of the sample is oxidized during a polarographic run, the intense absorption of these organic free radicals probably accounts for the visible color. No quantitative data were gathered on this point, but the existence of the yellow color only within the pH range of maximum semiquinone stability complements the polarographic evidence for the one-electron oxidation wave. A factor which contributes to the stability of the semiquinone is the presence of a large excess of the unoxidized diamine (*14*). Such conditions are inherent in a polarographic reaction where only a small fraction of the bulk concentration is reacted.

Figure 4 indicates that the ortho isomer is similar to the para in 1*M* hydrochloric acid, but the split into two waves is far less pronounced in this case. The color of the oxidized solutions indicates a change in the reaction at about the same pH as for the para isomer. While much might be speculated on the poorer definition of the split waves for ortho, no real conclusions can be reached from the data of Figure 4.

Essentially identical waves for *o*- and *p*-phenylenediamine were obtained in 1*M* sulfuric and perchloric acids. However, the split waves are best defined in the hydrochloric acid medium. Reproducible results have been obtained over a long period using several different platinum electrodes.

Considerable work was also done on the meta isomer. In 1*M* hydrochloric acid two waves are usually obtained. The $E_{1/2}$ of the first wave is always at about +0.95 volt *vs.* S.C.E., and appears to shift only slightly with increasing pH. However, much difficulty was experienced in reproducing the second wave. The reasons for this behavior are not known. No further data for the meta isomer are reported here.

Anodic Waves of *o*- and *p*-Phenylenediamine in Buffer Media. Typical oxidation waves for the ortho and para isomers in Britton and Robinson buffers are shown in Figure 5. In general, the limiting current plateau for the para wave was better defined (flatter) than the ortho. Polarograms were taken over the pH range from 1.7 to 10 for each isomer. Figure 6 shows the variation of $E_{1/2}$ with pH compiled from the polarographic data. The slopes of the individual portions of the curves are given.

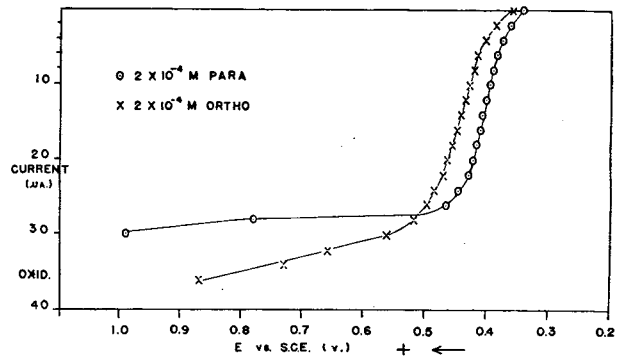

Figure 5. Typical oxidization waves in Britton-Robinson buffers

The shape of the curves for $E_{1/2}$ *vs.* pH can be interpreted in terms of the equations developed by Clark and others in their classical potentiometric studies of organic oxidation-reduction systems (*3*). These equations, which involve an expression for the sum of all the oxidant and reductant species in terms of hydrogen ion concentration, can be applied to polarographic practice with slight modifications (*16*). The following information can be derived from such a curve.

Each inflection represents an acidic dissociation constant (pK_a') of either an oxidized or reduced electroactive species. An inflection followed by a steepening of the curve is due to the dissociation of an oxidized form. Similarly, if the curve is flattened with increasing pH, it is due to the dissociation of a reductant. For a two-electron oxidation process, each dissociation constant alters the slope by a factor of 0.03 volt per pH unit. If the pK of an oxidant form is equivalent or very close to that of the reductant, then no change or only a very slight bend is seen at this point (*3*, *13*). Only those dissociation constants which fall within the experimental pH range and are modified by the oxidation-reduction process are detected. A complete interpretation of the curve usually involves correlation with the structural properties of the molecule and some pK data obtained from independent methods.

To evaluate the curves of Figure 6, it is first necessary to establish the number of electrons involved in the oxidation process at the rotating platinum electrode. The slopes of the individual ortho and para waves (log *i* *vs.* *E* plots) indicate a two-electron process. However, the irreversibility of the phenylenediamine oxidation leaves much to be desired in such a calculation. A further check, which is felt to be more reliable in this case,

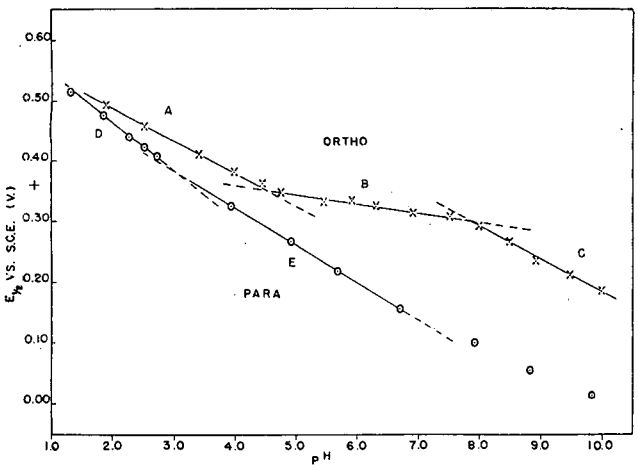

Figure 6. Curves of $E^1/_2$ *vs.* pH for *o*- and *p*-phenylenediamine

Slopes of individual portions of curves:

A.	0.056	*D.*	0.081
B.	0.017	*E.*	0.060
C.	0.054		

is indicated by the data of Figure 7. Here the oxidation waves for equimolar concentrations of the ortho and para isomers are compared with an equivalent concentration of hydroquinone, all in the same buffer medium. The oxidation of hydroquinone at the platinum electrode is a well-established two-electron process. The limiting currents for all three compounds are equal within 3 to 4%. These particular data were taken at pH 6.8; checks at other pH values gave similar agreement. There appears to be little doubt that the oxidation of *o*- and *p*-phenylenediamine at the rotating platinum electrode is a two-electron process in buffer media, corresponding to the diamine-diimine redox system. The two-stage univalent process (semiquinone formation) occurs only in a fairly acidic solution, as discussed earlier.

Returning to Figure 6, the interpretation of the curve of $E_{1/2}$ *vs.* pH for the ortho isomer can be given as follows. The first break occurs at a pK_a' of about 4.5 and is due to the dissociation constant of a reductant form. This value checks well with the recent values of 4.6 and 4.74 for the pK_{a2}' of *o*-phenylenediamine given by Vanderbelt, Henrich, and Vandern Berg, using electrometric titration and ultraviolet absorption methods, respectively (*17*). The pK_{a1}' of the ortho is about 0.6 (*17*), and is outside the range of detection in the present method. The next break in the curve appears at a pK_a' of 7.8 and is due to the dissociation constant of the oxidant (diimine). No other dissociation constants are discernible over the pH range from 1 to 10. The slopes of the end portions of the curve (0.056 and 0.054 volt per pH unit) are as close to the theoretical value of 0.059 as can be expected. The middle portion should have a slope of 0.030 volt, whereas the experimental value is only 0.017. This is understandable from an examination of the individual oxidation waves in this pH region. Starting at pH 6, the waves for the ortho isomer become more drawn out; this probably gives rise to a positive error in the $E_{1/2}$ value. Hence, the slope of the curve of $E_{1/2}$ *vs.* pH in this region is less than the theoretical value. Oddly enough, beyond pH 8, the waves tend to become more reversible in shape (less drawn out). It appears that this particular pH range represents an unstable region for one or more of the electroactive species. The over-all curve for $E_{1/2}$ *vs.* pH is represented by the general equation:

$$E_{Pt} = E'_{1/2} + 0.03 \log \frac{(K_{r_1}K_{r_2} + K_{r_1}[H^+] + [H^+]^2)}{K_O + [H^+]} - 0.06 \text{ pH}$$

where

K_{r_1} = first acidic dissociation constant of reductant (diamine)

K_{r_2} = second acidic dissociation constant of reductant (diamine)

K_0 = acidic dissociation constant of oxidant (diimine)

In the curve for the para isomer (Figure 6), the first break indicates a pK_a' for the reductant of 3.0. This corresponds to the first dissociation exponent of *p*-phenylenediamine given as 2.7 and 2.8 (*17*). No clean break is observed for the second dissociation of the reductant, where $pK_a' = 6.2$ (*17*), and therefore the effect must be counterbalanced by a dissociation of the oxidant. Thus, a pK_a' value of 6 can be assigned as the second dissociation exponent of the oxidant. The first dissociation of the oxidant is indicated by the 0.081 slope in the pH range from 1 to 3. Hence, an estimated value for this first oxidant dissociation exponent is < 1. This latter value could be more precisely established by extending the curve to lower pH values, when the 0.081 slope should shift back to 0.06. However, the split waves noted in the earlier section interfere with half-wave potential measurement in this region.

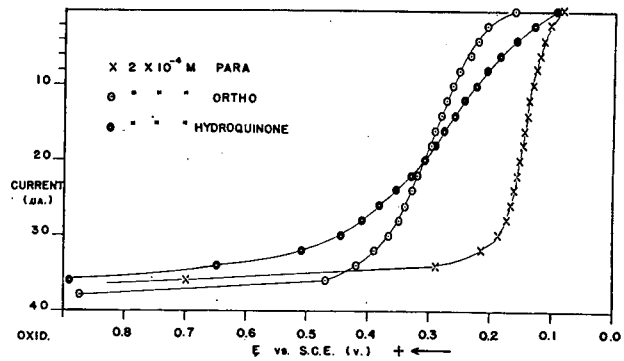

Figure 7. Anodic waves of *o*- and *p*-phenylenediamine and hydroquinone

The general shape of the curve for the para isomer checks well with potentiometric data of Michaelis and Hill on a variety of alkyl-substituted *p*-phenylenediamines whose pK values (reductant) correspond closely with the unsubstituted compound (*13*). The generalized expression for the curve of $E_{1/2}$ *vs.* pH or the para isomer is:

$$E_{Pt} = E'_{1/2} + 0.03 \log \frac{(K_{r_1}K_{r_2} + K_{r_1}[H^+] + [H^+]^2)}{(K_{01}K_{02} + K_{01}[H^+] + [H^+]^2)} - 0.06 \text{ pH}$$

An equivalent expression for potentiometric studies was first developed by Clark and others (*4*).

Regarding the reliability of the pK data obtained by the present method, it can be seen that the values for the reductants compare well with the independent measurements. The instability of the unsubstituted diimines makes it impossible to obtain corresponding independent data for the oxidants. Attempts to apply the classical potentiometric methods to the unsubstituted phenylenediamines were unsuccessful because of this instability (*4, 12*). In such a situation the polarographic method is of decided advantage. The value for the pK_0 of the ortho isomer may be considered quite reliable. The values for the oxidant species of *p*-phenylenediamine was approximated as indicated. The entire curve of $E_{1/2}$ *vs.* pH for each compound was rechecked three times over a 2-year period using at least five different platinum electrodes. Consistent values for the apparent dissociation constants (within 0.5 pK unit) were obtained. Constant ionic strength was not maintained in the buffer solutions used. Elving and others have shown that ionic strength differences may considerably influence half-wave potential measurements (*5*). Such an effect might very well account for the scatter of points at the high pH region in the curve for the para isomer (Figure 6).

An indication of the degree of correlation between the present

method and corresponding potentiometric studies is afforded by the data of Figure 8, which is a curve of $E_{1/2}$ *vs.* pH for *o*-tolidine. The potentiometric data were taken from the study of Clark, Cohen, and others (*4*), and recalculated *vs.* S.C.E. The more positive potentials in the low pH range can be attributed to the irreversibility of the *o*-tolidine oxidation wave. In this case, the polarographic method cannot be extended beyond a pH of about 6, because the waves are too drawn out for accurate $E_1/_2$ measurement. Even in this unfavorable case, the slope changes and hence the pK values check well with the potentiometric data.

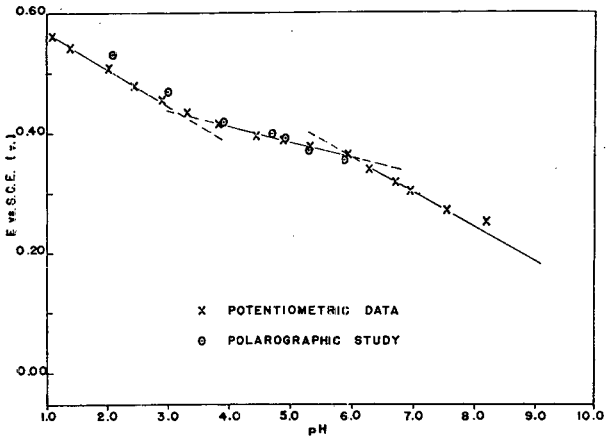

Figure 8. Potentiometric and polarographic data for *o*-tolidine

Quantitative Determination of Phenylenediamines at Rotated Platinum Electrode. Table II illustrates the linear relation between limiting current and concentration for *p*-phenylenediamine. The average reproducibility of these five measurements is 3.6%; however, 2 to 3% appears to be a more reliable estimate based on a larger number of determinations. Similar relations for both the ortho and para isomers can be obtained over the entire buffer range and in acidic solution. No concentration study of the meta compound was undertaken. In general, best results are obtained with the para isomer because the limiting current plateau is flatter than that of the ortho. Limiting currents for ortho and para were found to be independent of pH within ±5% (exclusive of the acidic region where two waves were obtained).

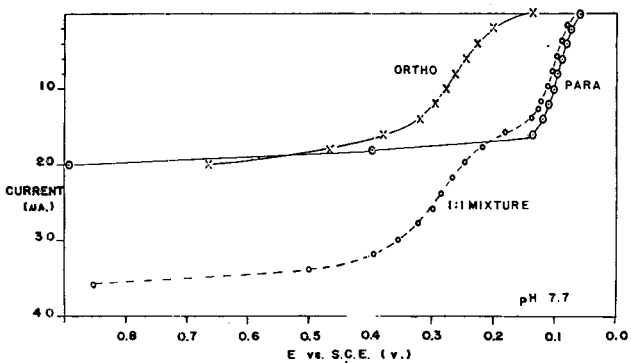

Figure 9. Polarograms of mixtures of *o*- and *p*-phenylenediamine at pH 7.7

From the data of Figure 6, it was hoped that the analysis of mixtures of *o*- and *p*-phenylenediamine might be possible at a pH of 7 to 8. The greatest separation of half-wave potentials occurs in this region. However, such a determination was only partially successful. Figure 9 shows the most favorable results obtained from a 1 to 1 mixture of $2 \times 10^{-4}M$ ortho and para isomers. With other ratios, the waves tend to merge and only semiquantitative results are obtainable. This is due in part to the irreversibility of both oxidation waves in this pH region.

Table II. *Limiting Current vs.* Concentration for *p*-Phenylenediamine

(Background, pH 7.7 buffer)

Concentration, $M \times 10^{-4}$	$i_{lim.}$, μa.	$i_{lim.}/C$
0.40	7.6	19.0
0.80	14.2	17.8
1.19	20.6	17.3
1.60	28.8	18.0
2.40	39.7	16.6
		Mean 17.7
		Dev. 3.6%

CONCLUSIONS

The application of solid electrode polarographic techniques to the measurements of the formal potentials of a labile organic redox system such as the diamine-diimine system has been shown to give valuable information which cannot be obtained by potentiometric techniques. This technique has already yielded very interesting information on the oxidation of sulfa drugs (*18*). In addition it has been shown that *o*- and *p*-phenylenediamine can be quantitatively determined at the 10^{-4} to $10^{-5}M$ level from limiting current measurements at the rotated platinum electrode. The analysis of mixtures is difficult and only semiquantitative results can be obtained.

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LITERATURE CITED

(1) Adams, R. N., Reilley, C. N., Furman, N. H., ANAL. CHEM. **25**, 1160 (1953).
(2) Bent, R. L., Dessloch, J. C., Duennebier, R. C., Fassett, D. W., Glass, D. B., James, T. H., Juilan, D. B., Ruby, W. R., Snell, J. M., Sterner, J. H., Thirtle, J. R., Vittum, P. W., Weissberger, A., *J. Am. Chem. Soc.* **73**, 2100 (1951).
(3) Clark, W. M., Cohen, B., others, Oxidation Reduction Studies I–X, Hyg. Lab., Bull. **151**, 1–363, 1928.
(4) *Ibid.*, IX.
(5) Elving, P. J., Komyathy, J. C., Van Atta, R. E., Tang, C., Rosenthal, I., ANAL. CHEM. **23**, 1218 (1951).
(6) Furman, N. H., *J. Electrochem. Soc.* **101**, 19c (1954).
(7) Gaylor, V. F., Conrad, A. L., Landerl, J. H., Abstracts, Pittsburgh Conference on Analytical Chemistry, March 1955; ANAL. CHEM. **27**, 310 (1955).
(8) Gaylor, V. F., Elving, P. J., Conrad, A. L., *Ibid.*, **25**, 1078 (1953).
(9) Hedenburg, J. F., Freiser, H., *Ibid.*, **25**, 1355 (1953).
(10) Julian, D. R., Ruby, W. R., *J. Am. Chem. Soc.* **72**, 4719 (1950).
(11) Lord, S. S., Rogers, L. B., ANAL. CHEM. **26**, 284 (1954).
(12) Michaelis, L., Hill, E. S., *J. Am. Chem. Soc.* **55**, 1481 (1933).
(13) Michaelis, L., "Oxidation Reduction Potentials," J. B. Lippincott, Philadelphia, 1930.
(14) Michaelis, L., Schubert, M. P., Granick, S., *J. Am. Chem. Soc.*, **61**, 1981 (1939).
(15) Müller, O. H., "Polarographic Method of Analysis," Chemical Education Publishing Co., Easton, Pa., 1941.
(16) Müller, O. H., "Polarography," chap. 28 in Weissberger's "Physical Methods of Organic Chemistry," Part II, Interscience, New York, 1949.
(17) Vanderbelt, J. M., Henrich, C., Vanden Berg, S. G., ANAL. CHEM. **26**, 726 (1954).
(18) Voorhies, J. D., Adams, R. N., private communication.

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Polarographic Behavior of Benzaldehyde Derivatives of Hydrazine, 1,1-Dimethylhydrazine, and Monomethylhydrazine

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In studying the determination of hydrazine and mono- and dimethylhydrazine in admixture, benzalazine, benzaldehyde dimethylhydrazine, and tribenzaldehyde-(bis)methylhydrazone were investigated at the dropping mercury electrode. In ethanolic solution containing quaternary ammonium salts as supporting electrolytes, well-defined diffusion currents were produced. Half-wave potentials and diffusion current constants were obtained in Britton and Robinson buffers over the pH range 3 to 12. The diffusion currents are a linear function of the concentration of the compound in the range 1 to 3 m*M*. In alkaline solution the reduction process for benzalazine and benzaldehyde dimethylhydrazone was found to be diffusion-controlled and irreversible. Values for the number of electrons involved in the polarographic reduction of one molecule were calculated from the Ilkovič equation. A 75% ethanol solution that was approximately 0.12*M* in tetra-*n*-butyl ammonium hydroxide and 0.1*M* in phosphoric acid was found best for determining benzalazine and benzaldehyde dimethylhydrazone in admixture. Solutions of tribenzaldehyde(bis)methylhydrazone hydrolyzed to benzaldehyde at too rapid a rate for precise analytical determination.

A PRELIMINARY survey of the behavior of the sulfates of hydrazine, methylhydrazine, and 1,1-dimethylhydrazine at the dropping mercury electrode indicated no direct reduction of the compounds. It was decided, therefore, to investigate the possibility of preparing the benzaldehyde derivatives of the hydrazines and estimating these polarographically as a means of determining the respective hydrazines.

The present report describes the polarographic behavior of the benzaldehyde derivatives of hydrazine, monomethylhydrazine and 1,1-dimethylhydrazine and presents a method for determining benzalazine and benzaldehyde dimethylhydrazone in admixture.

APPARATUS AND MATERIALS

Apparatus. A Sargent Model XXI recording polarograph and a Fisher Elecdropode were used in these studies. Half-wave potentials *vs.* the saturated calomel electrode (SCE) were determined with the Elecdropode (*5*).

The solutions used in these studies had cell resistances of less than 500 ohms, as determined with a Wheatstone bridge, so the *iR* drop correction was negligible in computing $E_{1/2}$ values (*6*). Apparent pH values of the solutions were obtained with a Beckman Model G pH meter.

Two capillaries were used throughout this work as dropping mercury electrodes. Capillary 1 had a drop rate of 4.90 seconds per drop at zero applied potential with a value of 1.60 mg.$^{2/3}$ sec.$^{-1/2}$ for $m^{2/3}t^{1/6}$ at a 92.7-cm. head of mercury. Capillary 2 had a drop rate of 7.20 seconds per drop at −0.90 volt in the "phosphate buffer" (pH 11 and 70% ethanol) with a value of 1.11 mg.$^{2/3}$ sec.$^{-1/2}$ for $m^{2/3}t^{1/6}$ at a 92.7-cm. head of mercury.

Small borosilicate glass beakers (30-ml.) were used as polarographic cells. A rubber stopper to which were attached the dropping mercury electrode, contact electrode, and a glass tube with a fritted disk was placed over the top of the small beakers. The cells were kept in a constant temperature bath at 30° ± 0.2° C. while the current-voltage curves were being determined.

Dissolved oxygen was removed from all solutions with pure nitrogen just prior to the polarographic examination. The nitrogen was passed through a portion of the solution being examined polarographically and finally through the solution in the polarographic cell.

Viscosities of solutions were determined by means of an Ostwald viscometer, and densities were obtained by means of a pycnometer.

Materials. Benzalazine was prepared according to the method of Hatt (*4*). The 1,1-dimethylhydrazone of benzaldehyde was prepared from dimethylhydrazine obtained from Aerojet General Corp., Azusa, Calif., by the method of Todd (*10*). It had a faint yellow color and boiled in the range 139–40° C. The yield was 68% of purified material.

Tribenzaldehyde(bis)methylhydrazone was prepared by the method of Harries and Haga (*3*). A white crystalline product was obtained that gave a melting point of 102–4° C.

Tetramethylammonium chloride (Eastman Organic Chemical Co. and Mallinckrodt Chemical Works, C.P. grade) was used as a supporting electrolyte. Occasionally with odd lots it was found that the salt had partially decomposed and a polarographic wave appeared at about −1.90 volts. In such cases it was necessary to recrystallize the salt from methanol-ether solutions before preparing the supporting electrolyte.

Table I. Effect of pH on i_d and $E_{1/2}$ of Benzalazine[a]

pH ("Apparent")	$E_{1/2}$ *vs.* SCE, Volts			$id/Cm^{2/3}t^{1/6}$		
	1st wave	2nd wave	3rd wave	1st wave	2nd wave	Wave Characteristics
3.5	−0.96	..	..	6.04	..	Poorly defined
4.8	−0.99	−1.21	..	2.44	3.09	Poorly defined
5.8	−1.05	−1.23	..	2.33	2.17	Fairly well defined
6.8	−1.07	−1.35	..	1.49	1.37	Poorly defined
8.0	..	−1.33	..	..	2.61	Well defined
8.8	..	−1.35	..	..	2.38	Well defined
9.7	..	−1.37	..	..	2.29	Well defined
11.4	..	−1.38	−1.92[b]	..	2.39	Well defined
12.4	..	−1.39	−1.92[b]	..	2.45	Well defined

[a] 1.0m*M* benzalazine, 0.5*M* tetramethylammonium chloride, 70% ethanol, 0.04 buffer, 0.001% gelatin, $m^{2/3}t^{1/6}$ = 1.60 mg.$^{2/3}$ sec.$^{-1/2}$.
[b] Obtained only in unbuffered-alkaline solution of $(C_4H_9)_4NOH$—70% ethanol, and with methyl red in place of gelatin.

Tetramethylammonium bromide was used in obtaining the quantitative data with benzalazine and benzaldehyde dimethylhydrazone. For most of this work Britton and Robinson buffers were used (*1*).

The phosphate buffer was prepared from orthophosphoric acid (Baker's C.P. analyzed) and tetra-*n*-butylammonium hydroxide (Southwestern Analytical Chemicals, Austin, Tex.). This buffer was approximately 0.12*M* in hydroxide and 0.09*M* in acid, with final adjustment to the desired pH made by adding hydroxide and checking with a Beckman Model G pH meter.

Stock solutions of the hydrazine derivatives were prepared in 95% ethanol. The 95% ethanol was obtained from LAC Chemicals, Inc., Culver City, Calif., and was not further purified. Redistilled mercury, C.P. grade, was used as the anode in all work.

Buffered solutions were added to the stock solutions of the benzaldehyde derivatives and the tetramethylammonium chloride solution so that final solutions placed in the polarographic cell were 1.0m*M* in reducible compound, 0.5*M* in tetramethylammonium chloride, 75% ethanol, and approximately 0.04*M* in buffer.

RESULTS AND DISCUSSION

Polarographic Studies. Benzalazine, benzaldehyde dimethylhydrazone, tribenzaldehyde(bis)methylhydrazone, and benzaldehyde were investigated in an alcohol-water system at the dropping mercury electrode.

BENZALAZINE. The behavior of benzalazine in Britton and Robinson buffered solutions is shown in Table I. Since the hy-

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drogen ions have a standard free energy that is different in alcoholic than in aqueous media, the pH of these solutions is only an "apparent" pH. A series of three waves was observed. Between pH 3.5 and 6.8 a wave was observed which increased in half-wave potential from −0.96 to −1.07 volts as the pH increased. Above pH 7.0 this low wave was not present. A second wave, well defined at a pH of 5.0, increased from −1.20 to −1.39 volts in the range pH 4.8 to 6.8 and remained constant throughout the alkaline range. A third wave appeared in tetra-*n*-butylammonium hydroxide solutions of benzalazine at pH 10 and above.

Table II. Effect of Mercury Column Height on i_d of Benzalazine[a]

Mercury Column (h), Cm.	i_d, μa.	$id h^{-1/2}$
92.1	7.51	0.790
75.6	6.80	0.791
64.8	6.26	0.790
52.1	5.70	0.802

[a] 70% ethanol, 0.5M tetramethylammonium chloride, 0.04M buffer, 0.001% gelatin, pH = 11, $m^{2/3}t^{1/6}$ = 1.60 mg.$^{2/3}$ sec.$^{-1/2}$.

In acidic solutions the diffusion currents (i_d) for both waves were difficult to reproduce precisely and varied considerably with change in pH. In basic solution (pH 11) the waves were well defined and diffusion currents could be measured precisely.

In order to test the electrode process in alkaline solution for diffusion control, the height of the mercury column was varied and the effect of i_d with this change was studied. The diffusion current was found to be proportional to the square root of the height of the mercury column and thus diffusion-controlled (Table II).

Table III. Effect of Concentration on i_d and $E_{1/2}$ of Benzalazine in Buffered and Unbuffered Solutions[a]

Benzalazine, mM/Liter	Buffered (Britton and Robinson, 0.04M)		Unbuffered [$(C_4H_9)_4NOH$]			
	$E_{1/2}$ vs. SCE, volts[b]	i_d, μa.[c]	$E^{1/2}$ vs. SCE[b]		i_d, μa.[d]	
			1st wave	2nd wave	1st wave	2nd wave
0.2	−1.39	0.70	−1.39	−1.94	0.73	1.18
0.4	−1.40	1.45	−1.40	−1.93	1.57	1.78
1.0	−1.40	3.75	−1.40	−1.92	3.73	3.67
1.4	..	..	−1.40	−1.92	5.17	4.90
2.0	−1.41	7.55	−1.41	−1.94	7.41	7.10
3.0	..	..	−1.39	−1.93	11.20	10.5

[a] pH = 11.2, $m^{2/3}t^{1/6}$ = 1.60 mg.$^{2/3}$ sec.$^{-1/2}$, 70% ethanol, 0.5M tetramethylammonium chloride.
[b] Potential of mercury pool vs. SCE measured individually for each concentration.
[c] 0.001% gelatin present.
[d] 0.001% methyl red present.

Most reductions of organic compounds at the dropping mercury electrode are irreversible processes, but it is possible to get an indication of the type of process by plotting log $(i/i_d - i)$ vs. E, where i is the current at a given potential, i_d is the limiting current, and E is the potential. If the electrode process is reversible, then the Nernst equation (*8*) should be applicable. If i and $i_d - i$ are used as indication of the relative concentration of the reduced and original material actually present at the electrode surface, the slope of the above plot should be $0.06/n$, if the process is reversible. It was found that such a plot did not give a straight line. Therefore, the electrode process is probably irreversible.

In acidic solutions maxima were present. These were suppressed by gelatin or methyl red (0.001%). No maxima suppressors were necessary in basic solution. The diffusion currents in alkaline media are a linear function of the concentration of benzalazine in the range 0.2 to 3.0 mM (Table III).

Table IV. Effect of pH on i_d and $E_{1/2}$ of Benzaldehyde[a]

pH ("Apparent")[b]	$E_{1/2}$ vs. SCE, Volts			$id/Cm^{2/3}t^{1/6}$		
	1st wave	2nd wave	3rd wave	1st wave	2nd wave	3rd wave
3.5	−1.05	..	..	1.32	..	..
4.4	−1.18	−1.36	..	1.03	1.94	..
5.7	..	−1.33	..	..	3.07	..
6.8	..	−1.36	..	..	3.14	..
7.8	..	−1.36	..	..	4.17	..
8.5	..	−1.38	..	..	3.95	..
9.5	..	−1.37	..	..	3.47	..
10.5	..	−1.46	−1.96	..	2.64	2.55
11.0	..	−1.46	−1.96	..	2.44	2.23
12.2	..	−1.48	−1.96	..	2.31	1.51

[a] 70% ethanol, 1.03mM benzaldehyde, 0.04M buffer, 0.5M tetramethylammonium chloride, $m^{2/3}t^{1/6}$ = 1.11 mg.$^{2/3}$ sec.$^{-1/2}$, 0.001% methyl red.
[b] Britton and Robinson buffers used for pH 3.5 to 7.8 and the "phosphate buffer" used from 8.5 to 12.2.

BENZALDEHYDE. The polarographic behavior of benzaldehyde has been studied extensively (*9, 11*). However, it seemed desirable to obtain additional benzaldehyde data as reference. Table IV gives the $E_{1/2}$ values and diffusion current constants for benzaldehyde in alcoholic solution over the pH range of 3 to 12. In neutral and slightly acidic and basic media only one well-defined wave was observed, while two well-defined waves were found for highly acidic and alkaline solutions.

BENZALDEHYDE DIMETHYLHYDRAZONE. Two waves were found for benzaldehyde dimethylhydrazone at the dropping mercury electrode. The diffusion current of the first wave (pH 3 to 7) decreases linearly with pH and goes to zero around pH 7. The second wave appears in the high alkaline pH range (pH 10 to 12) at a half-wave potential of −1.90 volts. Table V shows the effect of pH on i_d and $E_{1/2}$ of benzaldehyde dimethylhydrazone. The test for a reversible process at the electrode, $[(\log(i/i_d - i)]$ vs. E, did not yield a straight line. Thus, it seems likely the reduction process for benzaldehyde dimethylhydrazone is irreversible. Maxima were present in acidic solution. These were eliminated by adding gelatin to a concentration of 0.006% in the final test solution. No maxima suppressor was necessary in alkaline media. In alkaline media (pH 11) the electrode process was diffusion-controlled and the diffusion currents were linearly dependent upon concentration in the range 0.2 to 3.0mM.

Table V. Effect of pH on i_d and $E_{1/2}$ of Benzaldehyde Dimethylhydrazone[a]

pH ("Apparent")[b]	$E_{1/2}$ vs. SCE, Volts		$id/Cm^{2/3}t^{1/6}$	
	1st wave	2nd wave	1st wave	2nd wave
3.5	−1.05	..	4.84	..
4.4	−1.13	..	2.80	..
5.7	−1.17	..	1.77	..
6.8	−1.21	..	0.40	..
10.5	..	1.92	..	2.53
11.0	..	1.89	..	2.56
12.2	..	1.91	..	2.60

[a] 70% ethanol, 1.0mM hydrazone, 0.04M buffer, 0.5M tetramethylammonium chloride, $m^{2/3}t^{1/6}$ = 1.11 mg.$^{2/3}$ sec.$^{-1/2}$, 0.006% gelatin.
[b] Britton and Robinson buffers used for pH 3.5 to 6.8 and the "phosphate buffer" used from 10.5 to 12.2.

TRIBENZALDEHYDE(BIS)METHYLHYDRAZONE. Preliminary studies with this compound indicated the presence of three waves over the pH range of 3 to 12. The $E_{1/2}$ values were approximately −1.0, −1.5, and −1.9 volts (vs. the mercury pool) respectively in buffered alkaline solution. However, it was found in freshly prepared solutions of the hydrazone that the −1.0-volt wave was not present and that the i_d value of the −1.5-volt wave was much smaller for a given hydrazone concentration. A study of these waves with time was made. It was found that the second wave increased steadily with time and at the end of 48 hours it had reached a wave height about two thirds of that reached when

Table VI. Determination of Benzalazine and Benzaldehyde Dimethylhydrazone in Admixture

(Determination of benzaldehyde dimethylhydrazone)

Benzalazine Present, Mg.	Benzaldehyde Dimethylhydrazone, Mg.		Benzaldehyde Dimethylhydrazone Recovered, %
	Present	Found	
3.00	1.99	2.00	100.5
3.00	1.00	1.01	101.0
2.00	2.99	2.92	96.5
2.00	1.99	1.94	97.5
1.00	2.99	3.06	102.3
1.00	1.89	1.87	99.0
1.00	0.95	0.91	96.0
0.00	2.84	2.91	102.3
0.00	0.95	0.93	98.0

Average, $\bar{x}$ = 99.2%
Standard deviation, s = 2.40% abs.
Standard deviation of mean (of 9), s_m = 0.80% abs.
Confidence range, 99.2% ± 1.85%

the solution was allowed to stand 3 weeks. The first wave was very small for the first 24 hours and then increased linearly with time. It seemed likely that the hydrazone was hydrolyzing at a moderate and measurable rate to form benzaldehyde. A second time study was made in which the stock hydrazone solution was prepared with absolute ethanol. This study yielded results similar to the first study; however, the second (presumably benzaldehyde) wave did not increase so rapidly, and the first wave did not appear so soon. Ultraviolet absorption spectra of old and new solutions of the hydrazone and of benzaldehyde showed that the characteristic absorption peak at 246 mμ for benzaldehyde was present to a very small extent in the new hydrazone solution, and was very strong in the old hydrazone solution. Thus, the rate of hydrolysis of stock solutions of tribenzaldehyde(bis)methylhydrazone to form benzaldehyde appeared too rapid to allow the determination of this compound in the presence of benzalazine and benzaldehyde dimethylhydrazone.

Determination of Benzalazine and Benzaldehyde Dimethylhydrazone in Admixture. From the data it would appear that the most favorable conditions for the simultaneous determination of these compounds would be in the alkaline range, where benzalazine yields two good waves and benzaldehyde dimethylhydrazone yields only one good wave. The one benzaldehyde dimethylhydrazone wave overlaps that of the second benzalazine wave. Thus, it should be possible to determine benzalazine from the first wave and then determine benzaldehyde dimethylhydrazone by measuring the total second wave (benzalazine plus benzaldehyde dimethylhydrazone) and then subtracting the benzalazine portion of this wave and referring the remaining wave height to a standard graph for benzaldehyde dimethylhydrazone. The benzalazine portion of the second wave is obtained by knowing the ratio of the two benzalazine waves.

Stock solutions of benzaldehyde dimethylhydrazone and benzalazine were prepared in 95% ethanol. Aliquots of these solutions were mixed with the supporting electrolyte and additional 95% ethanol, so that the final solutions for polarographic analysis were 75% ethanol, 0.5M tetramethylammonium bromide, buffered with the phosphate buffer to an "apparent" pH of 11.5, and from 0 to 3mM in benzalazine and benzaldehyde dimethylhydrazone combinations.

Table VI presents data for the determination of benzaldehyde dimethylhydrazone with varying amounts of benzalazine; the results indicate a precision of about 24 parts per thousand. Table VII presents data for the determination of benzalazine in the presence of varying amounts of benzaldehyde dimethylhydrazone; the results indicate a precision of about 12.5 parts per thousand. As it is necessary to measure two diffusion currents in the determination of benzaldehyde dimethylhydrazone, the precision is not so good as in the determination of benzalazine.

The indices of precision used in the statistical analysis of data presented in the tables are as follows:

Standard deviation (estimate), $s = \sqrt{\Sigma(x - \bar{x})^2/(n-1)}$
Standard deviation of mean of n, $s_m = s/\sqrt{n}$
Confidence range (fiducial limits) = $\bar{x} \pm ts_m$
In the above
$\bar{x}$ = mean of n observations of x
t = Student's t (*2*) for the significance level desired and $n - 1$ degrees of freedom. For the 1-in-20 significance level and means of 11(10 degrees of freedom) t = 2.23.

Table VII. Determination of Benzalazine and Benzaldehyde Dimethylhydrazone in Admixture

(Determination of benzalazine)

Benzaldehyde Dimethylhydrazone Present, Mg.	Benzalazine, Mg.		Benzalazine Recovered, %
	Present	Found	
2.99	2.00	1.95	97.5
2.99	3.00	2.96	98.7
1.99	2.00	1.99	99.5
1.99	3.00	3.07	102.3
1.89	1.00	1.00	100.0
0.95	1.00	1.00	100.0
0.95	2.00	2.00	100.0
1.00	3.03	3.00	101.0
0.00	1.00	1.00	100.0
0.00	2.00	2.01	100.5
0.00	3.00	2.97	99.0

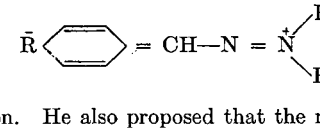
Average, $\bar{x}$ = 99.9%
Standard deviation, s = 1.24% abs.
Standard deviation of mean (of 11), s_m = 0.37% abs.
Confidence range, 99.9% ± 0.82%

Mechanism of Reduction. In basic solution (pH 11) it was possible to apply the Ilkovič equation ($n = i_d/607D^{1/2}Cm^{2/3}t^{1/6}$) to the determination of the number of electrons involved in the reduction of one molecule of benzalazine or benzaldehyde dimethylhydrazone. Diffusion coefficients were calculated by means of the Stokes-Einstein equation

$$D = \frac{2.96 \times 10^{-7}}{\eta(V_m)^{1/3}} \text{ sq. cm. per second at } 25° \text{ C.}$$

where η = solution viscosity in dyne-seconds per square centimeter and V_m = molar volume, molecular weight/density.

Although more accurate results for values of n may be determined by the mechanical stirring method (*12*) or coulometric measurement (*7*), the data obtained were sufficient to give an indication of the final product at the electrode. Values of 2.3 electrons were obtained for both benzalazine waves and for the one benzaldehyde dimethylhydrazone wave (−1.90 volts). The $E_{1/2}$ values of the third benzalazine wave (Table I) and the second benzaldehyde dimethylhydrazone wave at a pH of 11 (Table V) are the same. Since the hydrazone structure is present in benzaldehyde benzylhydrazone and benzaldehyde dimethylhydrazone, the reduction process at the dropping mercury electrode here could be the formation of 1,2-dibenzylhydrazine and 1,1-dimethyl-2-benzylhydrazine, respectively. The shift of half-wave potentials to more negative values with an increase in pH in acid solution (Tables I, IV, and V) may indicate the formation of an intermediate ion. As suggested by one of the reviewers of this manuscript, the high negative half-wave potential of benzaldehyde dimethylhydrazone in the alkaline "phosphate buffer" (Table V) may be explained by the presence of a resonance form (*10*)



in this solution. He also proposed that the more positive half-wave potentials for benzalazine suggest a 1,4- reduction in alkaline medium and that the double wave in acidic medium may possibly involve a similar reduction followed by rearrangement of

the compound to benzaldehyde benzylhydrazone, which produces the second wave. If this proposed mechanism were correct, in alkaline solutions one should obtain azobenzyl ($C_6H_5CH_2$—N=N—$CH_2C_6H_5$). In analogy to azobenzene one might expect azobenzyl to reduce below −1.0 volt polarographically. However, both waves observed for benzalazine in alkaline media (Table I) are considerably more negative. The proposed mechanisms should be verified by controlled potential electrolysis by which the products could be isolated and identified. Some of this work is now in progress.

From the similarity of half-wave potentials of benzaldehyde and benzalazine, it might be assumed that what one was really reducing at the dropping mercury electrode with solutions of benzalazine was actually benzaldehyde formed by hydrolysis. In order to show that the reaction was a reduction of the benzalazine itself, several tests were made. The classical aldehyde qualitative tests (silver mirror and 2,4-dinitrophenylhydrazine) showed no aldehyde to be present in the benzalazine solutions. In polarographic work it was observed that at a pH of 6 there was a slight separation of half-wave potentials between benzalazine solutions and benzaldehyde solutions. Ultraviolet absorption spectra measured for old and new solutions of tribenzaldehyde(bis)methylhydrazone and solutions of benzalazine, benzaldehyde dimethylhydrazone, and benzaldehyde showed that the characteristic absorption peak at 246 mμ for benzaldehyde was not present in the benzalazine and benzaldehyde dimethylhydrazone solutions, but was present to a very small extent in new tribenzaldehyde(bis)methylhydrazone solutions and was very strong in old solutions. Thus, it appears that the benzalazine molecule rather than benzaldehyde is the species which undergoes reduction at the dropping mercury electrode.

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LITERATURE CITED

(1) Britton, H. T. S., Robinson, R. A., *J. Chem. Soc.* **1931**, 1946.
(2) Fisher, R. A., "Statistical Methods for Research Workers," 10th ed., Oliver and Boyd, Edinburgh, 1946.
(3) Harries, C., Haga, T., *Ber.* **31**, 62 (1898).
(4) Hatt, H. H., "Organic Syntheses," Coll. Vol. II, pp. 6–395, Wiley, New York, 1947.
(5) Kolthoff, I. M., Lingane, J. J., "Polarography," 2nd ed., p. 70, Interscience Publishers, New York, 1952.
(6) *Ibid.*, p. 374.
(7) Lingane, J. J., *J. Am. Chem. Soc.* **67**, 1916 (1945).
(8) Nernst, W. Z., *Physik. Chem.* **2**, 613 (1888).
(9) Pasternak, R., *Helv. Chim. Acta.* **31**, 753 (1948).
(10) Todd, David, *J. Am. Chem. Soc.* **71**, 1353–5 (1949).
(11) Tokuoka, M., *Collection Czechoslov. Chem. Communs.* **7**, 392 (1935).
(12) Whitnack, G. C., Nielson, J. M., Gantz, E. S. C., *J. Am. Chem. Soc.* **76**, 4711–14 (1954).

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Separation of Acetylated Neomycins B and C by Paper Chromatography

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A semiquantitative method for distinguishing and determining neomycins B and C is presented. The procedure is directly applicable to fermentation samples or, preferably, after a preliminary purification by adsorption and elution on an ion exchange resin. The method is based on the separation by paper chromatography of the neutral *N*-acetyl derivatives of neomycins B and C and their detection through conversion to the *N*-chloro derivative followed by color development with starch–potassium iodide spray reagent.

THE use of paper chromatography as a criterion for the homogeneity of neomycin preparations has been reported from several laboratories (*4, 5, 10*), but subsequent to the actual isolation and characterization of neomycin B and neomycin C (*2*), it was found that none of these methods was capable of resolving a mixture of the two neomycins. The most recent report (*8*) still admits the inability to resolve the neomycins satisfactorily by paper chromatographic procedures. In view of the chemical nature of the neomycins (*2*), these two antibiotics may be considered as iosmeric oligosaccharides with a number of amino groups. Conceivably, they differ from each other only in minor structural features, possibly a difference in the configuration of one of the glycosidic linkages. Experiences in this laboratory indicate that the basicity of these two neomycin isomers probably dominates their paper chromatographic behavior and can account for the difficulty encountered in their resolution. It was therefore reasoned that if the basicity were neutralized by acetylation, the minor structural difference might be able to manifest itself as a difference in the mobility on a paper chromatogram. This proved to be the case.

EXPERIMENTAL

Detection of *N*-Acetyl Neomycins. For the reasons just discussed, paper chromatography of the crystalline *N*-acetyl neomycins (*2*) was tried. Because the *N*-acetyl neomycins are biologically inactive and do not respond to the ninhydrin reagent, an effective method for detecting them on paper chromatograms had to be developed first. It was found that a modification of the method developed by Rydon and Smith (*7*) for detecting peptides and amino acids was very effective for this purpose.

The modified procedure employs the following reagents.

Sodium hypochlorite. Add 1 part of Clorox (5.25% sodium hypochlorite in water) to 20 parts of water.

Ethyl alcohol, 95%.

Starch-iodide reagent. Mix equal volumes of a 1% soluble starch solution and a 1% potassium iodide solution. The starch solution should not be used for longer than 1 week.

The procedure consists of three steps. The developed paper chromatogram is sprayed with the sodium hypochlorite. When dry, it is sprayed with 95% ethyl alcohol. Finally, after the ethyl alcohol has evaporated, the chromatogram is sprayed with starch-iodide reagent. The acetylated neomycins show up as deep blue spots against a colorless background.

The principles involved consist of converting a substituted amide with hypochlorite into the chloramide, destroying the excess hypochlorite with a mild reducing agent, and oxidizing the iodide to iodine by the chloramide. Other mild reducing agents—e.g., a 5% glucose solution or a 1% formaldehyde solution—also destroy the excess hypochlorite without attacking the chloramide. In the original method of Rydon and Smith (*7*) chlorine gas was employed and the excess chlorine later removed by aeration.

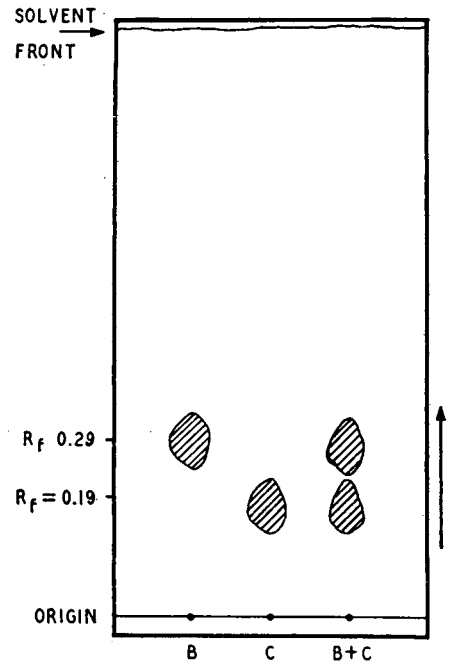

Figure 1. Paper chromatograms of *N*-acetyl neomycins B and C

B. *N*-Acetyl neomycin B
C. *N*-Acetyl neomycin C

This procedure has two disadvantages: The background is also somewhat blue and, in addition to amides and peptides, many other compounds including amino and ammonium compounds also show up as blue spots. The present modification eliminates these disadvantages and, with a few exceptions, is specific for monosubstituted amides. With glycylglycine, as little as 0.3 γ on a spot 12 mm. in diameter can be detected. Use of benzidine or tolidine in place of the starch-iodide reagent, as reported by Reindal and Hoppe (*6*), is, of course, also effective but the color is not as deep.

Development of Paper Chromatogram. Because the *N*-acetyl neomycins were expected to behave like neutral oligosaccharides, the solvent system (butyl alcohol–pyridine-water, 60 to 40 to 30) developed by Jeanes, Wise, and Dimler (*3*) for sugars was first selected for test. Descending chromatography on Whatman No. 1 paper for an overnight period—i.e., approximately 16 hours for a 40-cm. run—effected a satisfactory resolution of the *N*-acetyl neomycins. A diagram of the paper chromatogram thus obtained is shown in Figure 1. The R_f of *N*-acetyl neomycin B is equal to 0.29 and the R_f of *N*-acetyl neomycin C is equal to 0.19.

Repeated tests showed that it was essential to prepare the solvent mixture fresh every day because re-use often led to unsatisfactory separation. The chromatographic procedure was also shown to be sensitive; a spot approximately 13 mm. in diameter can be obtained with 6 γ of *N*-acetyl neomycin B or C, although quantities as small as 2 to 4 γ can also be detected.

Acetylation of Neomycins. In order to apply the method directly to neomycin samples, including fermentation broths, a procedure was developed for converting the neomycins to the *N*-acetyl derivatives. Originally (*2*) these had been prepared by acetylating the free bases in a methanol solution with acetic anhydride. It was found that *N*-acetylation could be readily carried out in buffered aqueous solutions.

To 1 ml. of a solution containing 1 to 2 mg. of neomycin per milliliter, is added 0.2 ml. of a 4.5*M* sodium acetate solution or 0.1 ml. of 3*M* dipotassium hydrogen phosphate. Then 0.1 ml. of acetic anhydride is added dropwise with shaking. When the resulting solution is spotted directly on paper and developed as described above, a satisfactory chromatogram is obtained as shown in Figure 2.

Figure 2 also shows the chromatogram of a sample acetylated in the absence of the buffer as well as that of the unacetylated neomycins. The acetylation in the absence of buffer apparently led to the formation of a mixture of partially aceylated products. Experiments with different sodium acetate concentrations demonstrated that 0.3*M* is the minimum required to ensure complete acetylation. Use of dipotassium hydrogen phosphate at the same concentration (0.3*M*) was found to be more advantageous, because a slight interference with the spot for *N*-acetyl neomycin C by sodium acetate could be eliminated.

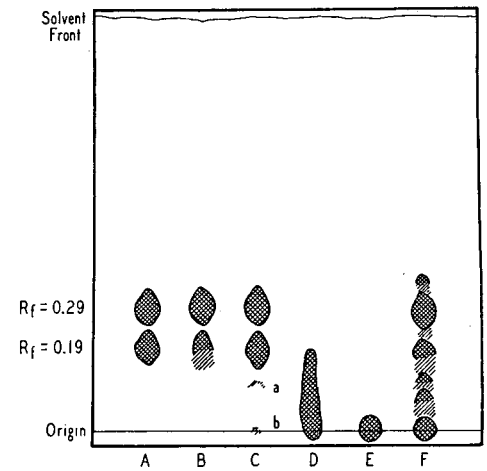

Figure 2. Acetylation procedure as studied by paper chromatography

A. Mixture of *N*-acetyl neomycin B and *N*-acetyl neomycin C
B. Mixture of neomycin B and neomycin C, acetylated with sodium acetate as buffer
C. Mixture of neomycin B and neomycin C, acetylated with dipotassium hydrogen phosphate as buffer
D. Mixture of neomycin B and neomycin C, acetylated in absence of buffer
E. Mixture of neomycin B and neomycin C, not acetylated
F. Fermentation broth sample acetylated directly with procedure given
a, b. Traces of color at *a* and *b* also appeared in acetylation mixture containing no neomycin with dipotassium hydrogen phosphate as buffer

The last example in Figure 2 represents a fermentation sample which has been acetylated by the procedure described above. It may be seen that there are a number of compounds other than the neomycins in the fermentation sample which also appear in the chromatogram. However, the characteristic spots of *N*-acetyl neomycins B and C are readily visible. Later experiments showed that the colored streak due to compounds other than neomycins can be eliminated if the neomycins in the fermentation sample are first purified by means of Amberlite IRC-50 (*9*). This paper chromatographic procedure is, therefore, applicable to the detection of neomycins B and C in fermentation samples.

SEMIQUANTITATIVE DETERMINATION

The areas of the spots, as determined by the method of cutting out and weighing, were found to be proportional to the logarithm of the sample weight within the range from 3 to 50 γ (*1*). How-

ever, because the position and slope of the standard curve are somewhat variable, it has been found necessary to include standard samples of the pure *N*-acetyl neomycins in each run. In this manner it is possible to estimate the percentage of neomycins B and C in a given sample. The error in the value for the percentage of the minor component, usually neomycin C, is of the order of ±30%. Although the method as developed permits only a semiquantitative estimation, it is useful for evaluating the ratio of neomycin C to neomycin B in various preparations and fermentation broths.

LITERATURE CITED

(1) Block, R. J., Le Strange, R., Zweig, G., "Paper Chromatography," p. 38, Academic Press, New York, 1952.
(2) Dutcher, J. D., Hosansky, N., Donin, M. N., Wintersteiner, O., *J. Am. Chem. Soc.* **73**, 1384 (1951).
(3) Jeanes, A., Wise, C. S., Dimler, R. J., ANAL. CHEM. **23**, 415 (1951).
(4) Leach, B. E., De Vries, W. H., Nelson, H. A., Jackson, W. G., Evans, J. S., *J. Am. Chem. Soc.* **73**, 2797 (1951).
(5) Regna, P., Murphy, F., *Ibid.*, **72**, 1045 (1950).
(6) Reindal, F., Hoppe, W., *Ber.* **87**, 1103 (1954).
(7) Rydon, H. N., Smith, P. W. G., *Nature* **169**, 922 (1952).
(8) Saitò, A., Schaffner, C. P., Abstract, p. 98, IIIrd International Congress of Biochemistry, Brussels, 1955.
(9) St. John, C. V., Flick, D. E., Tepe, J. B., ANAL. CHEM. **23**, 1288 (1951).
(10) Waksman, S. A., "Neomycin," p. 75, Rutgers University Press, New Brunswick, N. J., 1953.

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Partition Chromatography of Aliphatic Acids

Quantitative Resolution of Normal Chain Even Acids from C_{12} to C_{24}

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Reversed-phase chromatography on a silane-treated silicic acid column, with 2,2,4-trimethylpentane (isooctane) as stationary phase and aqueous methanol as eluting solvent, allows the quantitative resolution of mixtures of even-numbered, normal-chain saturated fatty acids in the range C_{12} to C_{24}. Less than 50 mg. of mixed acids are needed. The elution and titration of the seven acids require about 5 hours. Individual components representing 10% or more of the mixture are determined with an error not exceeding 2.5%. An almost comparable degree of accuracy is attainable for minor components (1 to 3% of the total mixture), when a procedure involving a complementary analysis is followed. A device for automatically and continuously changing the composition of the eluting solvent and a semiautomatic, motor-driven microburet are described. The method is applicable to the determination of the relative proportion of each carbon series represented in a fat sample and the relative proportion of saturated to unsaturated acids in each carbon series.

IN 1951 the present authors undertook to devise a quantitative chromatographic method for the estimation of even saturated fatty acids from C_{12} to C_{24} which could be used to analyze fats of marine origin. It was known that two mixtures of saturated acids could be obtained from such fats, the first resulting from complete hydrogenation of the sample and the second, from removal of all unsaturated components through oxidative degradation. Each of these mixtures would be chromatographed and the results combined to yield the respective proportions of saturated and unsaturated acids within each carbon series. Although no detailed information concerning individual unsaturated components would be obtained, a true picture of the fat structure would emerge.

One proposed application of such a procedure was the study of changes occurring in the fatty acid composition of fat in fishes and marine mammals during their life cycles. As this would require routine analysis of many samples, detection of relatively small changes, and estimation of small amounts of certain acids, a highly standardized and accurate method was needed.

Only the methods of Howard and Martin (*8*) and of Boldingh (*3*) were known at the time this investigation was undertaken. Mealorub powder could not be secured to try the method of Boldingh; the other method was found unsatisfactory over the range C_{12} to C_{24}. Successive modifications of the latter have led to the method described herein.

Others have described separations of even saturated acids of high molecular weight (*9, 10, 14*). These procedures covered sections of the range C_{12} to C_{24}; the recoveries were not fully quantitative.

Applications of the method of Howard and Martin involving combination of two complementary chromatograms (*4, 5, 11, 12, 15*) all had one point of similarity with the proposed method: One of the chromatograms used was that of a mixture of saturated acids derived from the original mixture, either by complete hydrogenation (*11, 15*) or by elimination of unsaturated components through oxidative degradation (*4, 12*). However, they all used the intact original mixture of acids for the complementary chromatogram to secure detailed information on all acid components. Moderately quantitative results were obtained with simple fats (*4, 5, 12*) or with simple fractions from fats (*11, 15*). Silk and Hahn showed that the chromatographic method used is semiquantitative only (*14*) in estimating the even saturated acids from C_{16} to C_{24}. It is believed that these applications could be improved by the use of the more accurate method described below. In separating the unsaturated acids from the original mixture, the method of Schuette and Dal Nogare (*13*) is used to eliminate acid oxidation products completely, thereby avoiding possible interference in the chromatogram of the saturated components.

OUTLINE OF METHOD

The present method has one basic feature in common with that of Howard and Martin (*8*) for the separation of acids from C_{12} to C_{18}. The column material is treated with dichlorodimethylsilane to render it unwettable by polar solvents. It has much in common with a method proposed by Vandenheuvel and Hayes (*16*) for the analysis of the monocarboxylic acids from C_2 to C_{12}: the type of Trubore glass column used; the commercial grade silicic acid used to prepare the column mixture; the proce-

dure for mixing and packing the column material using the same packing tools; the procedure for loading the column; and the method for detecting band boundaries. The latter, which consists in timing the neutralization by acid eluate of successive alkali increments, requires the maintenance of a constant rate of elution; this is achieved by using a constant hydrostatic head of solvent instead of the constant pressure used in the method for the lower acids.

There are other differences from the Vandenheuvel and Hayes method: Only one packing is used to cover the entire range of acids; the unknown acid mixture is loaded on the column as a chloroform solution; the silicic acid is silane-treated; and the phases are reversed, 2,2,4-trimethylpentane being the stationary phase and aqueous methanol the eluting solvent. A simple arrangement ensures the continuous and automatic change from an initial 75% methanol to practically 100% methanol towards the end of the analysis. The receiver used for the continuous collection and titration of eluate is fitted with electrodes connected to a pH meter. This modification was rendered possible by the polar nature of the effluent; it is of great assistance in detecting band boundaries by the method of successive titrations in permiting easy and accurate determination of end points. For the last modification a semiautomatic, motor-driven piston microburet is used instead of the common type.

An analysis involving seven acids requires about 50 mg. of test material and takes less than 5 hours. Major components ($\geq$ 10% by weight of the mixture) are determined with an error smaller than 2.5% by a single analysis. Under the same conditions, the error on minor components (1 to 3% of the mixture) may attain 20%. However, the performance of a complementary analysis reduces this error to an estimated 5% and decreases the error on major components in about the same proportion.

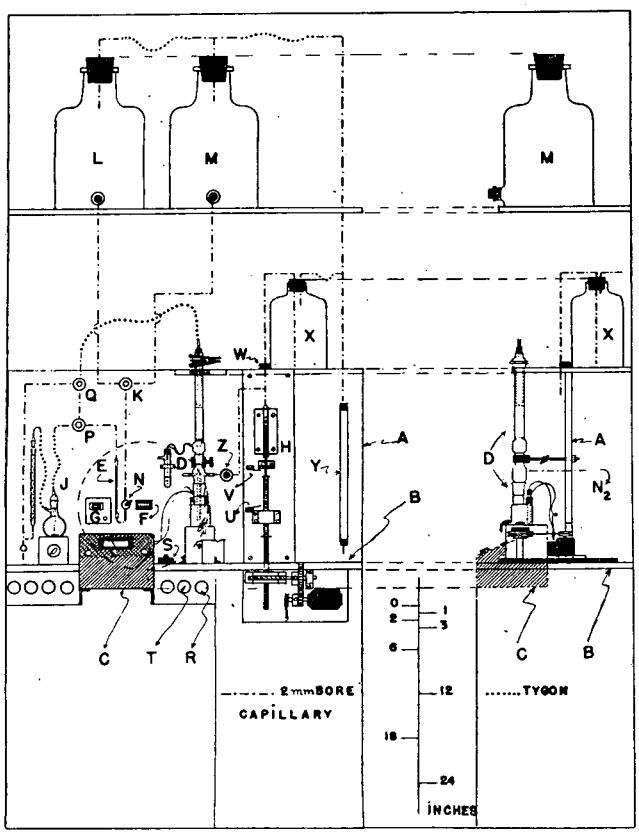

Figure 1. General layout of equipment

A. Wood panel
B. Table
C. pH meter (Beckman Model H-2)
D. Column assembly
E. Flow meter (Emil Greiner No. G-9142 Flowmeter)
F. Counter, Type U. S. Magnetic, lever reset, 110-volt a.c. (Veeder-Root, Hartford, Conn.)
G. Chronometer (Lab-Chron, Laboratory Industries, Inc., Chicago, Ill.)
H. Piston buret, motor-driven
J. Solvent mixer
K. Stopcock, straight-bore, three-way
L. Reservoir for methanol A, 20-liter
M. Reservoir for methanol B, 20-liter
N. Needle valve (Fisher packless)
P. Stopcock, straight-bore, three-way
Q. Stopcock, straight-bore, three-way
R. Selector switch
T. Switch
S, U, V. Microswitches
W. Microvalve size 0–1 and special rectifier (International Instrument Co., Los Angeles, Calif.)
X. Reservoir for alkali, 2-liter, brown glass
Y. Tube, contains soda lime and indicating carbon dioxide absorbent
Z. Stopcock, Teflon-plugged, two-way capillary

APPARATUS

General Layout. Most of the apparatus (Figure 1) is permanently fastened to wood panel *A* or table *B*. Sitting in front of panel *A*, the operator has all the controls within easy reach; under his direct vision are the only parts which require his attention: pH meter *C*, column assembly *D*, flow meter *E*, counter *F*, and chronometer *G*. Motor-driven piston buret *H* and solvent mixer *J* require no attention.

The position of stopcock *K* determines whether solvent will flow to the column from *L* (methanol A) or from *M* (methanol B). The rate of flow is adjusted by the opening of valve *N* with the help of flow meter *E*. On changing the position of stopcocks *P* and *Q* solvent will flow to the column either directly or through solvent mixer *J*.

Operation of the buret piston is commanded by switches *R*, *S*, and *T*. The position of selector switch *R* determines whether the piston is to move upward (delivery) or downward (filling). Pressing microswitch *S* energizes the drive motor, inducing movement of the piston only for the duration of the contact. Switch *T*, by shorting the leads to switch *S*, allows sustained, unattended filling or delivery; microswitches *U* and *V* interrupt the motor when the buret is filled or empty. When selector switch *R* is on "fill," valve *W* opens, admitting alkali from reservoir *X* to the buret. The amount of alkali delivered by the buret is registered by counter *F*.

Reservoirs *L*, *M*, and *X* are connected to tube *Y*, containing soda lime and indicating carbon dioxide absorbent. Stopcock *Z* on the alkali delivery line from the buret is turned off when the latter is being refilled.

Column Assembly. Figure 1 shows column assembly *D* set up for a run. Figure 2 shows the various borosilicate glass parts of this assembly. During a run, column I is fitted with adapters II and III. The latter is connected to the column by clamping securely the two parts of the common ball joint. The column itself fits on the 24/40 standard taper joint of adapter IV, while receiver V is connected to the latter through a 39/45 standard taper joint.

Solvent is led to the column through adapter III which is connected to the solvent dispensing system by small-section Tygon tubing. The effluent from the column reaches the receiver through adapter II, which passes through adapter IV. This arrangement leads the eluate directly to the receiver, avoiding splashing and evaporation and thus preventing the higher acids from crystallizing out before reaching the receiver.

The body *A* of the column proper (Figure 2), has a coarse fritted-glass disk, *B*, sealed at the bottom, followed by outlet tube *C*, and ended by tip *D*. Vent *E* prevents tube *C* from filling up. A small piece of rod, *F*, is sealed on the inside of *C*, its extremity touching the center of disk *B;* this prevents drops of eluate from stagnating under *B*. *G* provides an outlet for the nitrogen bubbled through the receiver; it is fitted with a length of small section rubber tubing leading the nitrogen to a water-filled bubble counter (Figure 1).

Adapter II is fitted to the column by inserting *D* through the hole in Teflon plug *H*. Vent *I* prevents the adapter from filling itself with liquid.

When adapter III is clamped in position, tip *K* is located 3 to 5 mm. above the silicic acid column. Inlet tube *L* is ring-sealed to the bottom of *J*. Cork *M* ensures the rigidity of *L*, thus protecting the ring seal in tip *K*.

Adapter IV (Figure 1) is clamped to vertical board *A*. This adapter supports the column and provides a permanent connection for the column assembly with both the nitrogen supply (Figure 2) through inlet *N* and with the alkali supplied by the buret through inlet *O*. Delivery tip *P* is removable for eventual cleaning.

Receiver V is a four-necked, flat-bottomed flask. Neck *Q*

allows the flask to be slipped on and off adapter IV, the normal position being as shown. Neck *R* is fitted with a calomel electrode and neck *S* with a glass electrode, both connected to the pH meter by 30-inch leads. The meter is carefully grounded and supplied with stabilized current (constant voltage transformer, 0.26 ampere, Sola No. 30804). The electrodes are fitted on necks *R* and *S* with the help of rubber sleeves, ensuring an air-tight seal yet allowing their easy removal; their lower ends are located about 10 mm. from the bottom of the flask.

Neck *T*, which is normally corked, allows the introduction of reagents in the course of a run.

Elastic band *U* maintains scale *V* in a position established by gaging the flask fitted with the electrodes and in operative position. The zero of this scale corresponds to a permanent mark, *W*, made on the flask after determining the lowest level of liquid which will ensure sufficient immersion of the electrodes when the stirrer is in operation.

Outlet *X* is connected to delivery tip *Y* by a piece of rubber tubing; pinch clamp *Z* allows liquid to be withdrawn.

The stirrer (Figure 1) is a 20-mm. length of 6-mm. diameter, Teflon-covered Alnico magnet rod. The entraining magnet is a 42-mm. length of 12-mm. diameter Alnico rod fastened to a pulley which is connected to a bicycle wheel race screwed to the bottom of a box; the latter can slide forward and backward, being guided by tracks screwed to the table. A stop is provided on the latter to limit the forward movement of the box and arrest it in its normal working position where it supports the receiver. The entraining magnet rod then coincides with the stirrer rod which should revolve in the front part of the flask (away from the electrodes). The pulley is belt-driven by a small motor fastened at the back of the box. A safe speed for the stirrer is 600 r.p.m.

Automatic Solvent Mixer. This apparatus, *J* in Figure 1, is shown in greater detail in Figure 3. It comprises mixing flask *A* and solvent saturator *B*. Flask *A* is made by adding outlet *C* to a long-necked, flat-bottomed flask. The body of *B* is constructed like a Vigreux column.

At the beginning of a run, flask *A* is completely filled with 75% methanol (methanol C); 100% methanol (methanol A) is introduced into the flask through adapter *D* at a constant rate while magnetic stirrer *E* is operating, driven by *F*. The solvent flowing out of the flask through *B* is thus gradually and evenly changing from the initial 75% methanol to increasingly more concentrated alcohol. The alcohol leaving the flask drips slowly down through a height *GH* of 2,2,4-trimethylpentane located in *B*. The 2,2,4-trimethylpentane–equilibrated alcohol collects under *G* before flowing through *I*, and from there, through *L* and *M*, to the column.

To prepare the device for a new run open stopcock *J* and remove adapter *H*, allowing *B*, *I*, and *M* to empty. Set stopcocks *K* and *L*, and allow methanol B to flow through *K*, *L*, and *I*; alternately closing and opening *J*, flush the base of *B*. Close *J* and allow alcohol to rise up to *G*. Adjust *L* to stop flow of alcohol. Fill *B* from *G* to the top with 2,2,4-trimethylpentane. Remove adapter *D*, empty the flask, rinse with methanol C, and fill with this solvent. Reconnect *D* and *H*.

Buret. The semiautomatic motor-driven buret (Figure 3) consists of a piston sliding in a tubing body, *N*, which is cemented in block *O*. *O* is fastened to the 0.25-inch aluminum base plate by leveling screws and aligned with the piston drive comprising piston shaft *P* and screw *Q*. Motion of the piston is induced when the drive is made to revolve; *Q* then screws or unscrews itself in nut *S*, thus causing the piston drive to move forward or backward, depending on the rotational direction. Motor *T* actuates this mechanism through a gear train: Gear *U*, directly on the motor shaft, is driving gear *UA*. The latter shares a common shaft with worm gear *UB* which drives worm gear *UC*. *UC* bears a square, central hole which fits over square bar *R*; *UC* thus induces the bar to revolve, allowing it to slide freely through the hole.

Details of the piston head are shown at the right in Figure 3. It is made of three $^1/_8$-inch thick Teflon disks sandwiched between washers and held together by screw *V*. The latter fits the threaded collar of *W*, a hollow knob presenting an off-centered slot *X*. Pin *Y* of piston shaft *P* can be inserted through the side of slot *X* but cannot be disengaged from *W* when the piston head is aligned with the piston shaft, since *Y* is larger than the centered part of the slot. However, it can revolve freely inside *W* without causing the piston head to rotate; the latter is thus merely pushed or pulled. Slack is provided in the centered part of the slot to allow for misalignment of tube *N*. *W* is used as a chuck when turning to size the Teflon disks on the lathe. Coasting of the motor is prevented by friction brake *Z* on the motor shaft.

A cam located at the end of the shaft common to gears *UA* and *UB* actuates microswitch *MS-C*, sending impulses to the alkali-recording counter. One count corresponds to $^1/_{40}$th of a turn of the screw or a $^1/_{546}$-inch movement of the piston; with the $^5/_8$-inch Trubore tube used, one count is equivalent to 9.70×10^{-3} ml. Checking the reproducibility of volume delivery has shown a maximum error of 3×10^{-3} ml. or approximately one third count; when 0.01*N* alkali is used, this is equivalent to 3×10^{-8} mole.

A wiring diagram of the buret is shown at the lower left of Figure 3. Parts *SW-R*, *SW-S*, *SW-T*, *MS-U*, and *MS-V* are switches denoted as *R*, *S*, *T*, *U*, and *V* in Figure 1. Parts denoted *HW* and *RW* are, respectively, the microvalve admitting alkali to the buret and the rectifier already described.

Reagents.

Methanol A. Nichols methanol, reagent grade, No. 1212. Reflux over sodium hydroxide. Distill, collecting from 64.0° to 65.0° C. Keep in reservoir *L*, Figure 1.

Methanol B. Mix 75 volumes of methanol A with 25 volumes of distilled water. Saturate by shaking with excess 2,2,4-trimethylpentane and pour into reservoir *M*, Figure 1, without removing excess hydrocarbon.

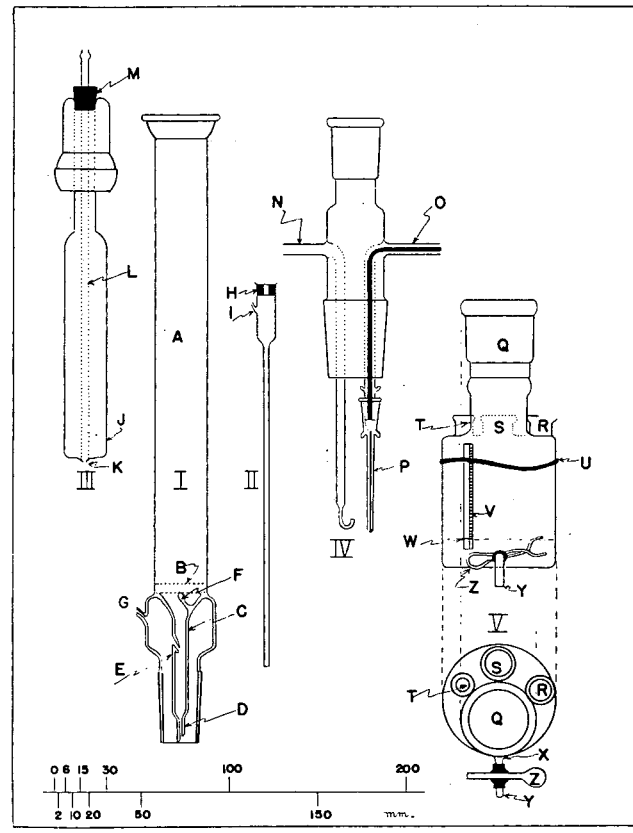

Figure 2. Parts of column assembly

I. Column proper
II, III, IV. Adapters
V. Receiver
A. Body of column, 1-inch Trubore tubing
B. Coarse fritted-glass disk, 5 mm. thick
C. Outlet tube, ring-sealed, funnel-shaped
D. Tip
E. Vent
F. Small piece of rod
G. Outlet for nitrogen bubbled through receiver
H. Teflon plug
I. Vent
J. Outer tube
K. Tip created by blowing out center of *J* and reducing hole produced to 1-mm. diameter outlet
L. Inlet tube
M. Cork
N. Nitrogen Inlet
O. Alkali inlet
P. Delivery tip. Bore corresponds to 47-gage steel wire
Q, *T*. Necks
R. Neck fitted with calomel electrode (Beckman 11-505-80A)
S. Neck fitted with glass electrode (Beckman 11-505-75)
U. Elastic band
V. Scale, thin strip of white, rigid plastic, engraved 1-ml. graduations
W. Permanent mark
X. Outlet
Y. Outlet
Z. Pinch clamp

METHANOL C. Mix 75 volumes of methanol A with 25 volumes of distilled water. Keep in a glass-stoppered bottle.

METHANOL RECOVERY. Reflux methanol used to pack, wash, and elute the column over sodium hydroxide. Redistill, collecting from 64° to 72° C. Use in the preparation of the silane-treated silicic acid.

CHLOROFORM. Distill Fisher certified reagent C-298. Collect from 60.0° to 60.8° C. Keep in brown glass-stoppered bottle. This reagent should be acid-free.

2,2,4-TRIMETHYLPENTANE. Phillips, pure grade. Redistill with 45-cm. Vigreux column, collecting from 98.0° to 98.6° C.

2,2,4-TRIMETHYLPENTANE RECOVERY. Shake repeatedly with water the 2,2,4-trimethylpentane used in the preparation of silicic acid; reflux with 1*N* sodium hydroxide for several hours; decant, dry, and fractionate as above. Check: when refluxed with water, the product should leave the water neutral to bromocresol green.

TITRATING SOLUTION. Reflux 1 liter of aldehyde-free 95% ethanol while bubbling nitrogen through the boiling liquid; let cool under nitrogen. Add 0.46 gram of clean sodium; let dissolve while bubbling nitrogen. Prepare 1 liter of 10% sodium chloride solution in distilled water; reflux the solution while bubbling nitrogen and let cool under the same conditions. Mix the two solutions in nitrogen-filled reservoir *X* (Figure 1) and stopper immediately. The strength of this solution will remain remarkably constant for several months.

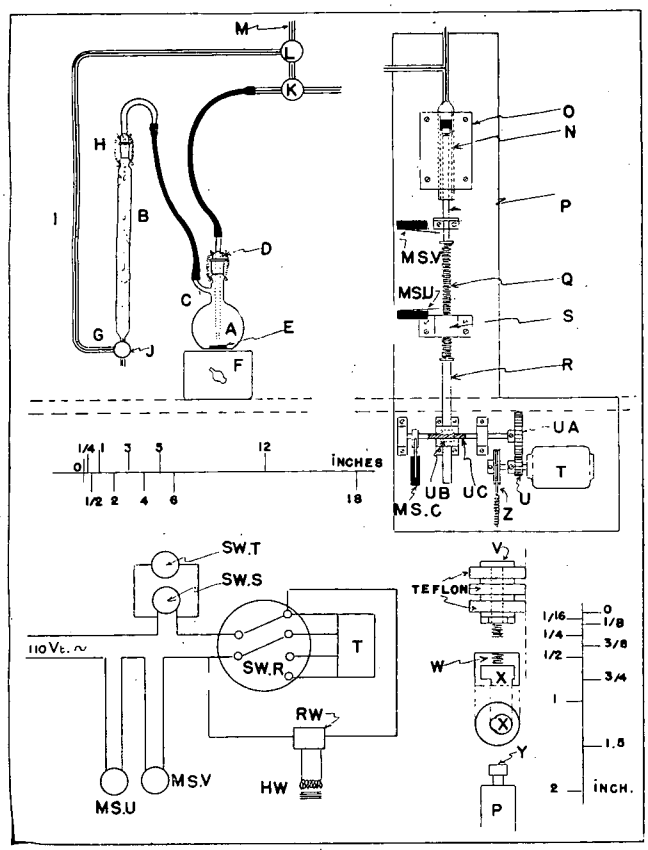

Figure 3. Solvent mixer (top left), semiautomatic buret (top right), wiring diagram of buret (bottom left), and buret piston (bottom right)

A. Mixing flask made by adding outlet *C* to long-necked, flat-bottomed, standard-taper 24/40 250-ml. flask
B. Solvent saturator constructed like Vigreux column
C. Outlet
D, H. Adapters
E. Magnetic stirrer driven by *F*
F. Motor, drives *E* (Mag-mix, Precision Scientific Co., No. 65904)
G. Lower level of 2,2,4-trimethylpentane
I. Capillary tubing
J, K, L. Stopcocks
M. Capillary tubing
N. Tubing body, 5/8-inch Trubore
O. Lucite block
P. Piston shaft P, 3/8-inch steel rod
Q. Screw, 0.5-inch 13-R.H.-bar
R. Square bar
S. Nut
T. Motor, Type NS-Y-12, 115-volt, a.c., 1/150 h.p. (Bodine)
U. Gear (Boston QBH-16)
UA. Gear (Boston QB-48)
UB. Worm gear (Boston LVHB)
UC. Worm gear (Boston G-1044)
V. Screw
W. Hollow knob
X. Off-center slot
Y. Pin of piston shaft *P*
Z. Friction brake on motor shaft
MS-C. Microswitch
SW-R, SW-S, SW-T, MS-U, MS-V. Switches, denoted *R, S, T, U,* and *V* in Figure 1
HW. Microvalve admitting alkali to buret
RW. Rectifier, 30-volt, d.c.

Silane-Treated Silicic Acid. SIEVE ASSEMBLY. This apparatus is made from parts of U. S. Standard sieves assembled in the following way: cover, No. 170, No. 230, and base.

FLASK. The center neck of a standard-taper, 1-liter, three-necked flask is fitted with a Trubore, all-glass stirrer (Scientific Glass Apparatus Co., Inc., No. J-2169). One of the side necks is connected, through a right-angle, stopcock-fitted, standard-taper connecting tube, to two 300-mm. high drying jars in series. The first is filled with calcium chloride, the second with soda lime. The other neck holds a 150-ml. dropping funnel fitted with a rubber stopper connected to a calcium chloride–filled drying tube.

PROCEDURE. With this procedure a 65-gram batch is prepared, enough to perform 60 successive analyses. The silicic acid is readily recovered and re-used. Usage of a batch is limited only by mechanical losses and by the formation of fines which have to be removed occasionally by sieving.

Sieve silicic acid (Mallinckrodt's No. 2847) using the prepared assembly. Three granular fractions are obtained: coarse (22%), medium (15%), and fine (63%), from which only the medium fraction is used. Spread 75 grams of the latter over a 15-cm. Petri dish, and dry over phosphorus pentoxide in vacuo, using a good mechanical pump and establishing the vacuum slowly to prevent sudden bursting of air pockets within the powder. Carry out the drying to constant weight, renewing the drying agent if necessary. Transfer the weighed powder to a wide-necked 250-ml. glass-stoppered bottle. Add rapidly a volume of water corresponding to 6% of the weight of silicic acid and stopper immediately. Shake and tap the bottle vigorously for 5 minutes and place for 15 minutes on a roller-mixer.

The rest of the operation must be carried out under a very efficient hood, using gas mask and gloves when handling aggressive dichlorodimethylsilane (Dow Corning). The latter should be cooled a few hours in the refrigerator before use.

In a glass-stoppered 200-ml. measuring glass, pour 6 ml. of silane for every milliliter of water used in humidifying the silicic acid. Add dry 2,2,4-trimethylpentane to the 100-ml. mark, stopper immediately, and shake cautiously.

Remove the dropping funnel from the prepared flask and pour 250 ml. of dry 2,2,4-trimethylpentane into the flask; switch on the stirrer and place a solids funnel over the open neck. Add the humidified silicic acid by portions and replace the dropping funnel. Pour the silane solution into the latter, stopper, and run the liquid regularly into the contents of the flask over a 30-minute period. Transfer the mixture, using 2,2,4-trimethylpentane for rinsing, to a 14-cm. Büchner funnel equipped with filter paper and a 2-liter suction flask connected to a water aspirator. Wash the product repeatedly to remove unreacted silane using approximately 400 ml. of 2,2,4-trimethylpentane in all. Remove the Büchner funnel, and add water to the filtrate to destroy silane. Transfer the cake to a 1-liter beaker containing 250 ml. of recovered methanol and stir vigorously to produce a slurry which is poured over a 14-cm. Büchner funnel equipped like the previous one; apply suction and filter slowly, taking care to keep the cake submerged by adding small portions of recovered methanol until the filtrate is no longer acid to bromocresol green (about 1 liter in all). Transfer the cake to a 15-cm. Petri dish; protect from dust while letting air-dry overnight.

Sieve the powder in the sieving assembly described above, retaining the medium fraction. Tenacious acidic impurities must be removed by applying to 24-gram batches of the powder the procedure described later under Column Mixture and Packing, then connecting the packed column to the methanol A reservoir and letting 400 ml. of this solvent percolate at the rate of 100 ml. per hour.

Remove adapter III (Figure 2); remove paper disks, and fill the column with recovered methanol; using the spiral stirrer, produce a slurry. Transfer to a Büchner funnel and filter under suction. Cover and let air-dry overnight. Keep 24 hours in a vacuum desiccator over phosphorus pentoxide.

To recover the powder after an analysis, follow the procedure described in the last paragraph.

ANALYTICAL PROCEDURES AND TECHNIQUES

Column Mixture. Place 8.0 ml. of 2,2,4-trimethylpentane in a 250-ml. glass-stoppered bottle. Add 23.5 grams of silane-treated

silicic acid and stopper. Alternately shake and tap the bottle for at least 5 minutes; place on a roller-mixer for 15 minutes.

Pour the powder obtained into a 250-ml. beaker containing 65 ml. of methanol B and stir rapidly to expel air bubbles.

Packing. Packing accessories such as paper disks, packing piston, and spiral stirrer have been described previously (*16*).

Pour the slurry into the glass column using a wash bottle containing methanol B to assist in the transfer. Insert spiral stirrer immediately and work it up and down in long strokes to homogenize the slurry. Then, starting from the bottom and gradually higher up, work the stirrer in short rapid strokes forcing air bubbles to rise and leave the slurry. Allow the slurry to settle by gravity; when settling has almost ceased, make sure that a height of at least 3 cm. of free supernatant liquid is left before inserting a blotting paper disk obliquely until it is completely submerged in the liquid. Insert the packing piston and lower it carefully, pushing the disk in firm contact with the packing. Remove the piston slowly. Add about 5 ml. of methanol B and place a second disk in a similar manner.

Table I. Part of Typical Record Chart

Alkali, Counts		Time, 0.01-Min. Increments			
Total (c)	Addition (δc)	Total (t)	Interval (δt)	$\delta t/\delta c$	Eluted Volume, Ml.
..	..	0	..	..	0
10	10	2648	2648	265	46
20	10	3080	432	43	...
40	20	3835	755	37.7	...
70	30	4613	778	26	...
100	30	4880	267	9	...
170	70	5107	227	3	...
270	100	5327	220	2	Peak
350	80	5571	244	3	...
370	20	5702	131	7	...
380	10	5806	104	10	...
390	10	6011	205	20.5	...
400	10	6611	600	60.0	115
410	10	7011	400	40.0	...
420	10	7249	238	23.8	Receiver emptied
440	20	7512	263	13	...
470	30	7701	189	6	...

Loading. Remove excess methanol B by suction, leaving about 1 ml. over the top paper disk. Allow the alcohol to drain from the paper disk by gravity. With an automatic micropipet (1809 self-adjusting transfer, Lambda-Pette-Research Specialties Co., Berkeley 7, Calif.) add 0.75 ml. of the sample solution. While this drains, place adapter III, unclamped, on the column and connect it to reservoir M. As soon as the sample has cleared the disk, add 0.75 ml. of methanol B, allow it to drain, add another 0.75 ml., and so on. In all, use four washings before connecting adapter III with the source of eluting solvent and clamping it on the column.

In the above sequence, the following rules are very important: The liquid should always be allowed to drain by gravity.

The sample solution, the methanol B immediately preceding, and that immediately following the addition of the sample should drain until the clearly visible edge of the descending meniscus just coincides with the upper edge of the paper disk. In each case, the subsequent addition of liquid must be made just when the edge of the meniscus fades out.

For the remaining two washings the next addition of methanol B is made when the preceding one has cleared the center of the paper disk, leaving the edge still wet.

All additions should be swift and deliberate. When the sample is being added, the tip of the pipet should be located centrally and as close as possible to the paper disk.

Development, Elution, and Titration. At the time this stage of the analysis is reached, the receiver is in working position, filled to mark W (Figure 2) with methanol B at pH 8.7; the stirrer and the pH meter are in operation. Nitrogen has been bubbling through the receiver for at least 0.5 hour and chronometer G (Figure 1) has been reset to zero.

As soon as the flow of eluting solvent is admitted to the column, chronometer G is switched on and 10 counts of alkali are added to the receiver. This is gradually neutralized by acid eluate causing the initial deflection of the pH meter needle, about 0.8 unit, to decrease. The time at which the pH meter needle reaches its initial position (8.7) is noted, and another 10 counts of alkali added to the receiver. Except for an occasional check of the rate of flow and for emptying the flask to mark W whenever the elution of an acid is completed, these two simple steps—adding alkali and noting the time corresponding to the end point—form the pattern of the whole analysis. After a new addition, before the recurrence of the end point, the operator determines for the preceding addition the time-alkali ratio, $\delta t/\delta c$, indicating the concentration of the acid eluted. Guided by this information, the addition of alkali is proportioned to the intensity observed. Thus, 30, 50, or even 100 counts of alkali may be added if the concentration is high and still rising; on the other hand, the size of the addition is reduced when, as indicated by the $\delta t/\delta c$ ratio, the concentration is low or declining. For convenience in calculating $\delta t/\delta c$ at a glance, all additions are made in multiples of 10 counts. Band boundaries corresponding to lowest acid concentrations are indicated by maxima observed in the $\delta t/\delta c$ values. These maxima must be determined as accurately as possible. Consequently, as soon as $\delta t/\delta c$ is observed to rise, each successive addition is limited to 10 counts only. Since periods separating successive end points become increasingly longer in the band boundary region, no difficulty is encountered in securing accurate time readings. No advantage is gained by reducing the size of single additions below 10 counts.

Table I shows the first section of a chart recorded during a typical run carried out on a mixture containing all even acids from C_{12} to C_{24}. Lauric acid appeared 26.48 minutes (the chronometer dial is marked in 0.01-minute graduations) after the onset of elution. The threshold volume (column 5) was 46 ml. as read on the receiver scale. After a few cautious additions of alkali, $\delta t/\delta c$ decreased to a low value, indicating a very strong band. Consequently, the subsequent additions were made larger and larger until the descending slope of the band, as indicated by increasing $\delta t/\delta c$, was reached. The successive additions were then reduced to 10 counts. As soon as the appearance of the C_{14} band became evident, the flask was emptied to mark W in a few seconds by simultaneously pinching clamp Z and the rubber tube on nitrogen outlet G, switching off the stirrer momentarily to permit adjustment of the level. Addition of alkali was then resumed, and the analysis of the following bands was carried out in an identical manner.

Two changes of the pH value taken as end point are made to take into account specific neutralization pH values. These have been determined for the different acids, under the conditions of the analysis, by using a procedure described below. Such changes should never be made in the band boundary regions but somewhere in the middle of the appropriate band where no great accuracy of $\delta t/\delta c$ is required. The first change is made in the C_{16} band (from 8.7 to 8.9), and the second in the C_{18} band (from 8.9 to 9.1).

Routine Analytical Procedure. Prepare the column mixture and pack the column as directed above.

Load with 0.75 ml. of a chloroform solution containing approximately 10 mg. of pure lauric acid per milliliter.

Elute with methanol B, directing the flow of this alcohol from reservoir M through clamped adapter III at a rate of approximately 2 ml. per minute, collecting the eluate in a beaker.

After approximately 120 ml. of eluate have been collected, prepare the receiver as follows: Fill to mark W with methanol B, adding a few drops of 20% sodium chloride solution in water. Put the 20-mm. Teflon-covered magnetic stirrer in place, and set the receiver in working position. Switch on the stirrer, direct the flow of nitrogen through the receiver, and switch on the pH meter.

After approximately 15 minutes, add enough alkali to bring the pH up to 8.7. Stop the stirrer and empty the flask to mark W. Switch on the stirrer and add 10 counts of alkali. Note the volume of eluate necessary to bring the pH back to 8.7. If this volume is less than 40 ml., empty the flask to mark W and add another 10 counts of alkali. When the volume of eluate necessary to bring the pH down to 8.7, after an addition of 10 counts of alkali, is 40 to 50 ml., the column conditioning is completed. The flow of methanol B is then arrested.

Unclamp adapter III and follow the directions given for loading and development, elution, and titration. After the column is loaded, the automatic solvent mixer is put into operation and the flow of eluting solvent directed through adapter III. The indicator in flow meter E (Figure 1) is set and maintained at 8.5, which corresponds to approximately 2 ml. per minute. Occasional small readjustments of the rate of flow should not be made in the region of the band boundaries.

After completing a run, refill the buret to avoid sticking of the piston to the glass body.

Empty the glass column and clean it, first by removing all silicic acid, then by filling it with 1N sodium hydroxide solution which is allowed to percolate through the fritted-glass plate; this is replaced by hot water, followed by 1N hydrochloric acid, more hot water, and finally recovered methanol. Dry in an oven.

Blank Curve and Alkali Standardization. Experience acquired over 2 years of almost daily use of the present method has shown a high consistency of the blank curve correction values and a

remarkable stability of the titrating alkali solution. Both require only an occasional check, which is effected in one single operation. The procedure is based on the fact that the threshold volumes are fairly constant, regardless of the composition of the mixture analyzed. Averages of these volumes for a considerable number of determinations are given below, along with the respective band-width volumes.

Acid	Threshold Vol., Ml.	Band-Width Vol., Ml.
C_{12}	45	70
C_{14}	115	70
C_{16}	185	75
C_{18}	260	75
C_{20}	335	60
C_{22}	395	60
C_{24}	455	55

For the determination of the blank curve, a blank analysis is made, using 0.75 ml. of chloroform to load the column. The complete procedure described above for loading, conditioning, and developing the column is applied in every detail. However, as soon as the volume of eluate corresponding to the threshold volume (115 ml.) for C_{14} is collected, the flow of eluting solvent is arrested by closing valve N, Figure 1. The total amount of alkali (counts) needed to maintain the pH at 8.7 up to that moment is the blank correction corresponding to C_{12}.

When the blank correction has been secured, the flow of nitrogen is substantially increased, the stirrer is switched off, neck T (Figure 2) is uncorked, and 0.75 ml. of a freshly prepared solution of pure lauric acid is added to the receiver contents with the aid of an automatic micropipet. Neck T is immediately stoppered, the flow of nitrogen reduced to normal, the stirrer switched on, and alkali added until the pH is restored to 8.7. The solution used is made from about 70 mg. of carefully weighed lauric acid dissolved in 5 ml. of methanol A.

From the weight of acid used and the amount of alkali necessary to bring back the pH to 8.7, the titer of the alkali, expressed in counts per millimole (or milligram) of acid, is determined. The receiver is emptied to mark W in the usual manner, valve N (Figure 1) is opened, and the elution resumed. This time, 75 ml. of eluate are collected. The procedure for the determination of the blank correction and alkali titer corresponding to the other acids is entirely similar, except that the standard acid solutions used are made in chloroform from C_{16} on. Also, the pH value taken for the end point is changed twice, as shown under Elution and Titration.

The volumes eluted correspond each time to the respective band-width volumes given below. Experience has shown that the normality of the titrating alkali, as determined under the above conditions, is not identical for each acid. Since these determinations are made under conditions duplicating those of the routine analysis, the titers corresponding to each acid should be used in calculating the analytical results. The problem is greatly simplified by the fact that a simple relationship exists between the different normalities. Expressing that found to correspond to palmitic acid as 100, the specific normalities for the acids from C_{12} to C_{24} are as follows:

Acid	Relative Sp. Normality of Alkali
C_{12}	99.5
C_{14}	100.5
C_{16}	100
C_{18}	99.5
C_{20}	99.5
C_{22}	97.5
C_{24}	97.5

It is thus only necessary to determine the titer corresponding to pure palmitic acid and to calculate the others from the above relative values.

The average value of the blanks resulting from a series of determinations is given below.

Volume Eluted, Ml.	Blank (Counts)
115	13
185	8
260	12
335	15
395	11
450	10
520	12

Plotting these values provides a blank curve allowing the correction for any given volume interval to be estimated.

Blank values were found to vary not more than 1 or 2 counts for each case examined and the above averages have been successfully used for months.

Specific Neutralization pH Values. These values were determined by using a procedure differing from that described for the determination of specific titer values only in that complete potentiometric titration curves were established for each acid. The specific pH values were then found from these curves in the usual manner. The use of these values in the detection of end points increases appreciably the sensitivity of the pH meter to changes in hydrogen ion concentration and is conducive to higher accuracy. Although the pH values found differed with each acid, only three values are used in the present procedure to take these differences into account. The selected values are close enough to the actual ones and the simplification does not involve any appreciable loss in sensitivity except in what concerns lauric acid. In this case, the initial pH value, 8.7, is appreciably different from the specific value, 8.2, found for the acid; results obtained for lauric acid are thus, as a rule, somewhat less reliable.

Calculations. The minimum alkali increment being 10 counts, the maxima for the $\delta t/\delta c$ ratios as observed do not correspond exactly to the band boundaries. The final c values are obtained by using a simple graphical interpolation procedure, plotting the inverse of $\delta t/\delta c$ against the corresponding c values obtained in the immediate neighborhood of the observed maxima. The interpolated c value corresponding to the example in Table I is 403 counts. The correct c values having thus been established, the amount of alkali corresponding to each band is obtained by difference; the blank correction corresponding to each band volume is then applied.

The resulting corrected amounts of alkali corresponding to each acid are expressed in counts. By using the specific titers and the appropriate general conversion factors, counts are converted to millimoles or milligrams of acid. Special cases, involving very small amounts of component acids are dealt with below.

EXPERIMENTAL

Acid Mixtures. Results obtained from synthetic acid mixtures (A and C) and from unknown mixtures (B and D) are presented below. Mixtures A and C were made using pure acids with corrected melting points and reported melting points (*6*) as follows:

Acid	Corrected M.P., ° C.	Reported M.P., ° C.
C_{12}	43.7	43.5
C_{14}	55.2	54.4
C_{16}	62.5	62.9
C_{18}	69.6	69.6
C_{20}	75.2	75.4
C_{22}	80.4	80.0
C_{24}	83.9	84.2

From C_{12} to C_{18} included, these acids were obtained by carefully distilling the corresponding Eastman Kodak (white label) methyl esters in a Podbelniak semiautomatic microanalyzer, saponifying the constant boiling fractions, and recrystallizing the recovered acid from aqueous ethanol and aqueous acetone. The C_{20} and C_{22} acids were both synthesized by repeatedly applying a known procedure (*17*) starting from pure stearic acid. Lignoceric acid was synthesized from pure behenic acid by converting it to its methyl ester, reducing it to the corresponding alcohol with lithium aluminum hydride, and applying from there on the method of Bleyberg and Ulrich (*1*). The final yield in pure lignoceric acid was slightly better than 80% calculated on the methyl behenate used (10 grams).

Mixture B was obtained by submitting about 5 grams of herring oil to methanolysis and treating the resulting methyl esters with an excess of potassium permanganate in boiling acetone. After

Table II. Results from Mixture A Containing Acids from C_{12} to C_{24} in Unequal Amounts[a]

Acids	Theory, Mg.	Found, Mg.	Recovery, %	Volume Eluted, Ml. Total	Band
C_{12}	1.08	1.12	103.7	102	60
C_{14}	5.31	5.32	100.2	195	73
C_{16}	11.08	11.09	100.2	244	69
C_{18}	17.52	17.30	98.7	311	67
C_{20}	18.18	18.13	99.7	368	57
C_{22}	9.72	10.02	103.1	428	60
C_{24}	3.94	3.91	99.2	485	57
Total	66.83	66.89			

[a] Total time, 4 hours, 13 minutes.

Table III. Results from Mixture B[a] and Mixture C[b]

Acids	Mixture B Run 1 Found, mg.	Mixture B Run 1 Composition, %	Mixture B Run 2 Found, mg.	Mixture B Run 2 Composition, %	Mixture C Found, mg.	Mixture C Composition, %
C_{12}	0.94	2.0	0.94	2.0	0.74	1.6
C_{14}	13.23	28.2	13.12	28.2	13.00	28.0
C_{16}	27.82	59.0	27.68	59.4	27.82	59.9
C_{18}	3.63	7.7	3.63	7.8	3.63	7.8
C_{20}	1.46	3.1	1.24	2.6	1.25	2.7
C_{22}	0	0	0	0	0	0
C_{24}	0	0	0	0	0	0
Total	47.08	100.0	46.61	100.0	46.44	100.0

[a] Mixture of saturated acids from herring oil.
[b] Synthetic mixture made to represent composition found for mixture B.

excess oxidant and manganese dioxide had been destroyed with potassium bisulfite, the mixture was acidified and extracted with chloroform and the dried chloroform solution was percolated through a column of alumina until acid-free. The chloroform solution then yielded on evaporation the methyl esters corresponding to the saturated acids originally contained in the sample of herring oil (*13*). Complete removal of unsaturated components was checked (iodine value); saponification then led to the free acids from which a 70 mg. per ml. solution in pure chloroform was prepared.

For mixture D a 1-gram sample of herring oil was submitted to methanolysis. The resulting methyl esters were completely hydrogenated in 1 liter of 95% ethanol at room temperature using platinum dioxide as catalyst. The acids were obtained from the saturated esters through saponification, and a 70 mg. per ml. solution of these acids in chloroform was prepared.

DISCUSSION

Results. The results shown in Tables II, III, and IV were obtained by performing the analyses in a room where the temperature was automatically maintained at 25° ± 1° C.

In illustrating a chromatographic method of this type, the presentation of results obtained with mixtures of components in about equal amounts is unrealistic and might be misleading. Cases corresponding to such mixtures are very rarely met in practice, but the performance of any method will be much better, generally speaking, with this type of mixture than with highly disproportionated ones, such as mixture A in Table II. In this example, the recovery obtained for lauric acid, 103.7%, although not uncommonly good for such small quantity, is admittedly better than average.

As stated previously, the error on minor components (1 to 3% of total mixture) is apt to reach 20% for a single determination, owing to small systematic errors which bear heavily on the results obtained for the minor components. If a degree of precision comparable to that obtained with major components is desirable, the following method is applied: A synthetic mixture, made to represent the composition found for the unknown, is prepared and analyzed. The results are then used to correct the previous ones. The method is based on the fact, illustrated by duplicate runs in Table III and Table IV, that the chromatograms from a given mixture are quantitatively reproducible within narrow limits. Experience has shown that the correction applied should be not the whole difference between "unknown" and "synthetic" compositions found but only half that difference. In the case at hand, the corrected composition for C_{12} is thus 2.2%.

In general, less complex mixtures lead to more accurate results. They also require lesser amounts of sample material. An average of 7 mg. per component acid has proved adequate in determining total sample size.

A comparison of volumes eluted (Tables II and IV) shows a good consistency of band volumes; with total and threshold volumes the consistency is even better.

When acids in the series are missing entirely or present in very small amounts (from 0 to about 0.5% of the total mixture), the band immediately preceding the missing or feeble one stretches somewhat beyond the average limits given above. If the following acid is completely absent, the band will stretch 10 to 12 ml. beyond the normal value. If a very small amount of the next acid is present, a distinct band may not form but trailing of the preceding band will occur. An example is given in Table IV, where the band volume for C_{22} is abnormally long. A systematic study of appropriate synthetic mixtures has established the following rule: If a well-defined band trails beyond the corresponding average band volume by about 10 ml. only, the next higher even acid is absent; any acid eluting immediately beyond this point is taken, less the appropriate blank correction, as the next higher acid. In the example at hand one is thus justified in taking as behenic acid that which has eluted with the first 72 ml. of effluent; the acid contained in the extra 12 ml. is then taken as lignoceric acid.

Table IV. Results from Mixture D[a]

Acids	Run 1 Found, mg.	Run 1 Composition, %	Run 1 Band volume, ml.	Run 2 Found, mg.	Run 2 Composition, %	Run 2 Band volume, ml.
C_{12}	0.73	1.3	60	0.52	0.94	61
C_{14}	3.11	5.6	52	3.14	5.7	62
C_{16}	14.93	26.8	64	14.93	27.1	68
C_{18}	11.18	20.0	61	10.88	19.8	63
C_{20}	12.08	21.6	62	11.76	21.4	62
C_{22}	13.77[b]	24.7	84[b]	13.77[b]	25.0	83[b]
C_{24}	...	<1	..	...	<1	..

[a] Made of hydrogenated total fatty acids of herring oil.
[b] Limiting to normal band volume, result is 13.26 mg. in each case.

The final composition obtained for mixture D as a result of the application of this rule is shown in the first column of Table V as the average for runs 1 and 2.

The composition of mixture B (run 2) was recalculated, using as described the data from synthetic mixture C. The corrected composition is shown in the second column. It was estimated from data obtained through analysis of synthetic mixtures that

Table V. Composition of Herring Oil Sample Calculated from Chromatographic Data Obtained from Mixture B[a] and Mixture D[b]

Acids	Mixture D (Run 2), %	Mixture B, %	Herring Oil, % Saturated	Herring Oil, % Unsaturated
C_{12}	2.20	1.12	0.43	0.69
C_{14}	28.30	5.65	5.55	0.10
C_{16}	59.1	27.00	11.58	15.42
C_{18}	7.8	19.90	1.53	18.4
C_{20}	2.6	21.50	0.51	21.0
C_{22}	0	24.00	0	24.0
C_{24}	0	0.79	0	0.79
Total	100.00	100.00	19.60	80.40

[a] Hydrogenated acids.
[b] Saturated acids.

the latter type of corrective procedure decreases the maximum error on minor components (1 to 3% of the total mixture) to less than 5% and that on major components (≥ 10% of the total mixture) to less than 1%. Discrepancies between original values (run 2, Table III) and corrected values (second column, Table V) are, except for lauric acid, smaller than 1%.

The third and fourth columns in Table V show the composition of herring oil fatty acids obtained by combining the values in the first two columns; the percentage of total saturated acids (19.6%) was determined by weighing the saturated acids (mixture B) obtained as described by the method of Schuette and Dal Nogare (*13*) from a 5-gram sample of oil.

Equipment. The capacity of the piston microburet, about 22 ml., was made larger than in commercial instruments to permit the use of weaker titrant. A higher degree of precision is thus attained and errors arising from alkali diffusion through the delivery tip are overcome without resorting to extremely fine, easily plugged orifices.

The buret piston and body are easily removed, cleaned, replaced (eventually by another size), and connected to any other titrant supply. Removing the column thus provides a convenient general purpose installation for cases requiring precision titration of small samples.

The complete apparatus applies, with minor modifications, to the chromatographic separation of the lower aliphatic acids (*16*).

Mixing flask *A* in the solvent mixing device used (Figure 3) is similar to one proposed by Bock and Ling (*2*) but was developed independently.

Band volumes (Tables II and IV) show the remarkable effect attained by the proper combination of initial solvent volume and concentration which brought the successive bands at almost regular intervals. The initial solvent volume used was measured by slipping the magnet stirrer in the empty flask, filling the flask to the brim with water, and fitting it with adapter *D*, the tip of which had been plugged. The volume of the water trapped in the flask up to the tip of the side arm was 262 ml. A careful check should be made of the initial solvent composition. While a small increase in the water content would not affect results unfavorably, the reverse would lead to poorer separations. The density of the methanol C used in the present work was 0.883 at 25° C. Methanol B must not be substituted.

Column Mixture. Early attempts to procure the promising Mealorub first described by Boldingh (*3*) met with obstacles also reported by other workers (*14*). The column mixture of kieselguhr and paraffin oil proposed by Howard and Martin (*8*), along with their procedure for silane treatment of the column material, did not seem to offer a degree of definition conducive to reproducibility. Consequently, a well-defined granular fraction from a controlled commercial silicic acid was selected as a starting material, and a standardized procedure for the silane treatment was evolved.

Selection of the adequate granular fraction of silicic acid resulted in columns sufficiently porous to allow the use of a relatively small hydrostatic head, thus eliminating all disadvantages inherent in the use of gas pressure. A practically constant head was provided by using a wide solvent reservoir. The change in level resulting from the withdrawal of the quantity of solvent required by one operation, about 500 ml., corresponded to about 1% of the initial liquid head. Under such conditions, the rate of flow is easily controlled and maintained to a fixed value by occasional resetting of the needle valve.

The amount of silicone material present in the final silicic acid powder is directly related to the amount of water added to the silicic acid prior to treatment with excess dichlorodimethylsilane. A systematic study of the properties of treated powders, corresponding to initial additions of water ranging from 3 to 11%, showed 6% water to be the optimum when separating power, permeability, ease of packing, and column stability were taken into consideration.

Packing and Loading. The sequence of operations described for loading the column with the sample solution resulted from a systematic study involving various solvents and solvent combinations both to make up the solution and to wash it down the column. Care in following the described loading schedule was found of major importance.

On developing the column, a translucent ring appears early at the top, progressing slowly down the first quarter of the column height. Correct manipulations will produce a thin, even ring; a jagged, broad, or lopsided ring indicates either defects in the disk size or shape, or a faulty, uneven loading of the column.

APPLICATIONS

The range C_{12} to C_{24}, covered in a single operation, makes the present method directly applicable to the vast majority of fats, a surprisingly large number of which contain acids belonging to one or both extremes of this range. Among these are the marine oils, fats from the brain, liver, and other organs of many land animals, and fats from the seeds of plants growing in the temperate, tropical, and subtropical zones. Carbon series below C_{12} or above C_{24} are comparatively rare. In the marine oils, they are very sparingly represented, when present at all. Fats containing keto-, hydroxy-, cyclic, and branched-chain acids would not be directly tractable, but these are of exceptional occurrence (*7*).

The method should be particularly useful in giving quantitative information concerning variations affecting the fatty acid composition of naturally occurring lipides of interest to science and economy; differences arising in the same species of plant or animal are of particular interest in that they may reflect important biological or pathological phenomena.

Among a host of accessible technological problems are those involving control and monitoring of fat fractionating processes, analysis and control of commercially produced fat mixtures, and detection of adulteration in fatty foods and of impurities in samples of fatty acids and derivatives.

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LITERATURE CITED

(1) Bleyberg, W., Ulrich, H., *Ber.* **64**, 2504 (1931).
(2) Bock, M. R., Ling, N.-S., ANAL. CHEM. **26**, 1543 (1954).
(3) Boldingh, J., *Rec. trav. chim.* **69**, 247 (1950).
(4) Crombie, W. M. L., Comber, R., Boatman, S. G., *Biochem. J. (London)* **59**, 309 (1955).
(5) Crombie, W. M. L., Comber, R., Boatman, S. G., *Nature* **174**, 181 (1954).
(6) Hilditch, T. P., "Chemical Constitution of Natural Fats," 2nd ed., Wiley, New York, 1947.
(7) Hilditch, T. P., *J. Sci. Food Agr.* **12**, 557 (1954).
(8) Howard, G. A., Martin, A. J. P., *Biochem. J. (London)* **46**, 532 (1950).
(9) Nykamp, H. J., *Anal. Chim. Acta* **10**, 448 (1954).
(10) Nykamp, H. J., *Nature* **172**, 1102 (1953).
(11) Popják, G., Tietz, A., *Biochem. J. (London)* **56**, 46 (1954).
(12) Savary, P., Desnuelle, P., *Bull. soc. chim. France* **1953**, 939.
(13) Schuette, H. A., Dal Nogare, S., *J. Am. Oil Chemists' Soc.* **28**, 229 (1951).
(14) Silk, M. H., Hahn, H. H., *Biochem. J. (London)* **56**, 406 (1954).
(15) *Ibid.*, **57**, 577 (1954).
(16) Vandenheuvel, F. A., Hayes, E. R., ANAL. CHEM. **24**, 960 (1952).
(17) Vandenheuvel, F. A., Yates, P., *Can. J. Research* **28B**, 556 (1950).

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Separation and Detection of Cyanamide *and Its* Derivatives and Determination of Urea by Paper Chromatography

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Cyanamide and its derivatives which were formed in the manufacture of dicyandiamide were chromatographed with 1-butanol–ethyl alcohol–water (4:1:1) and with methyl ethyl ketone–petroleum ether–water (9:4:3) on Whatman No. 3 and No. 3MM filter paper. Sixteen spots were detected on the chromatograms; cyanourea, biguanide, melamine, three salts of guanidine, urea, guanylthiourea, thiocyanate, thiourea, dicyandiamide, and cyanamide were characterized. Quantitative analysis for urea gave a recovery of 95%.

DURING the manufacture of dicyandiamide from cyanamide, by-product formation occurs from the reaction of these substances with water or with impurities introduced into the reaction mixture from the raw material. In a continuous process, these by-products and their decomposition products are allowed to remain in the system, and further reaction may occur slowly with cyanamide and dicyandiamide to consume further amounts of both substances.

Much effort has been devoted to the development of methods of analysis of the compounds likely to be formed (*6*, *14*), but these procedures as well as unpublished modifications of them have yielded results of questionable accuracy.

The use of paper chromatography as an aid in studying the nature and quantities of these compounds in solutions of unknown composition has not been reported, although the development of urea, guanidine, and guanidine derivatives on paper chromatograms has been described (*1–3*, *10*, *12*). When the work reported here was nearly complete, it came to the authors' attention that Hübener and others (*7*) had quantitatively determined urea on paper chromatograms. Measurements of urea involved the addition of *p*-dimethylaminobenzaldehyde to the chromatogram to form a yellow complex which was eluted with pyridine and measured spectrophotometrically at 449 mμ. This procedure was modified recently by Bode and Ludwig (*5*).

EXPERIMENTAL

Apparatus. Borosilicate glass chromatography jars with paper support racks and solvent assemblies, and a Chromatocab were purchased from the Schaar Co., Chicago.

Reagents were sprayed on the developed chromatograms with a laboratory constructed glass atomizer.

A Beckman Model DU spectrophotometer with Corex cells of 1.002-cm. light path was used for the urea determinations. The slit width was kept constant at 0.16 mm.

Developing Solvents. Numerous solvent mixtures consisting of various combinations of 1-butanol, methyl ethyl ketone, ethyl alcohol, water, petroleum ether, ammonia, and acetic acid were tested as the mobile phase on Whatman No. 3 filter paper. This paper was used because of its high capacity for solids. The paper was not prewashed and the chromatograms were developed over a temperature range of at least ±5° C. from room temperature without any noticeable change in the resolution of the various cyanamide derivatives. The solvent which gave the best separation of most of the compounds was a solution of 1-butanol–ethyl alcohol–water (4:1:1). The organic layer of a mixture of methyl ethyl ketone–petroleum ether–water (9:4:3) was employed, however, to separate dicyandiamide, thiourea, and the thiocyanate ion which formed only one spot with the former solvent. Considerable streaking occurred with this solvent on Whatman No. 3 filter paper, but this was partly corrected by using Whatman No. 3MM.

The discovery of these combinations of solvents was fortunate because of the desirability to use neutral solvents, since the substances under investigation were subject to chemical reaction in an acid or basic medium.

Reagents. Alkaline ferricyanide–nitroprusside (FCNP), ammoniacal silver nitrate (AmAg), *p*-dimethylaminobenzaldehyde (DAB), and 2,6-dichloroquinonechloroimide (DCC) were prepared according to the procedures of Berry and others (*2*, *3*). Sullivan's reagent, 1,2-naphthoquinone-4-sodium sulfonate (NQS) (*11*), for detecting guanidine on paper chromatograms, was adapted from the procedure of Bertrand and Myers (*4*) for determining guanidine in eluates from ion exchange resins.

Preparation and Use of Reagents. All solvents and reagents for spraying chromatograms were analytical reagent grade.

REAGENT FERRICYANIDE-NITROPRUSSIDE. Equal volumes of 10% sodium hydroxide, 10% sodium nitroprusside, and 10% potassium ferricyanide were mixed and diluted with 3 volumes of distilled water. Allowed to stand for 20 minutes, the dark brown solution turned pale yellow and the reagent was ready for use. This reagent was unstable at room temperature, but could be kept for 2 or 3 weeks in the cold without deterioration. Colored spots occurred immediately after spraying with the reagent.

REAGENT AMMONIACAL SILVER NITRATE. Equal volumes of 0.1*N* silver nitrate and 5*N* ammonium hydroxide from previously prepared stock solutions were mixed. After spraying with this reagent, some areas appeared immediately and further development was obtained by heating the chromatogram at 100° C. for 10 minutes.

REAGENT *p*-DIMETHYLAMINOBENZALDEHYDE. Two grams of *p*-dimethylaminobenzaldehyde were dissolved in 100 ml. of 1.2*N*

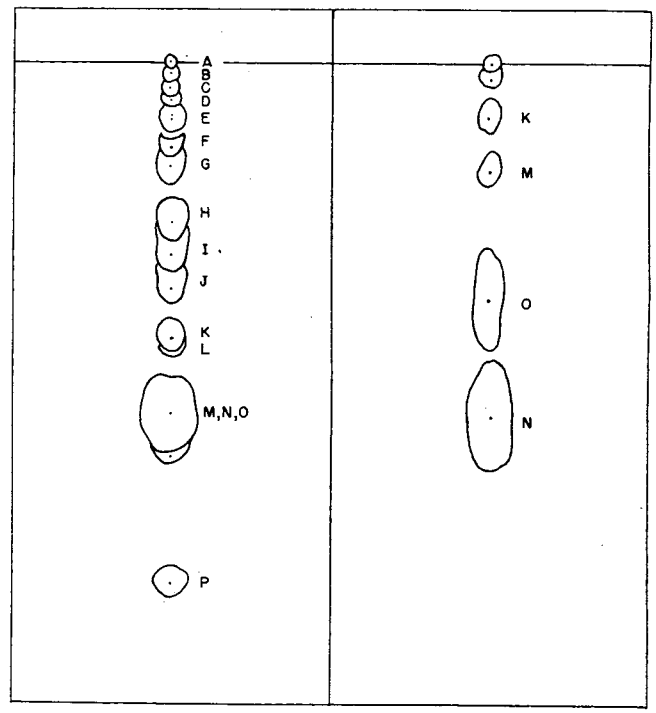

Figure 1 (*left*) and Figure 2 (*right*). Chromatograms of cyanamide and its derivatives

Developed with 1-butanol–ethyl alcohol–water (4:1:1) for 23 hours

Developed with methyl ethyl ketone–petroleum ether–water (9:4:3) for 30 hours

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hydrochloric acid. This reagent could be kept for 3 to 5 days in the cold without deterioration. Yellow colors appeared immediately after spraying.

REAGENT DICHLOROQUINONECHLOROIMIDE. One gram of 2,6-dichloroquinonechloroimide was dissolved in 100 ml. of absolute ethyl alcohol. Storage in the cold kept the reagent for 2 to 3 weeks. The colors produced by this reagent reached their maximum intensity after 2 to 3 hours.

REAGENT 1,2-NAPHTHOQUINONE-4-SODIUM SULFONATE. Four volumes of a freshly prepared 1% solution of 1,2-naphthoquinone-4-sodium sulfonate and 1 volume of 4*N* sodium hydroxide were mixed immediately before use. After being sprayed with this solution, the chromatogram was heated in an oven for 2 minutes at 100° C. and then sprayed consecutively with a 2.5% solution of urea, concentrated hydrochloric acid, and concentrated nitric acid (density 1.42).

Method. PREPARATION OF CHROMATOGRAMS. Fifty microliters of dicyandiamide mother liquor were applied in the normal manner to an area 1 cm. in diameter on the starting line of a sheet of Whatman No. 3 paper, 6 × 22.5 inches. The chromatogram was developed for 23 hours with a solution of 1-butanol–ethyl alcohol–water (4:1:1) by the descending method, and after drying in a well-ventilated fume hood, the chromatogram was sprayed with the desired reagent. Development on Whatman No. 3MM paper with the organic layer of a mixture of methyl ethyl ketone–petroleum ether–water (9:4:3) required only 5.5 hours for the solvent front to reach the bottom of the paper, but for good resolution the chromatogram was developed for 30 hours. One spray alone did not reveal the position and size of all the spots developed with 1-butanol–ethyl alcohol–water and the chromatogram illustrated by Figure 1 was prepared from the combined information derived by spraying chromatograms with ammoniacal silver nitrate, 1,2-dichloroquinonechloroimide, and alkaline ferricyanide-nitroprusside. The latter reagent was used to spray the chromatogram illustrated by Figure 2.

Tables I and II list R_f and R_t values of the spots and the colors developed with the reagents. R_t values (distance a compound moved relative to thiourea) were of greater aid than R_f values in characterizing the compounds, as only a partial separation had occurred when the solvent front had reached the bottom.

Table I. Comparison of Constituents in Dicyandiamide Mother Liquor with Authentic Cyanamide Derivatives

Paper. Whatman No. 3
Developing solvent. Solution of 1-butanol–ethyl alcohol–water (4:1:1)
Sample. 0.05 ml. mother liquor

Compound	R_f	R_t	FCNP	AmAg		DAB	DCC	NQS[a]	
				After spraying	After heating			After heating	After final spraying
A		0.00	+ Orange-red	−	+ Light gray	+ Blue-green	+ Purple-gray	−	−
B		0.03	+ Green-blue	+ Light gray	+ Gray	−	+ Orange	−	−
C		0.07	−	−	+ White	−	+ Purple	−	−
D		0.10	−	−	+ Light gray	−	Faint orange	−	−
E		0.17	+ Orange	−	++ White	+ Yellow	++ Purple	−	−
Potassium cyanoureate	0.04	0.19	+ Orange	−	++ White	+ Yellow	++ Purple		
R		0.22	++ Red	−	++ White	−	+ Purple	++ Yellow	++ Yellow
Biguanide	0.08	0.21	++ Orange-red			−		++ Yellow	++ Yellow
G		0.29	−	−	++ White	+ Yellow	+ Gray	−	−
Melamine	0.08	0.22	−	−	++ White	+ Yellow	+ Gray		
Guanylurea carbonate	0.18	0.52	++ Purple	−	+ Dark brown	−	+ Gray brown	−	−
H		0.45	++ Orange	−	++ White	−	+ Purple	++ Red-blue	++ Red
I		0.54	++ Orange	−	Light brown with dark brown trim on the bottom	−	+ Brown gray	++ Red-blue	++ Red
J		0.64	+ Light orange	−	+ Light brown	−	−	++ Red-blue	++ Red
Guanidine carbonate		Streaked from 0.22–0.55	++ Orange	−	++ Dark brown with white top	−	++ Purple	++ Red-blue	++ Red
K		0.77	++ Red	−	+ Light brown	++ Yellow	−	−	−
Urea	0.35	0.79	+ Red	−	+ Light brown	++ Yellow	−	−	−
L		0.82	+ Blue-green	+ Brown	+ Brown	−	+ Red-brown	−	−
Guanylthiourea	0.35	0.80	++ Green turned yellow after 1 min.	++ Brown	++ Dark brown	−	+ Red-brown		
M, N, O		1.00	++ Blue, turned yellow after 1 min.	++ Brown	++ Gray-brown	+ Yellow	++ Light brown	Red-blue	+ Red
Ammonium thiocyanate	0.38	0.86	++ Blue	−	++ White	+ Red on standing	++ Red-brown		
Thiourea	0.49	1.00	++ Blue, turned yellow after 1 min.	++ Brown	++ Gray-brown	+ Yellow	++ Red-brown		
Dicyandiamide	0.51	1.05	++ Magenta	−	++ White	−	−		
P		1.47	++ Magenta	++ Yellow	++ Yellow	++ Yellow	Slight gray	++ Orange-red	+ Yellow
Cyanamide	0.61	1.50	++ Magenta	++ Yellow	++ Yellow	++ Yellow	Slight gray	++ Orange-red	+ Yellow

$R_t = \frac{\text{distance moved by compound}}{\text{distance moved by thiourea}}$.
Negative test. −
Positive test. +
Very positive test. ++

[a] Compound P and cyanamide gave orange-red color after spraying with alkaline solution of 1,2-naphthaquinone-4-sodium sulfonate. No colors were produced with other compounds.

Table II. R_f Values of Constituents in Dicyandiamide Mother Liquor Developed with Organic Layer of a Mixture of Methyl Ethyl Ketone–Petroleum Ether–Water (9:4:3)

Paper. Whatman No. 3MM.
Sample. 0.02 ml. mother liquor

Compound	R_f	FCNP	AmAg		DAB
			After spraying	After heating	
Unknown	0.00	++ Orange	–	+ White	+ Yellow
Unknown	0.02	++ Red	+	+ White	–
K (urea)	0.15	+ Red	–	+ Gray	++ Yellow
M (thiocyanate and another compound)	0.30	+ Buff	–	+ Gray	+ Red on standing
O (dicyandiamide)	0.66	++ Magenta	–	++ White	–
N (thiourea)	1.00	++ Blue, center turned yellow in 1 minute after spraying	++ Brown	Brown-gray center, gray ring	+ Yellow

A comparison of the mobilities and color reactions of the constituents of the mother liquor with those of authentic cyanamide derivatives indicated that compounds E, F, G, K, L, M, N, O, and P in Figures 1 and 2 and Tables I and II were cyanourea, biguanide, melamine, urea, guanylthiourea, thiocyanate, thiourea, dicyandiamide, and cyanamide, or salts thereof. The spots listed in Table II as unknown were those compounds which had low mobilities in the methyl ethyl ketone–petroleum ether–water solvent and did not separate in the manner illustrated by Figure 1. Compounds H, I, J, guanidine carbonate, and guanidine nitrate gave a positive test with Sullivan's reagent, which indicated that the three unknowns were probably different salts of guanidine. Compound J could not be identified as guanidine nitrate, although this salt had the similar R_f value of 0.65, since no nitrate ions were introduced into the system. One of the salts was presumed to be guanidine carbonate, however, even though this compound formed a long streak from the starting line, and another was thought to be guanidine cyanate. The presence of cyanate ions in the mother liquor was indicated from the number of equivalents of conjugate acids with pK_a values between 4 and 5. All of these acids could not be accounted for by melamine and cyanourea (*8*).

Quantitative Determination of Urea. Approximately 6 mg. of urea in a weighed aliquot of the solution (0.3 to 0.4 gram) were distributed over 10 spots on the starting line of a sheet of Whatman No. 3 filter paper, 13 × 22.5 inches. Three guide strips, one of which was located at each end of the chromatogram and one in the middle, were spotted with the solution and the chromatogram was developed for 30 hours with 1-butanol–ethyl alcohol–water (4:1:1). After drying, the guide strips were sprayed with alkaline ferricyanide-nitroprusside to locate the urea, and the urea-containing zones in the unsprayed sections of the chromatogram were cut out and then cut diagonally in half to form two triangles. These papers were hung vertically between glass rods above a large funnel fitted into a 25-ml. volumetric flask and washed with about 22 ml. of water. The eluate was then made up to volume.

Table III. Analysis of Standard Urea Solutions

% Urea		Recovery, %
Found	Theoretical	
0.92	0.97	95
1.69	1.76	96
2.18	2.29	95.5

A 10-ml. aliquot of the eluate was mixed with 10 ml. of a solution of *p*-dimethylaminobenzaldehyde (*13*), diluted to 25 ml. with distilled water, and after standing for 20 minutes the intensity of the yellow solution was measured with a Beckman DU spectrophotometer at 420 mμ. The urea concentration was determined from a calibration curve obtained with standard urea solutions. Beer's law was obeyed, with an absorptivity at 420 mμ of 3.96.

Table III shows that urea solutions varying in concentration from 1 to 2.3% could be analyzed by paper chromatography with a recovery of 95%.

Table IV. Determination of Urea in Presence of Other Cyanamide Derivatives

Sample	% Urea	
	Chromatographic method	Precipitation method
Dicyandiamide mother liquor	1.81	1.81
	1.79	1.80
		1.82
	1.93	1.95
	1.96	1.97
Cyanamide solution	1.31	1.39
	1.38	1.40
	1.80	1.70
	1.79	1.66
	1.67	1.63
	1.74	1.67

When a chromatogram of the constituents in commercial dicyandiamide liquors was sprayed with the *p*-dimethylaminobenzaldehyde reagent, yellow areas developed with the cyanoureate ion, melamine, urea, thiourea, and cyanamide. Only urea and cyanamide, however, formed a complex which showed any appreciable absorption at 420 mμ. Table IV gives the results obtained for the determination of urea in a number of liquors by the chromatographic method and by a procedure that involved the removal of cyanamide and thiourea with silver nitrate prior to adding the reagents for spectroscopic measurements. The data obtained by chromatography were corrected on the basis of the per cent recovery shown in Table III.

DISCUSSION

Paper chromatography has been useful for elucidating the nature of the principal compounds which were present in solutions in various parts of the dicyandiamide process, and an estimate of the number of compounds in trace amounts has been obtained. The method should also be suitable for detecting and identifying cyanamide and its derivatives in other sources. Because a large number of compounds can be formed theoretically from cyanamide, the possibility remains, however, that other compounds were present which did not give a color reaction with

the reagents used in this work. Indeed, from an inspection of the contour of biguanide, it appeared that a compound was present between cyanourea and biguanide.

With the exception of dicyandiamide, urea, and thiourea, the other compounds were acids and bases and it is possible that a compound could be located in more than one spot in order to preserve electrical neutrality of the spots. Compounds H, I, and J were characterized in part as the guanidinium ion, but the identity of the anions was not determined with certainty. The nature of compound B, which appeared from its color reactions to contain sulfur and which was not thiourea, guanylthiourea, or thiocyanate, is open to speculation. An ultraviolet absorption spectrum of an aqueous eluate of this zone gave a band at 236 mμ which is the wave length maximum reported for thiourea (*9*), indicating the presence of a closely related molecule.

Only the areas corresponding to the guanidinium salts underwent the Sullivan reaction. However, an orange color was formed by cyanamide when an aqueous solution of 1,2-naphthaquinone-4-sodium sulfonate and alkali was sprayed on the chromatogram and this color disappeared in successive stages of the Sullivan reaction. These two reactions suggest specific methods of analysis for cyanamide and guanidine in mixtures of related compounds.

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LITERATURE CITED

(1) Adachi, S., *Kagaku* **23**, 582 (1953).
(2) Berry, H. K., Biochemical Institute Studies IV, pp. 88–92, Univ. Texas Publ. 5109, Austin, Tex., May 1, 1951.
(3) Berry, H. K., Sutton, H. E., Cain, L., Berry, J. S., *Ibid.*, pp. 22–55.
(4) Bertrand, M., Myers, J. L., *Can. J. Chem.* **31**, 1252 (1953).
(5) Bode, F., Ludwig, U. M., *Schweiz. med. Wochschr.* **84**, 629 (1955).
(6) Bourjol, S., Teindas, Mrs., *Mém. poudres* **31**, 51 (1949).
(7) Hübener, H. J., Bode, F., Mollat, H. J., Wehner, M., *Hoppe-Seyler's Z. physiol. Chem.* **290**, 136 (1952).
(8) Kennerly, G. W., unpublished data.
(9) Mason, S. F., *J. Chem. Soc.* (*London*) **1954**, 2071.
(10) Roche, J., Van Thioai, N., Hatt, J. L., *Biochem. et Biophys. Acta* **14**, 1, 71 (1954).
(11) Sullivan, M. X., *Proc. Soc. Exptl. Biol. Med.* **33**, 106 (1935).
(12) Tuppy, H., *Monatsh. Chem.* **84**, 342 (1953).
(13) Watt, G. W., Chrisp, J. D., ANAL. CHEM. **26**, 452 (1954).
(14) Williams, H. E., "Cyanogen Compounds," Edward Arnold & Co., London, 1948.

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Rapid Paper Chromatography of Carbohydrates and Related Compounds

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This method is useful for the rapid separation and tentative identification of carbohydrates and related compounds in biological fluids and extracts. No desalting or other purification is required. Interference by inorganic salts is prevented by a simple "overspotting" technique using pyridinium sulfate. Ascending one-dimensional chromatograms are completed in 2 hours. Spots of carbohydrates, polyhydric alcohols, aldonic and uronic acids, nucleosides, phosphate esters, and other derivatives are semiquantitatively detected by improved specific color reagents. R_f values are highly reproducible; this makes possible an analysis of some of the causes of variation in R_f, especially the systematic variations due to interference by inorganic ions and to varying loads. The method is not suitable for the complete separation of structurally similar carbohydrates or of a large number of carbohydrates and closely related compounds on one chromatogram; it is designed for the rapid identification of a small number of carbohydrates in complex biological systems containing considerable quantities of many other materials.

THE basic objective of the work reported in this paper has been to develop a rapid method of separation and tentative identification of carbohydrates and related compounds in biological fluids without preliminary purification. This research was instigated by the discovery that an isopropyl alcohol–pyridine-water–acetic acid solvent, used previously for paper chromatography of alkali and alkaline earth cations (*5*), has the useful property of moving sugars, polyols, uronic acids, purine, and pyrimidine ribosides, and their phosphate esters all within the R_f range of 0.1 to 0.9. The solvent tends to separate carbohydrates and their derivatives into groups of similar structure, all moving within a narrow R_f range—e.g., pentoses have an R_f of 0.70 to 0.77, and hexuronic acids have an R_f of 0.33 to 0.38. This is useful for a preliminary classification of unknowns; more specialized solvents, with higher resolving power, can then be used to separate and identify individual compounds.

The solvent has other useful properties. Inorganic salts normally do not interfere with the movement of organic compounds on the chromatogram, and desalting is usually unnecessary. The low viscosity of the solvent allows it to ascend rapidly, and an ascending one-dimensional chromatogram can be completed in 2 hours. The high volatility of the solvent makes possible complete drying of the chromatogram in 15 minutes. The solvent power is high, and loads of several hundred micrograms of most carbohydrates move as compact spots, without streaking. The commonly occurring inorganic ions (potassium, sodium, calcium, and magnesium) present in the unknown solution are well resolved and can be easily identified.

A second objective has been to study the systematic variation of spot size and R_f with the quantity of substance spotted on the chromatogram. If paper chromatography is done with great care, R_f values are reproducible to ± 0.01; the variation of R_f with load can then be clearly demonstrated, especially for ions (*5*). Such "load effects," together with interference effects due to other substances, are important causes of the R_f fluctuations which have led many workers to run known standards simultaneously with unknowns, and to replace R_f by R_g (the ratio of the distance traveled by the substance to the distance traveled by glucose).

A third objective has been to determine which of the many spray reagents described in the literature are most specific and sensitive, and to apply these reagents to a large number of carbohydrates and related compounds, on developed chromatograms, and over a hundredfold range of concentration.

REAGENTS

Paper-Washing Solvent. Distilled water, pyridine (spectroscopic grade) and glacial acetic acid (ACS reagent grade) are mixed in the volume ratio 80 to 15 to 5.

Developing Solvent. Isopropyl alcohol (98 to 99%, Eastman 212), pyridine (spectroscopic grade), glacial acetic acid (ACS reagent grade), and distilled water are mixed in the volume ratio 8 to 8 to 1 to 4.

Solution CD-1 (0.1*M* 2-aminobiphenyl hydrogen oxalate). Dissolve 1.69 grams of 2-aminobiphenyl (Eastman 2523) and 0.9 gram of anhydrous oxalic acid (or 1.26 grams of oxalic acid dihydrate) in a mixture of 5 ml. of glycerol, 10 ml. of distilled water, and 84 ml. of C.P. acetone. This solution keeps indefinitely. It is a reagent for all carbohydrates that can be pyrolyzed to furfural derivatives.

Solution CD-2 [0.1*M* *N*-(1-naphthyl)-ethylenediamine dihydrochloride]. Dissolve 1.3 grams of *N*-(1-naphthyl)-ethylenediamine dihydrochloride (Eastman 4835) in a mixture of 2.5 ml. of glycerol, 5 ml. of distilled water, and 42 ml. of C.P. acetone. The solution keeps indefinitely at 0° C. It is a fairly specific reagent for ketoses.

Solution CD-3-A (0.1*M* periodic acid). Dissolve 228 mg. of periodic acid (H_5IO_6, G. F. Smith Chemical Co.) in 10 ml. of distilled water. This stock solution is relatively stable, and may be kept for several weeks at room temperature, with only slight loss of periodic acid. It keeps for months at 0° C.

Solution CD-3-B (0.005*M* periodic acid). Dilute 1 ml. of solution CD-3-A with 19 ml. of C.P. acetone. This solution is unstable, and should not be used for more than 3 hours after being prepared. It is a fairly specific oxidant for 1,2-glycols.

Solution CD-3-C (0.01*M* benzidine). Dissolve 184 mg. of benzidine in a mixture of 0.6 ml. of glacial acetic acid, 4.4 ml. of distilled water, and 95 ml. of C.P. acetone. The solution is yellow and keeps indefinitely. It detects unreacted periodic acid on paper chromatograms.

Solution CD-4-A (0.3*M* potassium permanganate). Dissolve 4.8 grams of potassium permanganate in water to a volume of 100 ml. This stock solution keeps indefinitely.

Solution CD-4-B (0.01*M* potassium permanganate). Dilute 1 ml. of solution CD-4-A with 29 ml. of C.P. acetone. The solution is stable for about 1 hour.

Pyridine, 10%, in Water. This is a useful solvent for extraction or dilution of carbohydrates and derivatives, especially acids.

Pyridinium Sulfate, 1*M*. Mix 20 ml. of pyridine, 50 ml. of water, and 10 ml. of 10*M* sulfuric acid; cool to room temperature and dilute to 100 ml. with water. This is a useful additive to eliminate interference on chromatograms by divalent cations such as calcium.

Pyridinium (Ethylenedinitrilo)tetraacetate, 0.65*M*. Dissolve 2.92 grams of (ethylenedinitrilo)tetraacetic acid (ethylenediaminetetraacetic acid) in 4 ml. of pyridine plus 10 ml. of water; cool, and adjust volume to 15.4 ml. with water. This is a good solvent for insoluble barium and calcium salts, and an additive useful to eliminate divalent cation interference on chromatograms.

Barium Acetate, 1.0*M*. Dissolve 2.73 grams of barium acetate monohydrate in water plus a few drops of glacial acetic acid to a volume of 10 ml. This is an additive to retain acidic substances near the starting spot on chromatograms.

APPARATUS

The chromatographic apparatus is that previously described for one-dimensional ascending chromatography using 1-inch-wide strips (*5*), except for one useful modification.

The suspension of the paper strips in the earlier design was by attachment to a paper clip inserted in the upper No. 26 cork on the glass chromatographic tube. This has been replaced by a glass hook, made by drawing out one end of a 12-cm. length of 5-mm.-diameter glass rod and bending it into a hook. The straight shaft of this hook is inserted in a small hole drilled through the center of a No. 26 cork; the fit should be loose enough to permit the rod to be pushed up and down easily. Whatman No. 4 paper rolls, 1 inch wide, are cut into strips 580 mm. long, which are folded in the middle to form "double strips" 290 mm. long; on each side, a pencil line is drawn 38 mm. from the fold (these are the "starting lines," to the center of which spots of solution are applied). The free ends of the double strip are then clipped together with a paper clip, and the strip is hung on the glass hook by the paper clip. The glass hook should be close to the lower end of the cork. A 1-inch length of glass rod is inserted in the fold at the lower end of the double strip, to act as a separator. The cork and double strip hanging from its hook are then placed in the chromatographic tube. The strip hangs from 2 to 4 cm. above the level of solvent in the solvent container at the base of the chromatographic tube. When all the strips that are to be run have been hung in the tubes, each strip is lowered into the solvent by pushing down the glass rod to which the hook is attached, until the strip is dipping about 10 mm. below the level of the solvent. This modified suspension system gives results identical to those of the earlier system, but is much more convenient and easy to adjust.

Many spray reagents require heating the chromatograms to 100° C. in an oven; the temperature and time are fairly critical, especially when minute quantities are to be detected. Many ovens fail to heat the paper uniformly, and give erratic results. Gravity-convection ovens with heaters in the lower section are unsatisfactory for rapid uniform heating; forced-circulation ovens tend to dry the paper too rapidly, unless the spray reagent contains glycerol as a humectant. The following special heating system, however, has given satisfactory reproducibility: A 600-watt Forma-Vac vacuum oven (Forma Scientific Co., Marietta, Ohio) is operated on a 750-watt, 0- to 130-volt variable autotransformer. The thermoregulator of the oven is set at its maximum or short-circuited so that the oven is on continuously. The oven is warmed up quickly by setting the voltage at 130 for 15 to 20 minutes; it is desirable to have this warm-up controlled by a time switch to safeguard the oven from overheating. When the temperature, indicated by a thermometer hanging inside the oven and visible through the glass window, is 100° to 110° C., the transformer is reset to 50 volts. This maintains a constant internal temperature of 110° to 115° C. The heater in this oven is distributed throughout the wall, so that there are no hot spots, and the heat flow is very uniform. This system provides constant infrared radiation and is superior to the interrupted heating obtained by the action of a thermoregulator.

PROCEDURE

Preparation and Development of Paper Strips. The only change from the method previously described is in the length of the paper strips, which are 40 mm. shorter because of the modified strip-suspension system used in the present work. The 290-mm.-long double strips are washed by ascending chromatography in the "paper-washing" solvent, to remove divalent cations, which might form complexes with and alter the mobility of carbohydrates or their derivatives. Washed strips should be handled carefully to avoid contamination by fingerprints, etc.

In preparing solutions or extracts for spotting strips, 10% pyridine in water is a useful solvent. Such solutions can be kept in a refrigerator for a week or two without danger of bacterial contamination; they must not be heated above 50° C., because hot pyridine can epimerize sugars. Strong acids, bases, or salts should not be added. For hot acid hydrolyses of polysaccharides or phosphate esters, a measured volume of 10*M* sulfuric acid should be added; this is neutralized and precipitated by adding an equivalent quantity of barium acetate. Insoluble complexes containing calcium, iron, and the like can often be dissolved in 0.65*M* pyridinium (ethylenedinitrilo)tetraacetate.

If the solution to be chromatographed contains a considerable amount of free, nonvolatile acid or base, it must be neutralized with pyridine or acetic acid before development of the chromatogram. Otherwise, as the solvent ascends over the initial spot, its composition will be altered by removal of pyridine or acetic acid to balance the excess acid or base. This will cause small but significant changes in the R_f of the spots. The presence of excess acid or base is easily checked by applying a 1-μl. spot of the solution to a test strip and treating with bromocresol purple solution (*5*). If addition of pyridine or acetic acid to the original solution is inconvenient, exposure of spots on paper strips to pyridine vapor or acetic acid vapor will effect the neutralization.

It may be desirable to add a precipitating or complexing agent to a solution before spotting, in order to eliminate mutual interference by incompatible substances, or to eliminate some of the compounds from a solution giving a too complex and full chromatogram, or to obtain additional information on the chemical nature of the spots revealed on a chromatogram of the untreated solution. A 1*M* pyridinium sulfate solution can be used to

precipitate large excesses of calcium, and also to minimize interference by most cations. A 0.65*M* pyridinium (ethylenedinitrilo)tetraacetate solution is a solubilizer and clarifier for solutions, and also minimizes cation interference; but because it is a zwitterion it may interfere with the movement of small amounts of anions. A 1*M* barium acetate solution will precipitate sulfate, phosphate, and many organic acids, or greatly retard their movement on the chromatogram; lead acetate has been used for this purpose (*5*), but excess lead forms a spot at *R* 70 on the chromatogram, while barium remains close to the starting line, below *R* 20, and is less likely to interfere with detection of carbohydrate spots.

If the addition of a precipitant or complexing solution to the original solution is undesirable, an overspotting technique can be used. This requires application of a spot of the precipitant solution, drying, and application of a spot of the solution to be chromatographed directly on the first spot. Soluble and insoluble products of the reaction can then be separated by allowing water to ascend the paper to a point above the spot. Insolubles remain in the original spot and solubles move to a new spot at the water front; the new spot can then be chromatographed in the usual way. This preseparation method, using lead acetate as precipitant, has been described (*5*). A simpler method has been used in the present work. A 1-μl. spot of the precipitant solution is applied at a point 0.5 to 1 mm. above the center of the starting line and allowed to dry for 2 to 3 minutes. A 1-μl. spot of the solution to be chromatographed is then applied at the center of the starting line, and allowed to dry. When developed, the chromatogram is practically identical to that obtained by single-spotting of a solution to which the same amount of precipitant has been added before application to the paper. The overspotting technique is a powerful analytical aid in one-dimensional paper chromatography. It makes possible the use of operations comparable to selective precipitation, filtration, washing of precipitates, thousandfold concentration, etc., all on one paper strip at room temperature, using microgram quantities in microliter volumes, without elaborate microchemical glassware (*4*, *5*).

Detection of Spots on Chromatograms. In the preliminary work many of the spray reagents described in the literature were tried, but most of them were not satisfactory, especially at levels below 10 γ. Sensitive reagents are needed to detect a small quantity of one substance mixed with large quantities of other substances, because the total load of all substances on the paper must not exceed a few hundred micrograms in order to yield small spots and good separations. Two reagents proved to be exceptionally good: the aniline hydrogen oxalate reagent of Partridge (*9*) and the periodate-benzidine reagent of Cifonelli and Smith (*2*). However, these reagents have a high water content and must be applied by spraying, which is an inconvenient operation requiring great care to ensure uniform coverage and to prevent smearing of the spots. The reagents were therefore modified by using acetone as the major component so that they could be simply poured onto the paper strips. The pouring technique is simple, clean, and quick and gives sharply defined spots. In modifying Partridge's aniline reagent, the aniline was replaced by 2-aminobiphenyl because aniline hydrogen oxalate is insoluble in acetone.

Application of a pouring reagent can be done neatly by filling a 1-ml. serological pipet, applying the tip to the paper strip at the solvent front, and allowing the reagent to flow out quickly so that the strip is saturated in a few seconds. The acetone evaporates within 30 seconds, leaving a uniform deposit of reagent. One milliliter saturates a length of about 200 mm. of a 25-mm.-wide paper strip.

Each of the spot-detector solutions used in this work has an identifying symbol—e.g., CD-1 is carbohydrate detector number 1—which is marked on each chromatogram to which it has been applied.

Solution CD-1 (0.1*M* 2-aminobiphenyl hydrogen oxalate) is poured on a strip, which is allowed to dry in air for 10 to 15 minutes. The strip is placed in an oven at 110° C. for 5 minutes. The background is pale yellow. Spots of pentoses are red, hexoses greenish brown, uronic acids purple. After several days the background darkens slightly, some spots become more intense, and colors change to shades of brown. It is desirable to outline the spots in pencil and note the intensity and color while they are fresh. The sensitivity of CD-1 is approximately the same as that of the aniline reagent from which it is derived, and is of the order of 0.01 μmole in a spot area of about 1 sq. cm. on a developed chromatogram.

Like all other primary aromatic amine reagents, CD-1 is relatively specific for compounds that readily form furfural derivatives on acid pyrolysis. Monosaccharides and disaccharides react strongly, trisaccharides and tetrasaccharides react weakly, and the higher polysaccharides give no color. Aldoses react more rapidly than ketoses, and Partridge (*9*) claims that glucose and sorbose can be differentiated by this means. It is true that, when equal amounts of sorbose and glucose are present, some color is developed by glucose before any color is developed by sorbose; but the glucose color intensity at this stage is much less than the maximum that can develop on further heating. If heating is continued until the glucose spot attains maximum intensity, the sorbose spot will also have developed color.

Solution CD-2 (0.1*M* *N*-(1-naphthyl)ethylenediamine dihydrochloride) is poured on a strip, which is allowed to dry in air for 10 to 15 minutes and placed in an oven at 110° C. for 4 minutes. At levels of 1 μmole, ketoses give reddish spots on a pale tan background, while aldoses and uronic acids give fainter yellow, red, or brown spots. At lower ketose levels the color is a characteristic golden yellow. The limit of sensitivity is of the order of 0.02 μmole per square centimeter. CD-2 is not markedly superior to other ketose-specific reagents described in the literature (*6*, *8*); it is somewhat less specific but more sensitive and gives clear, well-defined ketose spots.

Solution CD-3-B (0.005*M* periodic acid) is poured on a strip, which is allowed to dry in air at room temperature for 3 to 4 minutes. Solution CD-3-C (0.01*M* benzidine) is then poured on and allowed to dry. As it dries, the background becomes deep blue with white or yellow spots. In 5 to 10 minutes, when the spots show maximum contrast, they are outlined in pencil or with a ball-point pen and the intensity is noted. The background color slowly fades to gray and the spots to grayish white. The limit of detection for many polyols is of the order of 0.005 μmole per square centimeter, but may be as high as 0.5 μmole per square centimeter for many compounds, especially polysaccharides, which react very slowly with periodic acid. CD-3 is much less specific than CD-1 but it is useful for many important compounds, such as glycerol, difficult to detect by any other reagent, except the unstable lead tetraacetate in benzene (*1*) or the extremely unspecific potassium permanganate. Although the periodic acid in CD-3 is not a powerful general oxidant, it reacts with 2-aminoethanol, serine, threonine, and strong reducing agents, and gives faint spots with amines and amino acids when they are present at levels above 0.2 μmole. Spots obtained with this reagent should therefore be interpreted with caution, and a variety of other tests (such as ninhydrin) applied if possible.

Solution CD-4-B (0.01*M* potassium permanganate) is poured on a strip, which is allowed to dry in air, and continuously observed for spot development during the first 10 minutes. The background is at first bluish purple, gradually fading to magenta, and finally to rose. Spots develop at markedly different rates, according to the quantity and the reducing power of the substance in the spot; the initial color is greenish yellow, and may change to yellow (manganese dioxide) and then to white (manganous salt). As each spot appears, the time in minutes after application of CD-4-B is marked on the paper near the spot; this time is a useful indication of both reducing power and quantity of the

Table I. Movement and Detection by Reagents of Carbohydrates in Isopropyl Alcohol–Pyridine–Water–Acetic Acid (8:8:4:1)

(2 hours, 30° C.)

Substance	Micromole[a] Spotted	Detection[b] by CD-1	CD-2	CD-3	CD-4	$R_f \times 100$ Values of Spot Center and Limits
(Ethylenedinitrilo)-tetraacetic acid	1.0	±W	..	±BL	1++W	8 (12-3)
		...	..	..	4±Y	20 (22-18)
	0.2	...	..	±BL	2++W	8 (11-4)
Mucic acid	0.2	...	..	++Y	3++Y	17 (23-9-0)
	0.02	...	..	+W	3+Y	18 (21-15)
Calcium saccharate	0.5[c]	...	..	++Y	2++W-Y	17 (24-9)
	0.1[c]	...	..	++Y	2++W-Y	16 (22-10)
	0.2[c]	...	..	+Y	2+Y	17 (21-13)
Potassium glucose-1-phosphate	0.25	+BR	±BR	±BL	3+BL-W	10 (15-7)
	0.25[c]	±BR	±	±BL	3±BL	18 (25-10)
	0.1	(±)	..	..	3±BL	22 (27-17)
	0.05[c]	...	..	..	...	
Calcium lactobionate	1.0[d]	...	..	++Y	3++Y	25 (37-12)
	0.1[d]	...	..	++Y	4±Y	26 (32-21-12)
	0.02[d]	...	..	+W	10(±)	22 (28-17)
	0.005[d]	...	..	±W	...	28 (32-23)
α-D-Galacturonic acid	1.0	++P	++P	++Y	2++Y	30 (41-21)
		+P	±P	..	±Y	18 (20-16)
	0.1	+P	..	++Y	2++W-Y	32 (40-25)
	0.02	+P	..	+W	4±Y	31 (37-25)
	0.005	(±)	..	(±)	...	32 (36-27)
meso-Inositol	0.5	...	..	±W	3±Y	31 (36-23)
	0.1	...	..	(±)	3±Y	31 (35-26)
Barium fructose-6-phosphate	0.25[d]	+BR	+Y	++W	3++Y	31 (37-24)
	0.10[d]	+BR	..	+W	3±Y	33 (38-28)
	0.02[d]	...	..	±W	4(±)	30 (35-25)
D-Glucuronic acid (+ lactone)						33, cf. Glucuronolactone, R_f 0.84
3-Adenylic acid	0.5	...	..	+BL	2+BL-W	33 (43-22)
	0.1	...	..	..	±BL-W	29 (36-21)
Tartaric acid	1.0	...	..	++Y	3++Y	36 (45-24-16)
	0.1	...	..	++Y	3++W-Y	30 (37-23)
	0.02	...	..	+W	3±Y	28 (34-23)
	0.005	...	..	±W	...	29 (31-27)
Scyllo-inosose	0.5	±BR	±Y-R	+W	1++W-Y	35 (43-27-20)
	0.1	±BR	..	++Y	2++Y	38 (43-33)
	0.02	...	..	+W	8±Y	38 (42-32)
	0.005	...	..	±W	...	38 (42-33)
D-Glucosamine hydrochloride	1.0	+BR	±R	++Y	1++W-Y	53 (62-45)
		±BR	..	+Y	2+Y	70 (78-62)
	0.1	...	..	++Y	2+Y	38 (44-32)
	0.02	...	..	+W	4±Y	37 (43-32)
	0.005	...	..	±W	...	40 (44-36)
Gluconic acid	1.0	...	..	++Y	2++W-Y	38 (48-26)
		...	..	+Y	±Y	(82-48)
		...	..	++Y	4+Y	89 (94-82)
	0.1	...	..	++Y	2+Y	38 (46-33)
		...	..	+Y	±Y	(78-46)
	0.02	...	..	+Y	3±Y	39 (44-33)
	0.005	...	..	±W	...	39 (43-35)
Calcium 2-ketogluconate	0.5	++P-BR	..	++Y	2++W-Y	(46-17)
	0.5[d]	++P-BR	..	++Y-W	2++W-Y	39 (48-31)
	0.5[c]	++P-BR	..	++W	1++W-Y	39 (48-30)
	0.1[d]	+P	..	++Y	...	40 (46-33)
	0.1[c]	+P	..	+W	2++Y	41 (47-35)
Sodium glycerophosphate	0.25	...	..	++W-Y	4+BL-W	20 (28-12)
	0.25[c]	...	..	++W-Y	3±BL	36 (46-27)
	0.1	...	..	±W	6±BL-W	40 (49-31)
	0.02	...	..	±Y	...	37 (41-33)
Gulonic acid (+ lactone)						cf. Gulonolactone, R_f 0.75
D-Melibiose	0.5	++BR	±Y	++W	4+Y	45 (53-36)
	0.1	+G-BR	±Y	+W	6±Y	46 (52-39)
	0.02	±BR	..	±W	...	44 (50-39)
Maltotetraose	0.2	±BR	..	+W	7±Y	46 (57-32)
	0.05	±Y	..	±W	...	46 (53-38)
Raffinose	0.5	++G-BR	+BR	±W	4+Y	46 (54-38-21)
	0.1	±BR	±Y	±W	...	44 (50-38)
	0.02	±BR	±Y	..	...	43 (50-36)
Isomaltose	0.5	++G-BR	±Y	++W	4+Y	47 (55-38)
Lactose	0.5	+G-BR	±Y	+W	±Y	47 (55-38)
	0.1	±G-BR	..	±W	...	46 (52-38)
	0.02	±BR	..	±W	...	46 (50-40)
L-Quinic acid	0.5	...	..	++W	8±Y	50 (61-39)
	0.1	...	..	+W	8±Y	50 (56-43)
	0.02	...	...	±W	...	50 (52-47)
Malto triose	0.5	+BR	±Y	+W	1±W	50 (59-40)
	0.1	±BR	..	±W	...	51 (57-42)
Calcium 5-keto gluconate	0.25[c]	++P-BR	+P-BR	++W	2++W-Y	51 (60-43-37)
	0.1[c]	+P	..	++W	2++W-Y	53 (60-45)
	0.02[c]	±	..	±Y	3±Y	53 (60-46)
D-Trehalose	0.5	...	..	±W	8±Y	52 (58-47)
Cellobiose	0.5	++G-BR	±Y	++W	7±Y	53 (62-43)
	0.1	±BR	..	±W	...	52 (58-46)
Melezitose	0.5	+BR	+Y-BR	±W	8±Y	54 (64-43)
	0.1	±BR	±Y	..	...	54 (61-47)
	0.02	±Y	±Y	..	...	54 (60-47)
Maltose	0.5	+BR	±Y	++W	3±Y	57 (65-49)
	0.1	±BR	..	±W	10±Y	58 (64-52)
Calcium glycerate	0.5[d]	...	..	++Y	2++W-Y	58 (66-48)
	0.1[d]	...	..	++Y	2++Y	60 (65-55)
	0.02[d]	...	..	±W	3±Y	58 (62-52)
Guanosine	0.1	...	..	±W	3±Y	60 (66-54)
D-Galactose	1.0	++G-BR	±Y	++Y-W	4+Y	60 (69-50)
	0.1	+G-BR	..	++W	6±Y	63 (67-59)
	0.02	±BR	..	±W	...	63 (67-57)
Quebrachitol	1.0	...	..	++W	4±Y	62 (69-54)
	0.1	...	..	±W	...	63 (67-59)
Sucrose	0.5	+BR	+R-BR	±W	8±Y	63 (70-55)
	0.1	±BR	±Y-R	±W	...	62 (68-55)
	0.02	±BR	±Y	..	...	61 (66-55)
Dulcitol (galactitol)	0.5	...	..	++Y	3++Y	51 (71-31)
	0.1	...	..	++W	3±Y	65 (70-60)
	0.02	...	..	+W	...	63 (66-60)
	0.005	...	..	±W	...	63 (66-59)
D-Sorbitol	1.0	...	..	++W	3++Y	62 (72-52)
	0.1	...	..	++W	3++Y	64 (71-57)
	0.02	...	..	+W	4±Y	65 (70-60)
	0.005	...	..	±W	...	65 (69-61)
Turanose	0.5	±Y	+Y	++Y	6±Y	61 (71-52)
	0.1	...	±Y	+Y	...	64 (69-56)
	0.02	...	..	±W	...	65 (69-60)
D-Glucose	1.0	++G-BR	±Y	++W	3++Y	63 (71-54)
	0.1	+G-BR	..	++W	...	65 (70-60)
	0.02	±BR	..	±W	...	65 (70-60)
Floridoside	1.0	...	..	+W	4+Y	65 (73-58)
L-Malic acid	0.2	...	..	±R	2++W-Y	65 (73-56)
	0.02	...	..	..	6±Y	61 (64-57)

[a] Where calcium salts are used, calculations are based on one half the molecular weight. The number of micromoles is therefore that of the acid, not the salt.

[b] Reagents used for detection are: CD-1, 0.1*M* 2-aminobiphenyl hydrogen oxalate; CD-2, 0.1*M* *N*-(1-naphthyl)-ethylenediamine dihydrochloride; CD-3. 0.005*M* periodic acid, followed by 0.01*M* benzidine; and CD-4, 0.01*M* potassium permanganate. Colors of spots are abbreviated as follows:

BL, blue
BL-W, blue-white
BR, brown
G-BR, greenish brown
P, purple
P-BR, brownish purple fading to brown
R, red
R-BR, reddish brown
R-Y, reddish yellow
W, white
W-Y, white center, yellow periphery
Y, yellow
Y-R, yellowish red
Y-W, yellow center, white periphery
No color symbol, color too faint to identify

Spot intensity is coded as follows:

++ maximum intensity
+ distinct spot, of less than maximum intensity
± faint spot, near or at limit of detection

For spots detected by CD-4, the intensity symbol is preceded by a number from 0 to 10. This is the number of minutes elapsed between the time of application of the reagent and the time of appearance of the spot.

[c] Solution or spot treated with pyridinium (ethylenedinitrilo)tetraacetate to eliminate interference by cations.

[d] Solution or spot treated with pyridinium sulfate to eliminate interference by cations.

Table I. Movement and Detection by Reagents of Carbohydrates in Isopropyl Alcohol-Pyridine-Water-Acetic Acid (8:8:4:1) (*Continued*)

(2 hours, 30° C.)

Substance	Micromole[a] Spotted	Detection[b] by CD-1	CD-2	CD-3	CD-4	$R_f \times 100$ Values of Spot Center and Limits
Mannitol	1.0	...	..	++Y	2++Y	60 (72-44)
	0.1	...	..	++W	3+Y	66 (72-58)
	0.02	...	..	+W	5±Y	65 (69-61)
	0.005	...	..	±W	...	65 (69-61)
Citric acid	0.5	...	..	+BL	2++W-Y	65 (75-55)
	0.1	...	..	±BL	3+W-Y	62 (66-57)
	0.02	...	..	..	4±Y	62 (66-58)
Xanthosine	0.5	++R	±Y	++W	2++Y	66 (76-55)
	0.1	+R	..	±W	2+Y	67 (74-59)
	0.02	±R	..	..	6±Y	64 (69-59)
Pinitol	1.0	...	..	+Y	4++W	67 (74-60)
	0.1	...	..	±W	10±Y	67 (72-62)
Inosine	0.5	±R	..	++W	3±Y	67 (75-60)
	0.1	±R	..	+W	8±Y	67 (74-61)
	0.02	...	..	±W	...	68 (71-65)
Adenosine	0.20	...	..	++W	8±Y	68 (75-60)
	0.10	...	..	+W	...	69 (74-63)
	0.02	...	..	±W	...	65 (68-61)
L-Arabinose	1.0	++R	±R	++Y	3++Y	69 (78-60)
	0.1	+R	..	+W	4±Y	70 (76-64)
	0.02	±R	..	±W	...	70 (74-66)
L-Sorbose	1.0	++G-BR	+R-Y	++W	2++Y	66 (76-56)
	0.1	±G-BR	±Y	+W	5±Y	69 (74-54)
	0.02	±BR	±Y	±W	6±Y	69 (73-65)
D-Fructose	1.0	+BR	++R-Y	++W	2++W-Y	66 (75-55)
	0.1	±BR	+Y	++W	5±Y	70 (76-64)
	0.02	...	±Y	+W	...	69 (74-64)
D-Mannose	1.0	++G-BR	±R	++W	3++Y	67 (75-58)
	0.1	+G-BR	..	+W	6±Y	70 (75-65)
	0.02	±G-BR	..	±W	...	70 (75-65)
L-Arabitol	1.0	...	..	++Y	3++Y	70 (79-62)
	0.1	...	..	++W	4±Y	73 (78-69)
	0.02	...	..	+W	...	72 (76-68)
	0.005	...	..	±W	...	74 (77-71)
Adonitol (D-ribitol)	1.0	...	..	++Y	2++W-Y	70 (79-60)
	0.1	...	..	++Y	3+Y	73 (78-67)
	0.02	...	..	+Y	8±Y	73 (77-69)
	0.005	...	..	±W	...	73 (76-69)
D-Xylose	1.0	++R	±R	++W	3++Y	71 (78-64)
	0.1	+R	..	±W	4±Y	73 (78-69)
	0.02	±R	..	..	...	74 (78-69)
DL-Glyceraldehyde	1.0	++BR	±R	++Y	2++Y	75 (87-64-45)
	0.1	+Y	..	±W	3±Y	70 (89-50)
L-Fucose	1.0	++G-BR	±R	++W	3++Y	74 (82-65)
	0.1	±G-BR	..	++W	6±Y	75 (79-70)
	0.02	±BR	..	±W	...	75 (78-71)
D-Lyxose	1.0	++R	±R	++W	1++W-Y	72 (81-61)
	0.1	+R	..	+W	3+Y	75 (82-67)
	0.02	±R	..	±W	6±Y	75 (79-70)
N-Acetylglucosamine	1.0	+BR	..	+Y	5±Y	71 (80-62)
	0.2	±G-BR	..	±W	...	75 (81-68)
	0.04	...	..	..	...	...
D-Ribose	1.0	++R	±R	++Y	2++W-Y	75 (84-67)
	0.1	+R	..	+Y	4+Y	76 (82-70)
	0.02	±R	..	±W	4±Y	77 (80-73)
Shikimic acid	0.5	...	..	++Y-R	0++W-Y	77 (85-67)
	0.1	...	..	++R	1++W-Y	78 (84-70)
	0.02	...	..	±R	1±Y	78 (82-73)
meso-Erythritol	1.0	...	..	++Y-W	3++Y	73 (80-66)
	0.1	...	..	++W	3±Y	77 (82-70)
	0.02	...	..	+W	...	77 (81-72)
	0.005	...	..	±W	...	77 (80-75)
Gulonic lactone (+ acid)	1.0	...	..	++W	3++W-Y	75 (85-64)
		...	..	++Y	3++W-Y	40 (49-30)
	0.1	...	..	±W	3±Y	80 (84-76)
		...	..	±W	3±Y	43 (47-38)
2-Deoxy-D-glucose	1.0	±BR	±R	++Y	1++Y	78 (90-69)
	0.1	...	..	±Y	10±Y	81 (86-77)
Glycerol	1.0	...	..	++W	3++Y	79 (86-69)
	0.1	...	..	+W	6±Y	82 (86-78)
	0.02	...	..	±W	...	82 (85-79)
L-Rhamnose	1.0	++BR	±R	++W	3++W-Y	81 (90-72)
	0.1	±BR	..	+W	9±Y	83 (88-76)
	0.02	...	..	±W	...	82 (86-77)
Lactic acid	0.5	...	..	..	2++Y	84 (90-79)
	0.1	...	..	..	2+Y	80 (84-75)
	0.02	...	..	..	4±Y	82 (86-78)
Uridine	0.5	...	..	++W	1++W-Y	82 (91-74)
	0.1	...	..	+W	1++W-Y	84 (89-78)
	0.02	...	..	±W	3±Y	84 (88-82)
L-Ascorbic acid	1.0	±BR	±R	++Y-W	0++W-Y	82 (91-71)
	0.1	...	..	+W	1+Y	85 (90-78)
	0.02	...	..	±W	4±Y	86 (91-82)
Dihydroxyacetone	1.0	+BR	+R	++W	1++W-Y	82 (87-77)
	0.1	±R	..	+W	4+Y	86 (91-82)
	0.02	...	..	±W	6±Y	86 (89-82)
Glucuronic lactone (+ acid)	1.0	++P	+R	++W	2++W-Y	84 (91-75)
		+P	..	++Y	3++Y	33 (38-28)
	0.1	+R	±R	+W	3±Y	86 (91-81)
		±R	..	±Y	7±Y	34 (38-30)
	0.02	±R	..	±W	8±Y	86 (90-82)
Gluconolactone						cf. Gluconic Acid, R_f 0.38
Dehydroascorbic acid	1.0	±BR	±R	++Y	0++W	85 (93-76-57)
	0.1	±BR	..	±W	1±Y	90 (95-85)
Thymidine	0.5	...	..	..	1++Y	90 (96-83)
	0.1	...	..	..	1+Y	91 (96-86)
	0.02	...	..	..	2±Y	89 (94-84)

[a] Where calcium salts are used, calculations are based on one half the molecular weight. The number of micromoles is therefore that of the acid, not the salt.

[b] Reagents used for detection are: CD-1, 0.1*M* 2-aminobiphenyl hydrogen oxalate; CD-2, 0.1*M* *N*-(1-naphthyl)-ethylenediamine dihydrochloride; CD-3, 0.005*M* periodic acid, followed by 0.01*M* benzidine; and CD-4, 0.01*M* potassium permanganate. Colors of spots are abbreviated as follows:

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Y-R, yellowish red
Y-W, yellow center, white periphery
No color symbol, color too faint to identify

Spot intensity is coded as follows:

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+ distinct spot, of less than maximum intensity
± faint spot, near or at limit of detection

For spots detected by CD-4, the intensity symbol is preceded by a number from 0 to 10. This is the number of minutes elapsed between the time of application of the reagent and the time of appearance of the spot.

[c] Solution or spot treated with pyridinium (ethylenedinitrilo)tetraacetate to eliminate interference by cations.

[d] Solution or spot treated with pyridinium sulfate to eliminate interference by cations.

substance. At about 10 minutes, the spots are outlined in pencil, and the color and intensity are noted. The background soon fades to a light tan on which many spots become undetectable. Some substances reduce permanganate only to manganese dioxide. Many substances quickly reduce it to manganese dioxide, and then very slowly reduce this to the manganous form; the spots are yellow at the end of 10 minutes, but fade to white after 1 or 2 days. Minute quantities of these substances may not give a perceptible yellow spot in 10 minutes, but may show up as white spots after 1 or 2 days. The sensitivity of detection for many substances is of the order of 0.01 μmole per square centimeter, but some substances (such as the nonreducing disaccharide trehalose) are almost undetectable even at 0.5 μmole per square centimeter.

The low specificity of permanganate limits its usefulness, for it detects alcohols, amines, amino acids, and many unsaturated and aromatic compounds. CD-4 is a useful auxiliary test on chromatograms of unknown mixtures of organic compounds.

In alkaline medium permanganate is a weaker and more specific oxidizing agent, and has been used to detect certain reducing amino acids such as methionine and tyrosine (*3*).

RESULTS AND DISCUSSION

Table I summarizes the data on the movement of carbohydrates and related compounds in the isopropyl alcohol–pyridine-water–acetic acid solvent. Mobilities are given in units of 100 R_f measured from the starting line to the estimated mass center of the spot, and the numbers in parentheses give a more complete description of the spot. For example, the numbering 26 (32-21-12) indicates that the estimated mass center is at R_f 0.26, the main mass lies between R_f 0.32 and 0.21, while a "tail" extends from R_f 0.21 to 0.12. Most of the spots show no tailing, however. Nearly all of the spots were detected by more than one carbohydrate-detector solution, and many were detected by all four detector solutions; the numerical description is usually identical for at least two of the detector solutions, but the value listed is always that of the more sensitive detection. The description, therefore, gives the actual location of the compound on a developed chromatogram, so that the possibility of separating and isolating in pure form varying quantities of two or more compounds can be checked.

R_f values can be reproduced very well. As a test, eight strips were spotted with 1 μmole of L-arabinose, and two strips each detected with detector solutions CD-1, CD-2, CD-3, and CD-4. Numerical (100 R_f) descriptions of the eight spots were: 69 (76-60), 68 (75-60), 68 (74-62), 69 (75-62), 69 (78-61), 69 (79-57), 69 (76-60), 68 (75-60). In careful work, R_f values can nearly always be duplicated to $\pm$ 0.01 unit. This makes possible a clear analysis of the nature of the systematic variations in R_f due to variations in the quantity of the substance in the spot (load effects) or in the quantity and quality of other substances that may affect its movement on the chromatogram (interference effects).

One important load effect shows up with compounds that are completely ionized. This has been previously described for cations like sodium or magnesium (*5*), and is also clearly shown in Table I by the data for tartaric acid (R_f 0.36) and other strong acids. The R_f values both for the mass center and for the upper limit of the spot decline, but the value for the lower limit is relatively constant, as the load is decreased. This forward elongation of the spot as the load increases may be due to a change in the water-concentration gradient along the strip caused by the competition for solvent water molecules between the moving ions and the stationary cellulose fibers (*5, 6*).

A second load effect is shown by most of the nonionized compounds listed in Table I—e. g., by adonitol (R_f 0.70). At the heaviest (1 μmole) load, the R_f values for the mass center and the lower limit of the spot are several units lower than the values at loads of 0.5 μmole or less; at the lower loads, all R_f values become relatively constant—i.e., the size and location of the spot do not vary with changes in load. It is clear that the upward-moving and outward-spreading of the original spot by the ascending solvent flow are unaffected by the load up to a critical value near 0.5 μmole; at loads above 0.5 μmole there is a backward-elongation of the spot because the solvent cannot carry the full load, and the excess load lags behind. The carrying capacity of the solvent for most substances seems to be about 0.5 μmole, but for some substances it is nearer 1 μmole (*cf.* L-fucose, R_f 0.74), and for others it is nearer 0.1 μmole (*cf.* dulcitol, R_f 0.65).

Ionic Interference Effects. The most serious interference effect is the interaction between cations and anions. The developing solvent normally moves cations as the acetate salts and anions as the pyridinium salts, but when the load of ions is heavy or the ions are polyvalent the solvent may be unable to effect separation (*5*). This failure is shown by glucosamine hydrochloride (R_f 0.38) at the 1-μmole level, where the glucosaminium and chloride ions move together to a higher level (R_f 0.53) than chloride ions alone (R_f 0.50). At the 0.1-μmole level, the ions are separated by the solvent and glucosamine moves as the acetate to its "true" R_f value of 0.38. A similar effect is shown by sodium glycerophosphate at 0.25 μmole, where the unseparated salt moves to R_f 0.20, a value lower than sodium ions alone (R_f 0.30). At 0.1 μmole, the solvent effects a separation, and pyridinium glycerophosphate moves to R_f 0.40. Apparently, ionic interference between monovalent ions can be corrected simply by lowering the load to 0.1 μmole or less.

With polyvalent ions the interionic attraction is greater, and a variety of chelate complexes and salts may be formed, so that a streak rather than a compact spot is likely to show up on the chromatogram—e.g., calcium 2-ketogluconate in Table I, R_f 0.40. This is best corrected (as described in the section on Procedure) by overspotting on pyridinium sulfate before development of the chromatogram. The sulfate spot stays near the starting line and holds polyvalent cations strongly, but does not affect the movement of anions. Pyridinium (ethylenedinitrilo)tetraacetate is also effective, but has several disadvantages, and should be used only when needed to dissolve insoluble salts. It has a higher R_f than sulfate and covers a larger useful area on the developed chromatogram. It reacts strongly with permanganate. Its positively charged nitrogen atoms attract and hold back some anions. It is a weak acid, and is less effective than sulfate when competing for calcium ions with strong acid chelating agents such as tartaric acid, at the pH (about 5) of the chromatographic solvent.

There is no mutual interference between neutral molecules (such as ribose, galactose, or lactose) and ions. The presence of 0.1*M* potassium, sodium, calcium, and magnesium in the carbohydrate solution has no effect on the R_f values. Overspotting a carbohydrate solution on pyridinium sulfate, pyridinium (ethylenedinitrilo)tetraacetate, or barium acetate does not alter the R_f values.

There are striking interference effects, however, in chromatograms of ionized molecules. In the presence of calcium and magnesium a lactic acid spot shifted from R_f 0.83 to 0.80, a citric acid spot from R_f 0.67 to 0.14, and a tartaric acid spot from R_f 0.35 to 0. This interference was rectified by overspotting the solution on pyridinium sulfate; the acids then moved as pyridinium salts to their normal R_f values, while the calcium and magnesium were retained by the sulfate spot. Overspotting the solution on barium acetate displaced the lactic spot to R_f 0.69, while the citric and tartaric acids were mostly held at R_f 0 with slight upward streaking to R_f 0.24. Glucono-δ-lactone also shows serious distortion in high-salt chromatograms, but the normal pattern is restored by overspotting on pyridinium sulfate; overspotting on barium acetate causes complete retention of the gluconic acid in the barium spot below R_f 0.20.

The most complete study of ionic interference was carried out with calcium 2-ketogluconate (cf. Table I, R_f 0.40). A spot containing 0.5 μmole of 2-ketogluconic acid plus 0.25 μmole of calcium gives a long streak from R_f 0.46 to R_f 0.17. When detected with CD-1, the upper portion of the streak is purple in color, and the lower portion is purplish red; chromatograms treated with 8-quinolinol to reveal calcium (*5*) show that the calcium is located in the lower portion of the streak. There is therefore an imperfect separation of the free acid and a calcium complex (probably the calcium 2-ketogluconate acetate). If the calcium 2-ketogluconate is dissolved either in 1*M* pyridinium sulfate or in 0.65*M* pyridinium (ethylenedinitrilo)tetraacetate instead of in water, the calcium is almost completely retained near the starting line, and all of the 2-ketogluconic acid moves as the pyridinium salt to a compact spot at R_f 0.39, which gives the characteristic purple color of uronic acids when detected by CD-1. Overspotting of a water solution of calcium 2-ketogluconate on either pyridinium sulfate or pyridinium (ethylenedinitrilo)tetraacetate gives chromatograms identical with those obtained by single spotting of a mixed solution of the salt and the

precipitant. In preliminary experiments using the overspotting technique, a 1*M* solution of (ethylenedinitrilo)tetraacetate in ammonium hydroxide was used as the prespotted precipitant; in this instance, the 2-ketogluconic acid moved as a compact spot to R_f 0.28. Presumably, the acid moved as the ammonium salt, which has a much lower mobility than the pyridinium salt. Overspotting the water solution of calcium 2-ketogluconate on 1 μmole of barium acetate leads to complete retention of the acid in the region from R_f 0.14 to R_f 0.03, while the calcium moves freely to its normal position.

Correlation of Molecular Structure and R_f Value. Many empirical generalizations can be derived from the data in Table I. Desoxyaldohexoses have R_f values from 0.75 to 0.83, aldopentoses from 0.70 to 0.76, ketohexoses from 0.69 to 0.70, aldohexoses from 0.63 to 0.70. These ranges are probably too narrow to include some of the rarer sugars—e.g., the aldohexose idose would probably have an R_f of 0.75, by comparison with the data given by Isherwood and Jermyn (*7*) for movement in an ethyl acetate–pyridine-water solvent. These authors have shown that the R_f values for carbohydrates follow the same sequence in all chromatographic solvents (except phenol). Galactose and its homomorphs (arabinose, fucose, dulcitol, galacturonic acid) have the lowest R_f values within the group to which they belong. Glucose and its homomorphs (xylose, sorbitol, glucuronic acid) have slightly higher R_f values. Mannose and its homomorphs (lyxose, rhamnose, mannitol, mannuronic acid) have still higher R_f values, near the median value for each structural group. The configuration of the hydroxyls within each homomorphic group apparently determines the relative water affinity and thereby the relative mobility of the molecule.

Monophosphoric esters have R_f values from 0.37 to 0.43 unit lower than those of parent carbohydrates such as glycerol, glucose, or adenosine. Carboxylic acids have R_f values 0.20 to 0.30 unit lower than the parent polyol, but usually also show a strong lactone spot with an R_f value 0.20 unit higher than the parent polyol. Amino sugars (the only one tested, glucosamine) have an R_f about 0.25 unit lower than the corresponding hydroxyl form.

LITERATURE CITED

(1) Buchanan, J. G., Dekker, C. A., Long, A. G., *J. Chem. Soc.* **1950**, 3162.
(2) Cifonelli, J. A., Smith, F., ANAL. CHEM. **26**, 1132 (1954).
(3) Dalgliesh, C. E., *Nature* **166**, 1076 (1950).
(4) Feigl, F., "Chemistry of Specific, Selective, and Sensitive Reactions," Academic Press, New York, 1949.
(5) Gordon, H. T., Hewel, C. A., ANAL. CHEM. **27**, 1471 (1955).
(6) Isherwood, F. A., *Brit. Med. Bull.* **10**, 202 (1954).
(7) Isherwood, F. A., Jermyn, M. A., *Biochem. J.* **48**, 515 (1951).
(8) Lederer, E., Lederer, M., "Chromatography, a Review of Principles and Applications," Elsevier, New York, 1953.
(9) Partridge, S. M., *Biochem. Soc. Symposia (Cambridge, Engl.)*, No. 3, p. 52 (1950).

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Determination of Pipecolic Acid in Biological Materials

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Because pipecolic acid has widespread occurrence in plants, a quantitative method for its determination in biological materials is desirable. A sensitive quantitative method utilizes the color formed between pipecolic acid and ninhydrin under acid conditions. The reaction is subject to interference by salts and alpha amino acids. These interfering materials are separated from pipecolic acid by chromatography on Dowex 50-X12 in the sodium form. The method has been applied successfully to urine and to plant extracts.

PIPECOLIC acid has been isolated from and identified in a number of plants (*3–5, 7, 17, 18*). In both plants and animals (*2, 8, 9*) pipecolic acid is formed from lysine. In order to study the possible utilization of pipecolic acid by animals, a method for its determination in animal tissues, feces, and urine was developed and has also been applied to plant tissues.

Since the inception of this work, Schweet (*11*) has published a method that has been applied to the analysis of proteins, but in nonprotein fractions salts and amino acids interfere. The method proposed here separates pipecolic acid from these interfering substances and is more sensitive than Schweet's.

The first attempts to measure pipecolic acid were made by the method for the quantitative determination of amino acids on paper (*13, 14*), but this reaction lacked sensitivity and reproducibility. The colorimetric method described here is based on the reaction of pipecolic acid in vitro with ninhydrin under acidic conditions. The pipecolic acid is first freed of interfering materials (amino acids and salts) by chromatography on ion exchange resins.

This report is conveniently considered in two parts: the production of color between pipecolic acid and ninhydrin and the separation of pipecolic acid from interfering materials.

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PRODUCTION OF COLOR BETWEEN PIPECOLIC ACID AND NINHYDRIN

Reagents. Phosphoric acid, 1*M*.
Distilled 1-butanol.
Ethyl acetate; practical grade is satisfactory.
Ninhydrin solution, 4% (weight/volume) solution of ninhydrin in a mixture of 9 parts of 1-butanol and 1 part of 1*M* phosphoric acid. This is prepared fresh just before use (Table I).

Procedure. A sample containing 1 to 20 γ of pipecolic acid is evaporated to dryness in a test tube (12 × 175 mm.) in flowing air at room temperature. One milliliter of ninhydrin solution is added and the tube is capped and shaken. After being heated in boiling water for exactly 15 minutes, the tube is cooled rapidly and the solution is diluted to a convenient volume (5 to 10 ml.) with ethyl acetate. During and after heating, the tube must be kept out of intense light because light causes fading of the color. The color is measured at 575 mμ (Figure 1) within 1 hour. The quantity of pipecolic acid is obtained from a standard curve made from the pure acid treated in the same way. If proline is also present, the color at 510 mμ (Figure 1) must also be measured. The quantities of proline and pipecolic acid can be calculated from the absorption coefficients of these two acids at the two wave lengths.

$D_{575} = A\text{Pi} + B\text{Pr}$.
$D_{510} = C\text{Pi} + D\text{Pr}$.
$D_{575} + D_{510}$ = density measurements at 575 and 510 mμ, respectively.
Pi and Pr are the micrograms of pipecolic and proline.
A = density from 1 γ of pipecolic acid at 575 mμ.
B = density from 1 γ of proline at 575 mμ.
C = density from 1 γ of pipecolic acid at 510 mμ.
D = density from 1 γ of proline at 510 mμ.

The blank color is negligible. Accurate determinations of pipecolic acid up to 100 γ can be made by further dilution with ethyl acetate, but larger quantities produce an insoluble precipitate.

Table I. Factors Affecting Amount of Color Produced in Reaction of Pipecolic Acid (10 γ) with Ninhydrin

(Conditions used are those recommended, except as specifically stated)

Treatment	Relative Amount of Color
Recommended procedure (see text)	1.00
Effect of water concentration on color development	
Complete absence of water—sample dry and ninhydrin solution prepared by adding concentrated phosphoric acid directly to butanol	0.90
Additional water—sample dissolved in 0.1 ml. of water	0.42
Drying procedure	
Sample dried in oven at 105° C.	0.85
Effect of various factors on efficacy of ninhydrin solution	
1*N* sulfuric acid used in ninhydrin solution instead of 1*M* phosphoric acid	0.36
2*M* hydrochloric acid used in ninhydrin solution instead of 1*M* phosphoric acid	0.21
6*M* phosphoric acid used in ninhydrin solution instead of 1*M* phosphoric acid	0.57
0.1*M* phosphoric acid used in ninhydrin solution instead of 1*M* phosphoric acid	0.98
2 ml. of ninhydrin solution used instead of 1 ml.	0.98
Ninhydrin concentration 8% instead of 4%	0.99
Three-day-old ninhydrin solution	0.66
Effect of different substances	
1 mg. of sodium chloride added to sample	0.82
0.1 mg. of sodium chloride added to sample	0.95
100 γ of phenylalanine or leucine added to sample	0.80
Ammonium phosphate equivalent in nitrogen content to 100 γ of leucine added to sample	0.93
100 γ of proline added to sample	1.09
100 γ of hydroxyproline added to sample	1.01
Color produced by other procedures	
Pipecolic acid reaction run by proline method of Chinard (*1*)	0.11
Pipecolic acid reaction run according to procedure of Schweet (*11*)	0.70

DISCUSSION

The described method is sensitive to water concentration (Table I), to drying procedure, to the type of acid used, and to the age of the ninhydrin solution. It is relatively insensitive to volume and concentration of the ninhydrin solution. At the reaction temperature specified (100° C.), 15 minutes' heating time gives maximum color (Figure 2). Of a number of solvents tried (Table II), ethyl acetate proved the most satisfactory both as to intensity and stability of color.

Both salts and amino acids interfere with color production (Table I). However, the interference by amino acids (Table I) is not entirely due to the ammonium salt produced in the ninhydrin reaction (*10*).

The error caused by proline (Table I) can be corrected for as shown above. Hydroxyproline does not yield enough color to interfere (Table I). Other methods for proline and pipecolic acid are less sensitive (Table I).

SEPARATION OF PIPECOLIC ACID FROM INTERFERING MATERIALS

In order to make the above method applicable to samples of biological origin, it is necessary to separate pipecolic acid from interfering substances such as amino acids and salts (Table I). Rothstein and Miller (*8, 9*) used consecutive columns of ion exchange resins (IR-4 and IRC-50) followed by the formation of copper salts to isolate pipecolic acid from rat urine. In this case the quantities of pipecolic acid were considerable and their methods are not applicable to most biological materials.

One-directional chromatography was unsuccessful because interfering substances were not removed satisfactorily. It was learned (*12*) that in subjecting amino acids and salts to the process of ion exclusion (*16*) by chromatographing them on an ion exchange resin, pipecolic acid is separated from all α-amino acids and salts (Figure 3). This separation is not accomplished in the presence of sugars and other nonionic materials, and these must be eliminated by first retaining ionic materials on ion exchange resins. Heavy metals which intefere with the ion-exclusion process are also removed in this step.

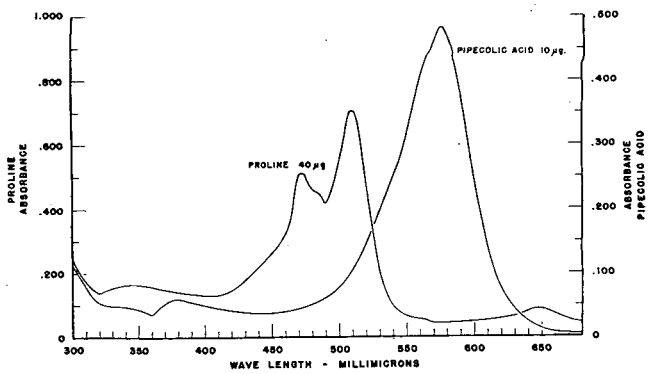

Figure 1. Spectral curves of compounds produced by reaction of ninhydrin with pipecolic acid and proline

Total volume of solution, 10 ml.

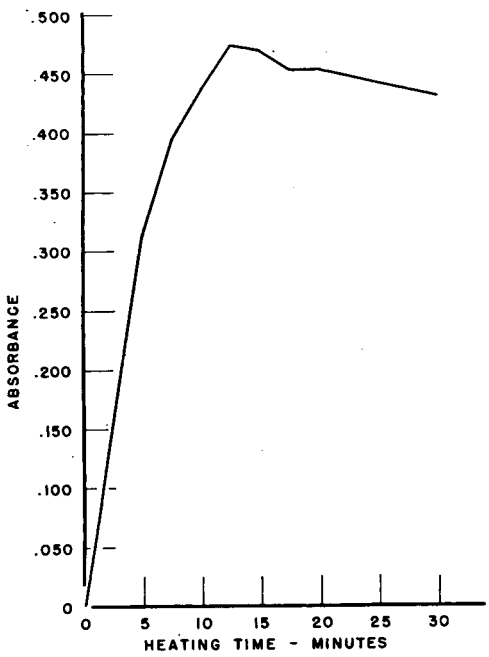

Figure 2. Effect of time on development of color between pipecolic acid and ninhydrin

Table II. Effect of Diluent on Intensity and Stability of Color Developed between Pipecolic Acid and Ninhydrin

Diluent	Relative Density as % of Standard	Relative Stability as % of Initial Color after 1 Hour
Ethyl formate	100.0	100.0
Ethyl acetate	100.0	100.0
Butanol–1*M* phosphoric acid, 9 to 1	80.0	47.7
95% ethyl alcohol	91.0	92.7
9:1 ethyl alcohol–acetic acid	85.0	90.2
1:1 ethyl alcohol–acetic acid	79.5	88.9
1:1:1 ethyl alcohol–acetic acid–water (*3*)	78.2	90.9
Methanol	83.2	95.0
Isoamyl alcohol	85.5	92.1
90% acetic acid	79.4	95.7

Preparation of Columns and Ion Exchange Resins. COLUMNS. Glass columns are required for two purposes—the separation of sugars from amino acids and the separation of pipecolic acid from the alpha amino acids and salts. Only in the latter case are the

dimensions of the column important. Glass tubes (0.9 cm. in inside diameter and 50 cm. long) are prepared with an opening of 3 to 4 mm. at one end.

RESINS FOR SEPARATION OF SUGARS FROM AMINO ACIDS. Ion exchange resin Dowex I-X2 (200–400 mesh) (Dow Chemical Co., Midland, Mich.) is prepared by treatment with excess carbonate-free 3*N* sodium hydroxide overnight and the base is removed by washing with pure water.

RESIN FOR SEPARATION OF PIPECOLIC ACID FROM SALTS AND α-AMINO ACIDS. The preparation of this resin is the most critical part of the whole method. Resin Dowex 50-X12 is treated with excess 3*N* hydrochloric acid on a steam bath for 24 hours. The excess hydrochloric acid is removed and the resin is neutralized with excess 3*N* sodium hydroxide on a steam bath for 24 hours. The excess base is removed by washing the resin with deionized water until the pH of the wash water reaches 10 ± 0.2. (Deionized water is prepared by passing distilled water through a mixture of strongly acidic and strongly basic ion exchange resin.) This resin is now in the sodium form.

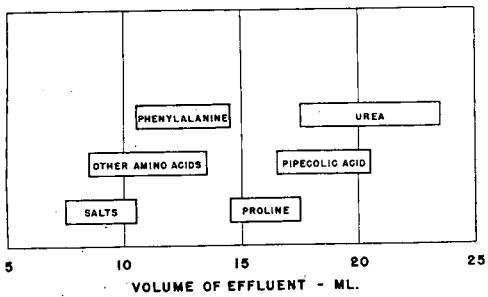

Figure 3. Pattern of elution of various substances

From column (0.9 cm. in diameter and 30 cm. in length) of Dowex 50 – 12% divinylbenzene in sodium form (200 – 400 mesh)

Table III. Recovery of Pipecolic Acid Added to Rabbit Urine and Turnip Leaf Extracts

Material	Pipecolic Acid Formed	Recovery, %
Rabbit urine, 5 ml.	3.77 ± 0.10[a]	....
Rabbit urine, 5 ml. + 10 γ of pipecolic acid	13.9 ± 0.80[a]	101 ± 7.4[a]
Turnip leaf extract	0.62 ± 0.0	....
Turnip leaf extract + 10 γ of pipecolic acid	10.73 ± 1.02[a]	101 ± 10.2[a]

[a] Standard deviation on three determinations.

PROCEDURE

Extraction of Samples. Fresh samples are ground with sufficient 95% alcohol to make the resultant alcohol concentration not less than 75%. The liquid is separated from the residue and the extraction is repeated with 75% alcohol as many times as is necessary to extract all amino acids. Dry samples are treated similarly, using 75% alcohol from the start. The insoluble material is discarded, because pipecolic acid does not occur in this fraction (*11*).

Samples containing carbonates should be acidified before application to resin columns, because carbon dioxide released in the resin disrupts the column.

Separation of Nonionic from Ionic Substances. Sample extracts are passed through a column of ion exchange resin, Dowex I, making sure that all amino acids are retained on the resins by testing for ninhydrin activity (*6, 15*) in the effluent. The resins are washed thoroughly with deionized water until tests for sugar are negative and the washings are discarded. The amino acids are eluted from the resins with 1*N* hydrochloric acid until the effluent is acid. The eluate is dried at room temperature and dissolved in water, the pH is adjusted to between 9.5 and 10.5, and the solution is made to a volume such that 1 ml. contains 1 to 20 γ of pipecolic acid.

Use of Dowex 50 for this purpose may result in difficulties with samples containing large amounts of urea, since this is retained on Dowex 50 and chromatographs with pipecolic acid (Figure 3).

Chromatographic Separation of Pipecolic Acid from α-Amino Acids and Salts. A column of resin Dowex 50-X12 in the sodium form is prepared by pouring a thick slurry of this resin into a glass column (0.9 cm. in internal diameter) using a filter paper disk at the bottom to retain the resin. Sufficient resins should be poured in so that the height of the settled resin is 30 cm. or more. Any resin above 30 cm. in height is removed.

A 1-ml. aliquot of extract from which nonionic materials have been removed is placed carefully on the top of the resin column and allowed to pass into the resins. The sides of the column are washed down with two successive 1-ml. portions of pure water, each time allowing the water to pass into the resin. Thirteen milliliters of pure water are placed on top of the resin and allowed to move into the resin. The corresponding effluent from the column contains the α-amino acids and salts and is removed. The next portion is collected in a test tube after 10 ml. of pure water are added to the top of the resin. This fraction is dried at room temperature and analyzed for pipecolic acid by the method given above.

DISCUSSION

Separation of Nonionic from Ionic Materials. Nonionic materials interfere with the separation of pipecolic acid from the α-amino acids and salts on the column of sodium resin and must therefore be separated from the amino acids and salts with ion exchange resins. The procedure also serves as a convenient method of concentrating an extract. The amount of resin used is not critical, as long as all amino acids are retained on the resin.

Separation of Pipecolic Acid from Other Materials. The preparation of the Dowex 50 -X12 in the sodium form is the most probable source of error in the whole procedure. If the acidic groups on the resin are not completely neutralized, pipecolic acid will be retained by the resin. Because of the high cross linkage of this resin, the penetration and consequent neutralization by sodium hydroxide are slow and require heat for a relatively long time.

Excessive washing of the resin may result in subsequent retention of amino acids. Sample solutions of low pH will act in the same manner. Insufficient removal of excess base from resin may result in incomplete separation of pipecolic acid.

The samples free of nonionic substances should be applied in a volume no greater than 1 ml. in order to obtain a sharp separation of amino acids in the effluent. The Dowex 50 -X12 column can be used only once, because impurities in the sample change the adsorption characteristics of the resin. One such test resulted in the loss of 44% of added pipecolic acid. Ornithine, lysine, and hydroxylysine which interfere in Chinard's method (*1*) and citrulline which interferes with Schweet's method (*11*) are retained by this resin.

The procedure presented here has not been found applicable to quantitative proline analysis.

Tests of Methods. The above procedures have been tested with pure pipecolic acid, urine, and nonprotein extracts of turnip leaves.

With standard pipecolic acid (15 γ) duplicates agree within 1%. The recovery of pipecolic acid added to aliquots of rabbit urine and turnip leaf extracts are presented in Table III. The replicate analyses of unsupplemented samples are good and the recovery of added pipecolic acid is within 10% of that added.

LITERATURE CITED

(1) Chinard, F. P., *J. Biol. Chem.* **199,** 91 (1952).
(2) Grobbelaar, N., Steward, F. C., *J. Am. Chem. Soc.* **75,** 4341 (1953).
(3) *Ibid.*, **76,** 2912 (1954).
(4) Harris, F., Pollock, J. R. A., *J. Inst. Brewing* **59,** 29 (1953).
(5) Hulme, A. C., Arthington, W., *Nature* **170,** 659 (1952).
(6) Moore, S., Stein, W. H., *J. Biol. Chem.* **176,** 367 (1948).
(7) Morrison, R. I., *Biochem. J.* **53,** 474 (1953).
(8) Rothstein, M., Miller, L., *J. Am. Chem. Soc.* **75,** 4371 (1953).
(9) *Ibid.*, **76,** 1459 (1954).
(10) Schlenker, F. S., ANAL. CHEM. **19,** 471 (1947).
(11) Schweet, R. S., *J. Biol. Chem.* **208,** 603 (1954).
(12) Thompson, J. F., Morris, C. J., unpublished data.
(13) Thompson, J. F., Steward, F. C., *Plant Physiol.* **26,** 421 (1951).
(14) Thompson, J. F., Zacharius, R. M., Steward, F. C., *Ibid.*, **26,** 373 (1951).
(15) Troll, W., Cannan, R. K., *J. Biol. Chem.* **200,** 803 (1953).
(16) Wheaton, R. M., Bauman, W. C., *Ind. Eng. Chem.* **45,** 288 (1953).
(17) Zacharius, R. M., Thompson, J. F., Steward, F. C., *J. Am. Chem. Soc.* **74,** 2949 (1952).
(18) *Ibid.*, **76,** 2908 (1954).

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Flame Photometric Determination of Calcium in Furnace Slag

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A flame photometer method was developed for a rapid determination of calcium in the range of 30% by weight in furnace slag. The method gives results comparable in reproducibility with a rapid chemical method previously employed. Total elapsed time required for the flame analysis is 2 hours. Previously the elapsed time using the chemical method was 24 hours.

THE routine determination of calcium in furnace slag by wet chemical analysis requires several hours. The method hereafter referred to as the "slow" chemical method consisted of solution of the sample in aqua regia, a conventional silica determination which resulted in silica removal, a fusion step on any insoluble, double ammonia treatment to remove R_2O_3 metals, and a calcium determination after double precipitation. The elapsed time required for this procedure was at least 48 hours.

The method referred to as the "fast" chemical method consisted of the following steps: solution of the sample with hydrochloric, nitric, sulfuric, and hydrofluoric acids; evaporation to dryness; a single ammonia separation; and a calcium determination after a single precipitation. The elapsed time required for this method was a minimum of 6 hours. In practice, though, this normally resulted in an elapsed time of 24 hours before results could be reported, because work was interrupted overnight.

It was desirable to increase the speed of determination so that the results might be available in time to serve more effectively in the controlling of actual furnace operation. A flame photometer method has been developed for this purpose. Others (*1–3, 5*) have recently reported investigations of flame analysis for calcium. This work illustrates the successful application of the flame method to a complex material of high calcium content. Others may find the technique useful when time is more important than high precision.

The time required for the determination is about 2 hours and the reproducibility is comparable to that obtained by a routine chemical method requiring about 24 hours. An additional 20 minutes per sample is required by the flame method when run in lots of two or more.

EXPERIMENTAL

Preparation of Sample Solutions. In the preparation of samples for analysis, three steps are required: (1) dissolution of sample, (2) removal of iron, and (3) dilution to bring the calcium content of the solution within the limits of the calcium standards, and adjustment of pH.

A 0.250-gram portion of the sample, preferably ground to pass 200 mesh, is placed in a quartz dish (Vitreosil opaque fused silica dish, 75-ml. capacity, $3^3/_4$ inches in diameter, $^{11}/_{16}$ inch deep, flat bottomed, glazed; Geo. D. Feidt & Co., Philadelphia, Pa.). Fifteen milliliters of water is added to wet the sample, followed by 10 ml. of concentrated hydrochloric acid. The dish is placed on a hot plate (medium setting) and the sample stirred with a glass rod to keep it from sticking to the surface of the dish. When solution is complete, 2 ml. of concentrated nitric acid is added to ensure oxidation of iron. The solution is then evaporated to dryness; this requires about 45 minutes. The sample is cooled and redissolved with heating (for about 1 minute) in 15 ml. of distilled water and 10 ml. of concentrated hydrochloric acid. The solution is then transferred to a 500-ml. volumetric flask, the dish is thoroughly rinsed, and the solution plus washings are made up to volume with distilled water. After the solution is thoroughly mixed, about 25 ml. are filtered through two sheets of Whatman No. 42 filter paper.

In this and the next step, the large dilutions were established to avoid any possible removal of calcium by silica or iron oxides as they are separated.

Ten milliliters of the filtered solution is transferred to a 100-ml. volumetric flask; 25 ml. of distilled water and 1 drop of methyl orange are added. The solution is then adjusted to the end point with 10% ammonium hydroxide solution. The flask is placed on a hot plate for 10 to 15 minutes, cooled, diluted to 100 ml. with distilled water, and filtered through Whatman No. 42 filter paper to remove iron.

A 25-ml. portion of the filtrate from the removal of iron is placed in a 50-ml. volumetric flask, and 0.1 ml. (2 drops) of 9.5% hydrochloric acid solution is added. The solution is made up to 50 ml. with distilled water. This solution, which contains 0.025 gram of sample per liter, is used for the calcium determination.

In this work each sample was run in triplicate and the average of the three values obtained was reported.

Preparation of Standards. BASE SOLUTION A. A calcium carbonate sample weighing 2.4974 grams is placed in a 250-ml. beaker and 15 ml. of distilled water added. The beaker is covered with a watch glass, then 10 ml. of concentrated hydrochloric acid is added slowly through the beaker lip. When solution is complete, 2 ml. of concentrated nitric acid is added and the solution is evaporated to dryness. The sample is then cooled and redissolved with heating in 15 ml. of distilled water and 10 ml. of concentrated hydrochloric acid. The solution is again cooled and transferred to a 1-liter volumetric flask, diluted to volume with distilled water, and filtered through a Whatman No. 42 filter paper. This solution contains 1000 p.p.m. of calcium.

Two analyses of the calcium carbonate and one of the actual standard base solution gave 55.5% calcium oxide. Calcium carbonate is 56% calcium oxide by theory.

BASE SOLUTION B. Twenty-five milliliters of Base Solution A is transferred to a 250-ml. volumetric flask, 2 drops methyl orange added, and the solution is adjusted to the end point with 10% ammonium hydroxide. The solution is diluted to volume with distilled water and filtered through Whatman No. 42 filter paper. This solution contains 100 p.p.m. of calcium.

CALCIUM STANDARDS. Ten milliliters of Solution B is transferred to a 100-ml. volumetric flask. Two drops (0.1 ml.) of 9.5% hydrochloric acid solution is added, and the solution is diluted to volume with distilled water. This standard contains 10 p.p.m. of calcium.

For the 5-p.p.m. calcium standard, 5 ml. of Solution B is

Table I. Per Cent Calcium Oxide in Daily Slag Samples

Sample No.		Flame	Chemical Methods: Rapid	Chemical Methods: Slow
SB-34		34.7	33.1	33.0
		33.6	33.4	
		34.7		
	Av.	34.3		
Check		32.5		
		32.5		
		33.0		
	Av.	32.7		
SB-35		35.2	32.4	31.7
		33.0	31.7	
		33.0		
	Av.	33.7		
SK-36		35.3	34.9	33.8
		35.8		
		36.4		
	Av.	35.8		
SB-37		33.6	32.3	31.7
		33.6	31.5	
		33.6		
	Av.	33.6		
1B-38A		38.1	35.9	34.9
		37.5	35.9	35.0
		37.5		
	Av.	37.7		
Std. dev.		0.43	0.86	0.21
% std. dev. (for mean CaO value of 30%)		1.43	2.87	0.70
Number of multiple determinations		7	8	8

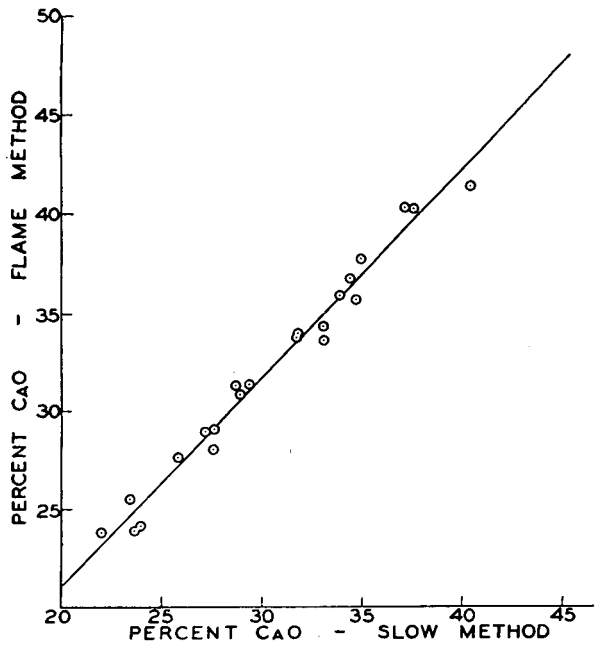

Figure 1. Correction curve for flame method *vs.* slow chemical method

treated as above; for the 3-p.p.m. and 1-p.p.m. standards, 3 ml. and 1 ml., respectively, of Solution B are used.

Standards are stored in polyethylene bottles.

The sample and standard solutions are prepared as just described to ensure the removal of iron and to maintain a constant acidity of the final solution. Both of these conditions are essential because tests have indicated that low results are caused by the presence of iron and that decreasing values are obtained from increasing acidity. Examples of each are noted below.

Spectrophotometer. A Beckman Model DU spectrophotometer equipped with a photomultiplier tube and the Model 9200 flame attachment with oxyhydrogen flame was used for measuring calcium emission intensity. Although the original work on this method was done with the blue-sensitive photocell and the oxyacetylene flame, the final tests and all of the comparative analyses were made using the photomultiplier tube and oxyhydrogen flame. It is likely that the added sensitivity of the photomultiplier was vital to the successful development of this analytical method.

Measurement Procedure. The instrument settings which were used in this method are as follows: oxygen pressure, 20 pounds per square inch; hydrogen pressure, 5 pounds per square inch; photomultiplier detector cell; wave length setting, 554 mμ; slit width, 0.1 mm.; selector range, 0.1; sensitivity setting, full counterclockwise; resistor, 22 megohms.

Plots of the flame emission curves for most common elements have been published (*4*). These have been found useful in predetermining possible interference.

For the 10-p.p.m. calcium standard, the instrument is adjusted for a reading of 100% emission by a small adjustment of the sensitivity control in a clockwise direction. Less than one complete turn of the knob was found sufficient to accomplish this. With this setting the corresponding emission readings are obtained for the 5-, 3-, and 1-p.p.m. calcium standards and for distilled water. Emission readings are then taken for the sample solutions.

Corrected emission readings are obtained by subtracting the background reading for distilled water from the readings for the standards and sample solutions. These corrected values for the standards are used in preparing an analytical curve. Data are plotted as per cent emission against parts per million of calcium. The corrected values obtained for the sample solutions are referred to this curve to determine calcium concentration.

RESULTS ON DAILY SAMPLES

Using the method described above, a test was carried out in which daily samples were analyzed over a period of 19 days. In Table I, representative results of this test and data on precision are listed showing values obtained for each of the three solutions prepared for the daily sample, average results, and the chemical analysis results performed by each of the two methods outlined above.

The multiple determinations were entirely different preparations run on different days. One duplicate pair for the rapid chemical method checked very poorly and, in the small group of duplicates available, had a large effect on the standard deviation determination.

With the flame procedure described, the error in scale reading on the Beckman instrument is equivalent to 0.5% calcium oxide. This is of the same order as the standard deviation (0.43%), and indicates that the present limits of the method are determined to a considerable extent by the instrument characteristics, over which there is no control.

DISCUSSION OF RESULTS

Inspection of the above results indicates that both the rapid chemical method and the flame method give higher results than the slow chemical method, which is believed to be the most accurate and has been used for reference throughout this work. The source of this constant error in the flame results is unknown.

Table II. Effect of Iron

Sample No.	% Calcium Oxide: Original solution	% Calcium Oxide: Iron removed
26	11.3	19.6
389	7.8	14.7
SB-6	20.5	23.7
SB-35	30.3	33.7
SB-39	37.9	41.4

A study of the variation in analysis of calcium with variation in concentration of other elements present, including zinc, lead, iron, cadmium, silicon, and sulfur, failed to establish any correlation.

If it is desirable to correct the accuracy of the flame results to correspond to those of the slow method, a correction curve like that shown in Figure 1 is satisfactory, where results from the flame photometer method are plotted against the slow chemical method. The straight line which fits the points was calculated by a least squares method. The line goes through the origin at 0% calcium oxide. (Note that the plot starts at 20%.) To correct an experimental flame determination, the point corresponding to the per cent analyzed is located on the vertical axis and corrected by going to the straight line plot and down to the horizontal axis to obtain the corresponding corrected lower value. For example, corrected values are shown below for the five results listed in Table I.

Sample	Flame: Original, %	Flame: Corrected, %	Slow Method, %
SB-34	34.3	32.6	33.0
SB-35	33.7	32.0	31.7
SK-36	35.8	34.0	33.8
SB-37	33.6	31.9	31.7
IB-38A	37.7	35.8	34.9

REPRESENTATIVE SLAG ANALYSIS

Representative weight percentages of some materials occurring in the slag samples of interest in this work are as follows:

	%
Silica	33
Calcium oxide	32
Iron	10
Zinc	3
Magnesium oxide	3
Sulfur	2
Lead	0.4

Table III. Effect of Acidity

Solution	Hydrochloric Acid Added, Ml.	Acid Concn. of Soln., %	% Calcium Oxide
A	1	4	23.8
B	2	8	21.7
C	5	20	20.3
D	10	40	14.7
E	15	60	12.5

EFFECT OF IRON

Several samples which were taken to solution at different times during this investigation were analyzed for calcium, using both the original solution and the same solution after removal of iron by precipitation with ammonium hydroxide. The results are listed in Table II.

EFFECT OF ACIDITY

For this test a sample was used which had been carried through the solution preparation procedure to include the removal of iron, but not the final dilution. Five 25-ml. aliquots were evaporated to a volume which permitted the addition of varying amounts of hydrochloric acid before adjusting the volume to 25 ml. The results of this test are shown in Table III.

CONCLUSION

The reproducibility of the flame method is better than that of the rapid chemical method, as indicated by the check analysis. Because the rapid chemical method has been used for furnace control work, it appears that the flame method is a satisfactory substitute from the point of view of reproducibility. Furthermore, it offers a rapidity of determination not previously available.

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LITERATURE CITED

(1) Baker, G. L., Johnson, L. H., ANAL. CHEM. **26**, 465–8 (1954).
(2) Chow, T. J., Thompson, T. G., *Ibid.*, **27**, 910–13 (1955).
(3) Curtis, G. W., Knauer, H. E., Hunter, L. E., *Am. Soc. Testing Materials*, Symposium on Flame Photometry, Spec. Tech. Pub. 116 (1952).
(4) Gilbert, P. T., Jr., *Ind. Laboratories*, p. 41 (August 1952).
(5) Hinsvark, O. N., Wittwer, S. H., Sell, H. M., ANAL. CHEM. **25**, 320–2 (1953).

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Absorptiometric Study of Certain Organic Fluorine Compounds

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Fluorinated 1,3-diones can be applied to the absorptiometric determination of some metal ions, in limited concentration range. A method for iron using thenoyltrifluoroacetone is developed in detail. Advantages are speed, simplicity, and a stable color reaction. Disadvantages are relatively low sensitivity and many interferences. Of the common methods used for determining acetone, only the 2,4-dinitrophenylhydrazine reagent is applicable to halogenated acetones. Methods for bromo-, chloro-, 1,3-dichloro-, and 1,1,1-trifluoroacetone are given. Absorption curves for the derivatives are presented.

THE substitution of fluorine into organic compounds has yielded a great variety of new products, some of which have properties of possible analytical interest. In general, these properties may be related to the high electronegativity of the fluorine. Thus, in aliphatic compounds several fluorine substituents will deactivate neighboring groups, such as hydrogen or another halogen. A nitro group is stabilized. Complete fluorination of an aliphatic chain gives the very inert fluorocarbons.

In contrast, fluorine increases the acidity of some groups and thus may enhance reactivity. Fluorinated nitriles are more easily hydrolyzed than simple nitriles. Acid strengths of organic acids, of compounds such as 1,3-diones, and of alcohols are increased. A carbonyl or an alcohol group near a perfluoroalkyl group forms a hydrate readily. The chelate compounds of 1,3-diones are more stable, more volatile, and lower melting when fluorine is present.

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This research aimed to examine some of these new compounds for their usefulness in analysis. An incidental and less practical objective was to extend generalizations on the effect of fluorine in organic analytical reagents.

A literature survey was made in order to find, if possible, good examples of organic fluorine compounds of the general kinds of organic analytical reagents listed by Welcher (*6*) with the types of applications summarized by Yoe and Sarver (*8*) and to locate any known analytical methods which involved use or determination of fluorine compounds. Of the vast number of fluorine compounds now known, many have little or no present analytical interest because of their high volatility, insolubility, or low reactivity. Of those of possible analytical usefulness, few have been examined. For present purposes, attention was directed only to compounds of possible absorptiometric interest. This interest finally centered on 1,3-diones and on halogenated acetones.

As possible analytical reagents, the 1,3-diones offered a wide field for study of chelate systems. Functioning as weak acids, they form salts with most metal ions. Possibly because of the large number of colored compounds, and consequently many possibilities for interference, they have not been used extensively in absorptiometric analysis. Pulsifer in 1904 (*5*) recommended acetylacetone as a reagent for iron(III). Though the sensitivity (0.003 mg.) compared favorably with that for the thiocyanate method, the latter has become a standard method while the former is seldom mentioned. Recently Cefola (*3*) suggested a new dione, thenoyltrifluoroacetone (TTA), as a sensitive reagent for the detection of iron(III). The red color formed in benzene served to detect 10 p.p.m. of iron(III). Other previous uses of the 1,3-diones are in the determination of microgram quantities of beryllium (*1*) by acetylacetone, using the ultraviolet spectrum of the complex and the determination of uranium by dibenzoylmethane (*7*, *9*).

Liquid-liquid extraction studies involving chelate salts of 1,3-diones have made available a number of fluorine-containing 1,3-diones. Commercially available examples are thenoyltrifluoroacetone (TTA), furoyltrifluoroacetone (FTA), tri- and hexafluoroacetylacetone (TAA, HAA), and 2-thenoyl- and 2-furoylperfluorobutyrylmethane (TPBM, FPBM). Numerous other compounds have been prepared in research quantities.

For examining the effect of a substituent on the determination of an organic compound, acetone offers some advantages. It is a simple compound. Because of clinical interest various accurate methods have been developed for its determination. Most of the possible halogenated derivatives have been prepared and characterized—e.g., as semicarbazones—but the accurate spectrophotometric methods for acetone had not been extended to them.

The most useful and accurate absorptiometric methods involve aldol type condensation, with salicylaldehyde, vanillin, furfural, or *o*-nitrobenzaldehyde, or formation of the 2,4-dinitrophenylhydrazone. These methods are sensitive to a few micrograms of acetone. The nitroprusside test and the ultraviolet absorption of acetone itself are not very sensitive nor accurate. The iodoform test is accurate and widely used as a titrimetric determination, but methods based on the absorption spectrum of iodoform or its colored reaction product with pyridine and strong base have less use.

EXPERIMENTAL WORK

The experimental work consisted of qualitative tests with a number of compounds, and from this information, the quantitative study of two kinds of methods.

Apparatus and Reagents. A General Electric recording spectrophotometer provided transmittance curves for the visible region, used mainly for qualitative screening. The Beckman B spectrophotometer was used for determinations at specific wave lengths, as in interference studies. Values read from these instruments are in per cent transmittance. A Cary recording spectrophotometer was used for ultraviolet absorption curves.

Measurement of pH was made with Beckman pH meters, either Model H-2 or C.

A standard iron(III) solution was prepared from iron wire, 99.8% pure, by dissolving 100.0 mg. in 10 ml. of 6*N* nitric acid and diluting to 1 liter. Similar standard solutions in hydrochloric or sulfuric acid were prepared. Except as otherwise indicated, the nitric acid solution is referred to below as stock iron(III) solution.

Table I summarizes certain information on the compounds used.

Solutions of thenoyltrifluoroacetone were prepared, 0.060*M* in absolute ethanol, and in 2-propanol, and 0.60*M* in 2-propanol. Ethanolic solutions, each 0.10*M*, of furoyltrifluoroacetone, thenoylperfluorobutyrylmethane, furoylperfluorobutyrylmethane, trifluoroacetylacetone, and hexafluoroacetylacetone were used for qualitative tests.

A series of solutions of reagent grade metal salts, mostly containing 10 mg. per ml. of the cation or the anion desired was used for the qualitative tests and for interference studies.

The stock solution of 2,4-dinitrophenylhydrazine was 0.1% in 2*N* hydrochloric acid. When a more concentrated solution was required, 0.20 gram was dissolved in 4 ml. of concentrated sulfuric acid and diluted to 20 ml. The former solution was stable for several months, the latter for only a few days.

Stock solutions of acetone, 1-chloroacetone, and 1,1,1-trifluoroacetone were prepared by adding a portion of the volatile liquid to a weighed quantity of water, obtaining the weight of the acetone, and diluting. Thus the following solutions were prepared: acetone, 0.100 gram per liter; 1-chloroacetone, 2.28 grams per liter; and trifluoroacetone, 1.65 grams per liter were prepared.

Solutions of 1-bromoacetone and 1,3-dichloroacetone, each 1.00 gram per liter, were prepared by weighing samples by difference from the pure material.

A 10% (volume) solution of salicylaldehyde in ethanol was used for the tests described. Other reagents were prepared as called for from materials of C.P. grade or better.

Table I. Data on Compounds Used

Compound	Abbr.	Source
4,4,4-Trifluoro-1-(2-thenyl)-1,3-butanedione (thenoyltrifluoroacetone)	TTA	Dow Chemical Co.
4,4,4-Trifluoro-1-(2-furyl)-1,3-butanedione (furoyltrifluoroacetone)	FTA	Midcontinent Chemical Corp.
4,4,5,5,6,6,6-Heptafluoro-1-(2-thenyl)-1,3-hexanedione (thenoylperfluorobutyrylmethane)	TPBM	Midcontinent Chemical Corp.
4,4,5,5,6,6,6-Heptafluoro-1-(2-furyl)-1,3-hexanedione (furoylperfluorobutyrylmethane)	FPBM	Caribou Chemical Co.
1,1,1-Trifluoro-2,4-pentanedione (trifluoroacetylacetone)	TAA	Caribou Chemical Co.
1,1,1,5,5,5-Hexafluoro-2,4-pentanedione (hexafluoroacetylacetone)	HAA	R. Levine, University of Pittsburgh
4,4,4-Trifluoro-1-(2-pyrryl)-1,3-butanedione (pyrroyltrifluoroacetone)		D. H. Campbell, Purdue University, Chem. Department
1,1,1-Trifluoroacetone		Prep. according to (*2*)
1-Bromoacetone		Eastman Organic Chemicals, Inc.
1-Chloroacetone		Eastman Organic Chemicals, Inc.
1,3-Dichloroacetone		

Table II. Qualitative Results of Fluorinated 1,3-Dione Chelation

Metal	Reagent	Color	Significant Wave Length, mμ	Sensitivity Estimated from Transmittance Curves
Cu(II)	All	Blue	500	1 mg. at 500 mμ
Cr(III)	TPBM	Red	580–590	5 mg. at 580–590 mμ; 0.5 mg. at 420 mμ
Co(II)	TPBM	Red	None	4.5 mg. at 420 mμ or 700 mμ
Ce(IV)	TPBM, FPBM	Red (in benzene)	None	
Fe(III)	All	Red	480–520 (shoulder)	See study below
Mn(II)	All	Yellow	No curve	
Ni(II)	All	Green	No curve	
Os(III)	TTA	Red, brown	None	1 mg. at 420 mμ
Ru(III)	TTA	Red, brown	None	1 mg. at 420 mμ
Th(IV)	All	White ppt.	No curve	
Tl(I)CS_2	All	Yellow orange	No curve	
UO_2(II)	All	Yellow	None	0.1 mg. at 420 mμ
Be(II)	HAA, TTA	...	380 (shoulder)	0.01 mg.
ZrO(II)	All	White ppt.	No curve	
Th(IV)	All	White ppt.	No curve	

Qualitative Screening Results. A few drops of the ethanolic solution of a 1,3-dione were added to a similar amount of a solution of metal ion (10 mg. of cation per ml.) on a spot plate. The positive results are shown in Table II. Effects of acid and base were also noted. In general, acid decolorized a test, and a base caused precipitation of a hydroxide. Where strong color was noted, a solution was prepared with about 0.5 ml. of the cation solution, 1 ml. of dione solution, and enough 2-propanol to dissolve the resulting precipitate, plus water to make 25 ml. In general, the spectrophotometric curves for the solutions had no transmittance minima in the visible region, and indicated rather low sensitivities. In most cases, where absorption in some region would allow a moderately sensitive determination, variation of transmittance with wave length was large enough to make the instrument setting a critical variable. Estimations of sensitivity from these curves are included in Table II. Color with a given ion was similar for all of the diones listed. With the diones containing an aromatic group, the color was usually stronger and the amount of precipitate greater than with the aliphatic compounds.

Addition of pyridine to a spot test with the diones gave some enhancement of the color with bivalent ions, such as copper(II), nickel(II), manganese(II), and cobalt(II), compared with solutions without pyridine.

With iron(II), furoylperfluorobutyrylmethane, and pyridine, a purple color formed. It was stabilized by hydroxylamine and excess pyridine and decolorized by acid. When both nitrate ion and hydroxylamine were present, the color shifted on acidi-

fication to that of the iron(III) complex. The action is reversible unless sufficient acid is added to decolorize the solution. Transmittance curves show a change with pH, with an isosbestic point at 554 mμ. This system follows Beer's law at the isosbestic point, and also at a transmittance minimum at 580 mμ when pH is held constant. As the sensitivity for determining iron is less than that with a 1,3-dione alone, a complete quantitative study was not made.

Pyrroyltrifluoroacetone gave results rather different from those using the related compounds studied. With cobalt(II), nickel(II), manganese(II), chromium(III), and thorium(IV) neither change in color nor a precipitate was observed. The color was bright orange with uranyl(II), light green with copper(II), and purple with iron(III). The transmittance curve for the last system resembles that of the above iron(II) complex with furoylperfluorobutyrylmethane and pyridine, rather than that of a complex of iron(III) with a dione alone. The system is pH-sensitive, but no reduction to iron(II) occurs. The transmittance curve shifts without having an isosbestic point. At pH up to about 2.3, the solution was green and the complex water-soluble. Above this value the solution was cloudy unless some organic solvent was added (2-propanol was used). The sensitivity of the reagent was about the same as that of the other diones, using either the green or purple solution.

The ultraviolet absorption of thenoyltrifluoroacetone, furoylperfluorobutyrylmethane, and trifluoroacetylacetone was increased by as little as 10 γ of beryllium. This sensitivity is of the same order as that with acetylacetone (*1*). No peak was found, and the greatest interest was near 200 mμ, the instrumental limit. No further work was done with this system.

Bromo-, chloro-, and 1,3-dichloroacetone gave positive iodoform tests, a very faint increase in color with nitroprusside, and a red color with salicylaldehyde solution and strong base. Trifluoroacetone gave none of these tests. However, in basic solutions, such as were used, the first three compounds hydrolyze to give other acetones; trifluoroacetone forms fluoroform and acetic acid, which does not react further.

Out of all these systems tested qualitatively, two were selected as being most promising to study on a quantitative basis. One was the determination of iron(III) by means of thenoyltrifluoroacetone, and the other was the application of the 2,4-dinitrophenylhydrazine test to the haloacetones.

DETERMINATION OF IRON(III)

The following quantitative work concerns a study of factors affecting the color reaction of thenoyltrifluoroacetone with iron(III) and the development of a recommended procedure for determining iron with this reagent.

Tentative Procedure. Qualitative tests showed that a red precipitate was formed when a few drops of stock iron solution (0.1 mg. per ml.) was mixed with thenoyltrifluoroacetone solution. For an initial quantitative method, 5 ml. of the stock iron solution was treated with 0.5 ml. of 0.06*M* thenoyltrifluoroacetone, the stoichiometric amount for a 1 to 3 iron-dione ratio. To this system were added 10 ml. of ethanol to dissolve the precipitate and then water to make 25 ml. Starting with this tentative procedure, a quantitative study was made of variable factors in order to establish the optimum conditions for a final recommended procedure. In general, spectrophotometric curves were determined for the visible region, with water in the blank cell.

Solvent for Precipitate. In addition to ethanol, acetone and 2-propanol were tried as solvents for the iron-thenoyltrifluoroacetone precipitate. A 10-ml. portion of ethanol or 2-propanol was sufficient to dissolve the maximum practical sample, but with acetone nearly 20 ml. was necessary. As the deepest color was obtained with 2-propanol, judged from the transmittance curves, this solvent was used subsequently.

Choice of Reagent. The aliphatic fluorinated diones, as well as a 0.1*M* ethanolic solution of acetylacetone used for comparison, gave lighter colors than those with aromatic groups. The latter all gave the same shape transmittance curve, and nearly the same color. The flat shoulder at 500 to 505 mμ (Figure 1) appeared at lower transmittances in the order: thenoyltrifluoroacetone, furoyltrifluoroacetone, thenoyl-, and furoylperfluorobutyrylmethane. For comparison, benzoylacetone gave a lighter color and a different curve. Because of its greater availability, thenoyltrifluoroacetone was used in this study. The results should be applicable to furoyltrifluoroacetone and thenoyl- and furoylperfluorobutyrylmethane because of their similarity to thenoyltrifluoroacetone.

Color Reaction. The reaction of complex formation was best in aqueous solution of iron(III). When thenoyltrifluoroacetone was added to an alcoholic solution of iron(III), the reaction was slow, and the color changed for hours. When the complex was allowed to precipitate from aqueous solution, then dissolved and diluted to volume, the color was stable for at least 24 hours. Although 2-propanol gave good results when used to dissolve the complex formed with ethanolic reagent, a reagent solution in 2-propanol gave immiscible drops or mixing with the aqueous sample. With 0.6*M* reagent, the drops did not readily dissolve in 2-propanol or in solvents used for extraction (below). With 0.06*M* reagent, the drops dissolved on warming with 2-propanol. An ethanolic solution did not present these difficulties. Although for this reason the ethanolic solution is recommended, the interference study used the 2-propanolic reagent.

The amount of reagent is not stoichiometric in the usual 1 to 3 ratio of iron(III) to dione. The transmittance at 502 mμ decreased with addition of reagent until about twice the stoichiometric amount was present. Further excess gave little change. Thus, 1 ml. (0.06 millimole) of reagent was sufficient for 0.5 mg. (0.009 millimole) of iron(III), the amount in a 5-ml. sample of the stock iron(III) solution. This amount gave below 10% transmittance at 502 mμ and was therefore near the upper limit for a determination.

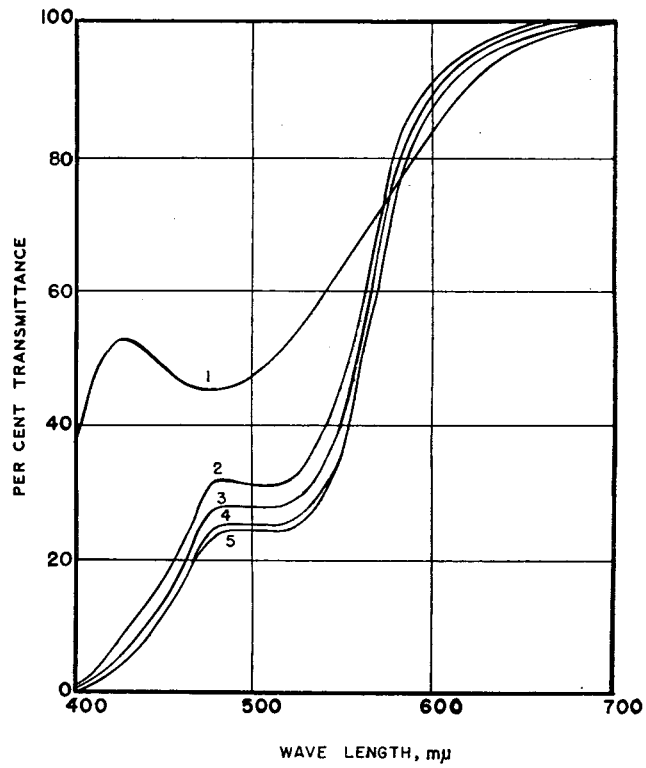

Figure 1. Spectral transmittance curves for complexes of iron(III)

1. Benzoylacetone
2. Thenoyltrifluoroacetone
3. Furoyltrifluoroacetone
4. Thenoylperfluorobutyrylmethane
5. Furoylperfluorobutyrylmethane

Effect of pH. The lowest transmittance was obtained when the aqueous sample was at pH 2.0. Only slight difference occurred with an initial pH of 1.6 to 2.1, but the transmittance rose sharply if the initial pH was outside these limits. The addition of a small amount of acid or base to the alcoholic solution of the complex after formation produced slow change, if any. A pH range of 1.9 ± 0.1 was obtained by adding small amounts of 1*N* sodium hydroxide or 6*N* nitric acid to the sample until the pH meter reading fell within that range. When several identical samples were thus prepared, the variation in transmittance was about ±1% for solutions transmitting 31%.

Conformity to Beer's Law. For samples of stock iron(III) solution run according to the recommended procedure (see below) the absorbance varies linearly with concentration. This was also true when a starting pH of 1.35 (that of the stock solution) was used and carefully reproduced. The variability was about the same (on a calibration curve) for either condition.

Extraction of the Complex. Samples containing 0.1, 0.2, and 0.3 mg. of iron(III) were treated with 1 ml. of 0.06*M* reagent, and the precipitated complex was extracted by three 8-ml. portions of an immiscible solvent. The extracts from a sample were run through absorbent cotton to remove water drops, combined, and diluted to 25 ml. with fresh solvent. Transmittance curves were taken. Benzene, carbon tetrachloride, isopropyl ether, and a 1 to 1 (volume) mixture of isopropyl ether and 1-hexanol were used. In each case the aqueous solutions were left colorless. The color in these solvents was about the same as in the 2-propanol solutions. As the shoulder at 500 to 505 mμ was not flat, a single wave length (500 mμ) rather than a range was used for measurement. The three points taken for each solvent did not plot straight lines of absorbance *vs.* concentration, but they did give consistent curves. The color was not enhanced by extraction, but it was as stable as before. No advantages were found for use of such an extraction procedure.

Statistical Test of Data. Data for 10 duplicate determinations were examined before doing interference tests, to set a standard value. These all had 0.2 mg. of iron(III) and used the 2-propanolic solution of thenoyltrifluoroacetone for color development. For these solutions the average transmittance, $\bar{X}$, was 31.0; the standard deviation, σ, was 0.30. The estimated true standard deviation, σ', is 0.32. Three-sigma control limits for transmittance of a series of determinations are at 31.0 ± 0.96, or, practically, 31.0 ± 1.0. This deviation, translated into amount of iron, allows ±3% error.

Interference by Diverse Ions. Samples for interference tests contained 2 ml. of stock iron(III) solution and up to 3 ml. of a solution (10 mg. per ml.) of another ion. The 2-propanolic reagent (0.06*M*) was used for color development. If, on addition of 10 ml. of 2-propanol, the colored precipitate did not dissolve completely, the immiscible portion was dissolved by warming on a steam plate. (All interference tests were not rerun using the ethanolic solution recommended, as on some trials no significant differences were found.) Large amounts of diverse ions were tried first, the amount being decreased if interference was noted, until the transmittance came within the acceptable range. (To test certain ions, amounts were increased by using samples of the solid salts.)

Effects of suspected interfering ions are given in Table III. The "amount added" and "error, %" columns give the extent of interference. Most of the cationic interferences were expected, qualitatively, from their complexing tendencies or from known compounds with thenoyltrifluoroacetone. One unusual case, mercurous ion, did not readily reduce ferric ion. The strongest anionic interferences were also expected from the nature of their iron complexes. Oxidizing agents gave no increase in color, indicating that all iron was in the ferric state. With reducing agents, time became a factor. Thiosulfate reduced the ferric ion immediately, but formate and thiocyanate could be present if the determination was made immediately. On standing, they also produced lighter solutions. Most interferences gave transmittance values above 32%, but thiocyanate (because of its iron complex), dichromate (its own color), cobalt, and chromium (color with reagent) gave transmittances below 30%.

Table III. Effects of Suspected Interfering Ions

Suspected Interfering Ion	Added as	Amount Added, Mg.	Error, %	Maximum Permissible Amount for 5% Error, Mg.
Acetate	$NaC_2H_3O_2$	75	0	75
Al^{+++}	$Al(NO_3)_3$	30	− 7.5	2
NH_4^+	NH_4NO_3	450	− 15	225
Ba^{++}	$Ba(NO_3)_2$	30	0	30
Be^{++}	$Be(NO_3)_2$	20	− 20	1
Bi^{+++}	$Bi(NO_3)_3$	0.5	7.5	0.2
BO_3^{---}	H_3BO_3	30	− 3	30
Br^-	KBr	30	− 6	20
Cd^{++}	$CdCl_2$	30	0	30
Ca^{++}	$Ca(NO_3)_2$	30	0	30
Citrate	$Na_3C_6H_5O_7$	0.5	− 7.5	0.2
ClO_3^-	$KClO_3$	30	3	30
Cl^-	NaCl	600	1	600
$Cr_2O_7^{--}$	$K_2Cr_2O_7$	5	27	1
Cr^{+++}	$Cr(NO_3)_3$	20	7.5	10
Co^{++}	$Co(NO_3)_2$	10	10	5
Cu^{++}	$Cu(NO_3)_2$	10	− 15	5
F^-	NaF	0.1	− 58	0
Formate	$NaCHO_2$	10	− 7.5	5
IO_3^-	KIO_3	20	− 30	10
I^-	KI	0.5	− 7.5	0.2
Pb^{++}	$Pb(C_2H_3O_2)_2$	30	− 10	15
Li^+	LiCl	30	2.5	30
Mg^{++}	$Mg(NO_3)_2$	30	0	30
Hg^{++}	$Hg(NO_3)_2$	5	− 18	1
Hg_2^{++}	$Hg_2(NO_3)_2$	15	Ppt., cannot determine	7.5
Ni^{++}	$NiCl_2$	30	− 7.5	20
NO_3^-	$NaNO_3$	600	− 3	600
NO_2^-	$NaNO_2$	5	− 3	5
Oxalate	$Na_2C_2O_4$	0.5	− 75	0
ClO_4^-	$KClO_4$	15	− 2.5	15
IO_4^-	KIO_4	1	− 58	0.5
PO_4^{---}	KH_2PO_4	0.2	− 68	0
K^+	KNO_3	400	− 3	400
Ag^+	$AgNO_3$	30	0	30
Na^+	NaCl	400	1	400
Sr^{++}	$Sr(NO_3)_2$	5	− 7.5	2
Tartrate	$Na_2C_4H_4O_6$	0.5	− 3	0.5
SCN^-	KSCN	10	20	5
$S_2O_3^{--}$	$Na_2S_2O_3$	1	−100	0
Th^{++++}	$Th(NO_3)_4$	2.5	− 10	1
Versenate	$C_{10}H_{20}N_2O_8$	0.1	− 12.5	0
Zn^{++}	$Zn(NO_3)_2$	30	0	30
ZrO^{++}	$ZrO(NO_3)_2$	0.2	− 12.5	0

Recommended General Procedure. SAMPLE. Obtain a representative sample containing 0.05 to 0.35 mg. of iron(III). This should be in solution as the chloride or nitrate but not sulfate. Any interfering ions present must be reduced to concentrations within the limits specified in Table III. Adjust a 5-ml. portion of the solution, preferably by using a pH meter, to pH 1.9 ± 0.1 with sodium hydroxide or nitric acid.

DESIRED CONSTITUENT. To the 5-ml. sample, add 1 ml. of 0.06*M* ethanolic thenoyltrifluoroacetone. When the precipitate has coagulated, in about 5 to 10 minutes, add 10 ml. of 2-propanol. When the precipitate dissolves, dilute to 25 ml. with water. Measure the absorbance at 502 mμ *vs.* water, and read the amount of iron from a calibration curve. The calibration curve for the recommended procedure of this study can be reproduced from the zero point and $A = 0.52$ for 0.20 mg. of iron(III).

DETERMINATION OF HALOGENATED ACETONES

This study was made to find the effects of halogen substituents on the colorimetric determination of acetone and, so far as possible, to correlate such effects with existing data on the effects of substituents. No new methods were developed. Qualitative tests showed that the presence of a halogen negated most common tests for acetone. Only one, the 2,4-dinitrophenylhydrazine method, proved applicable to all of the available halogenated acetones. This reagent was tested according to the established procedure of Greenberg and Lester (4).

Test Procedure. A 2- to 5-ml. portion of 0.1% 2,4-dinitrophenylhydrazine in 2*N* hydrochloric acid was mixed with the aqueous sample of an acetone (0 to 5 ml.). To this, 10 ml. of carbon tetrachloride was added. The solution was shaken for 15 minutes (for acetone). Then the carbon tetrachloride was sepa-

rated and washed with two 25-ml. portions of distilled water and with 5 ml. of 0.5*N* sodium hydroxide solution. It was run through absorbent cotton to remove water drops and spectrophotometrically examined. With acetone, the solution is yellow and the determination may be made by measuring the absorbance at 420 mμ.

Modified Procedure. As the above procedure was not entirely satisfactory for the halogenated acetones, some variations were made in order to apply it to them.

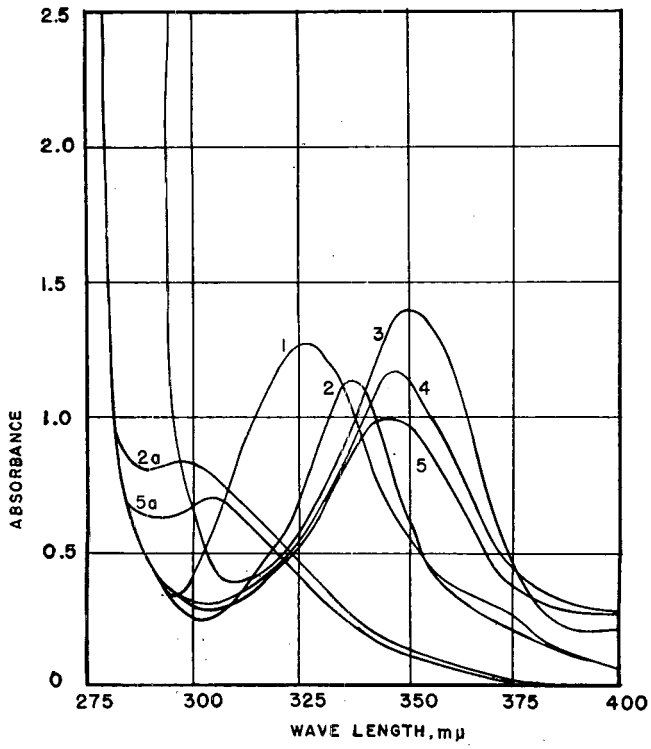

Figure 2. 2,4-Dinitrophenylhydrazones

1. 1,1,1-Trifluoroacetone
2. 1,3-Dichloroacetone before sodium hydroxide wash
2a. 1,3-Dichloroacetone after sodium hydroxide wash
3. Acetone
4. 1-Bromoacetone
5. 1-Chloroacetone before sodium hydroxide wash
5a. 1-Chloroacetone after sodium hydroxide wash

In all cases a reaction period of more than 15 minutes was used. One hour was allowed for 1-bromo-, 1-chloro-, and 1,3-dichloroacetone. This was sufficient, although intermittent shaking was used rather than continuous mechanical shaking. With 1,1,1-trifluoroacetone, a reaction time of about 2 days produced apparently complete reaction. A concentration series prepared at one time and given the same reaction time followed Beer's law. Another series, done separately, likewise followed Beer's law, but did not produce the same calibration curve as the first.

The 0.1% solution of 2,4-dinitrophenylhydrazine proved satisfactory except for trifluoroacetone. The very long reaction time in that case made it necessary to use a more concentrated reagent. A 1% reagent solution in dilute sulfuric acid proved successful when 1 hour was allowed for the reaction.

For 1-bromo-, 1-chloro-, and 1,3-dichloroacetone the basic wash produced a change in the ultraviolet spectrum of the 2,4-dinitrophenylhydrazone. This shift is probably attributable to the formation of 1-hydroxyacetone-2,4-dinitrophenylhydrazone from 1-bromo- and 1-chloroacetone, as the shifted peak falls at the same wave length. Then, by analogy, the shift with 1,3-dichloroacetone is probably attributable to hydrolysis to the 1,3-dihydroxyacetone derivative. With trifluoroacetone the sodium hydroxide wash caused no change in peak wave length. Spectrophotometric measurement was made of the solutions both before and after the basic wash.

Spectral Characteristics of Hydrazones. The absorption curve of acetone-2,4-dinitrophenylhydrazone contains a shoulder near 410 to 420 mμ and a peak near 350 mμ. The method of Greenberg and Lester uses the absorption of the shoulder in the visible region, by measuring with a filter photometer. The 2,4-dinitrophenylhydrazones of the halogenated acetones have peaks in the ultraviolet, but do not show the shoulder in the visible region. Measurement of the complete ultraviolet absorption curves therefore was made (Figure 2). Values for calibration curves were taken at the peaks. The peak is a rather more sensitive indicator than the shoulder for acetone as well as for the halogenated acetones.

Conformity to Beer's Law. Using the stock solutions described, concentration series were prepared for acetone and the halogenated acetones. In each case, there was approximate conformity to Beer's law. The allowable concentrations for bromo-, chloro-, and 1,3-dichloroacetone were about the same, 0.3 mg., while limits for acetone and trifluoroacetone are about 0.1 mg. Conformity to Beer's law was also found with the peaks ascribed to hydroxy- and dihydroxy-acetone-2,4-dinitrophenylhydrazones. Therefore, these could be used to determine the corresponding halogenated acetone but with a slightly different calibration curve than before the sodium hydroxide wash. The peaks and molar absorptivities are shown in Table IV.

Recommended General Procedure. SAMPLE. The sample for determination by this procedure should be representative. It should be aqueous and contain no reactive carbonyl compounds other than the desired constituent. The limiting amount depends on the constituent to be determined, 0.3 mg. if it is 1-bromo-, 1-chloro-, or 1,3-dichloro-acetone, and 0.1 mg. for 1,1,1-trifluoroacetone.

Desired Constituent. To this sample add 2 ml. of 2,4-dinitrophenylhydrazine solution, 0.1% in 2*N* hydrochloric acid for 1-bromo-, 1-chloro-, or 1,3-dichloroacetone, or 1% in 7*N* sulfuric acid for 1,1,1-trifluoroacetone. Add a 10-ml. portion of carbon tetrachloride (free from active carbonyl impurities) to the sample and shake the mixture intermittently for 1 hour. Separate the carbon tetrachloride layer and wash twice with 20-ml. portions of water. For 1,1,1-trifluoroacetone add a 5-ml. portion of 0.5*N* sodium hydroxide and shake the mixture for 3 minutes. Pour the carbon tetrachloride through dry absorbent cotton. Measure the absorbance at a suitable point in the ultraviolet region (Table IV) and read the quantity of constituent from a calibration curve.

Table IV. Absorptiometric Data for Haloacetones

Compound	Curve Peaks for 2,4-Dinitrophenylhydrazone Derivative, mμ	Molar Absorptivity of Derivative, ε
Acetone	345–350	13,000
1-Bromoacetone	344	11,700
1-Chloroacetone	340–344	8,800
1,3-Dichloroacetone	337	10,700
1,1,1-Trifluoroacetone	327	7,000
Hydroxyacetone	307	7,500
1,3-Dihydroxyacetone	297	10,700

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LITERATURE CITED

(1) Adam, J. A., Booth, E., Strickland, J. D. H., *Anal. Chim. Acta* **6**, 462–71 (1952).
(2) Blatt, A. H., "Organic Syntheses," Coll. Vol. II, p. 88, Wiley, New York, 1943.
(3) Cefola, M., others, *Mikrochemie ver. Mikrochim. Acta* **3**, 439–42 (1950).
(4) Greenberg, D., Lester, L. A., *J. Biol. Chem.* **154**, 177–9 (1944).
(5) Pulsifer, M. B., *J. Am. Chem. Soc.* **26**, 967 (1904).
(6) Welcher, F. J., "Organic Analytical Reagents," Van Nostrand, New York, 1948.
(7) Will, F., III, *Dissertation Abstr.* **14**, 761 (1954).
(8) Yoe, J. H., Sarver, L. A., "Organic Analytical Reagents," Wiley, New York, 1941.
(9) Yoe, J. H., Will, F., III, Black, R. A., ANAL. CHEM. **25**, 1200 (1953).

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Determination of Hydrofluoric Acid in Nitric-Hydrofluoric Acid Mixtures

Development of a Field Test

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The selection and adaptation of a colorimetric method to the rapid, accurate, and precise determination of hydrofluoric acid in nitric-hydrofluoric acid mixtures are described, introducing an application of an ion exchange technique to the separation of interfering cations from this mixed acid system. The two-stage method was developed for application as a field test and can be performed by technicians. Analyses of aliquots containing 8.00 mg. of fluoride ion were made within ±0.10 mg.

AN INITIAL review of the chemical literature revealed a large number of methods for determining the fluoride ion concentration in natural products, commercial chemicals, and water. However, no direct method for determining hydrofluoric acid in nitric-hydrofluoric acid mixtures was found which could be applied in the presence of cation impurities—e.g., iron, aluminum, chromium, and nickel. Certain ideal characteristics of the method being sought included the requirements that it should be as rapid, precise, and accurate as possible, but capable of being performed by technicians under field conditions—i.e., without the benefit of many of the facilities available in a chemical laboratory.

Although instrumental methods were precluded from consideration by these initial requirements, a study of them revealed many of the difficulties which can be encountered in fluoride determinations. Such information was obtained from the amperometric method of Castor and Saylor (*4*), the fluorometric method of Willard and Horton (*6*), and the spectrophotometric method of Bumstead and Wells (*3*).

The most promising method for determining fluoride in uncontaminated nitric-hydrofluoric acid mixtures appeared to be some colorimetric method which would take advantage of the unique bleaching action of the fluoride ion on certain metal–organic dye complexes, or lakes. The ASTM thorium nitrate–sodium alizarin sulfonate method (*1*) was chosen for development. This method includes four essential steps: (1) adjustment of the pH of an aliquot of sample and of a distilled water reagent blank, using sodium alizarin sulfonate reagent as the indicator; (2) titration of the aliquot with thorium nitrate to the characteristic red-purple color of the lake; (3) addition of exactly the same amount of thorium nitrate to the blank as was required for the aliquot titer; and (4) titration of the blank with standard sodium fluoride until the color of the blank exactly matches the color of the aliquot.

A back-titration with standard sodium fluoride, rather than a direct titration with thorium nitrate, has three advantages: Analytical grade thorium nitrate may be used in place of specially purified or standardized reagent; greater precision and accuracy are possible by back-titrating to a color match, thus avoiding the relatively poor end point obtained by direct titration with standardized thorium nitrate; this procedure does not require a separate blank.

All of the colorimetric methods reviewed in the literature were subject to interference from a large number of anions or cations, or both; therefore, separation of the fluoride ion from these contaminants was first required. Classically, this separation is accomplished by a steam distillation procedure first introduced by Willard and Winter (*7*), and subsequently modified by Boruff and Abbott (*2*) and Shell and Craig (*5*). Because these steam distillation procedures are long and require a fair degree of skill to obtain consistent results, they did not seem adaptable to the requirements of this problem.

Ion exchange techniques appeared to merit investigation as a more rapid means of removing the cation contaminants. A sample of Amberlite IR-120(H) cation exchange resin was evaluated and proved satisfactory for this purpose.

REAGENTS

Amberlite IR-120(H), a nuclear sulfonic acid-type cation exchange resin, Rohm & Haas Co., Philadelphia, Pa. The (H) designates an analytical grade of this resin marketed by the Fisher Scientific Co. with a moisture content of approximately 40%.

Reagent grade chemicals were used, except where otherwise noted, and the following solutions were prepared.

Sodium hydroxide, 1*N* and 0.1*N*.

Hydrochloric acid, concentrated, used as received.

Hydrochloric acid, diluted 1 to 250.

Fuming nitric acid (General Chemical Division, Allied Chemical and Dye Corp., New York). This acid contains negligible quantities of metallic ion contaminants which did not interfere in the determinations.

Alizarin Red S (National Aniline indicator No. 203), 0.05% aqueous solution.

Thorium nitrate, 0.1*M*. Dissolve 55.2 grams of thorium nitrate tetrahydrate in water, filter, and dilute to 1 liter.

Fluoride standard. Dissolve 4.4247 grams of sodium fluoride in water; dilute to 1 liter. The solution contains 2.00 mg. of fluoride ion per milliliter.

Fluoride stock solution. Dissolve 17.6988 grams of sodium fluoride in water; dilute to 1 liter. The solution contains 8.00 mg. of fluoride ion per milliliter.

Stock solutions were also prepared to contain 1 mg. of ferric ion per milliliter; 0.1 mg. of aluminum ion per milliliter; 0.1 mg. of nickelous ion per milliliter; and 0.1 mg. of chromium ion per milliliter. These were aqueous solutions prepared from the nitrates of the various cations, with the exception of the ferric solution, which was prepared from iron wire dissolved in nitric acid, then diluted with water.

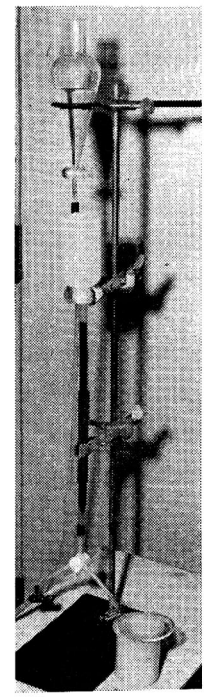

Figure 1. Ion exchange column for removal of cation contaminants from nitric-hydrofluoric acid mixtures

APPARATUS

A plastic column similar to the one shown in Figure 1 is charged with 24 grams of Amberlite IR-120(H) resin, as received. The column consists of a reservoir made from a 250-ml. polyethylene bottle; a 15-inch length of Kel-F tubing (chlorotrifluoroethylene, M. W. Kellogg Co., New York), 5/16 inch in inside diameter; and a Teflon Y-tube (Du Pont) which permits downward elution and reverse washing of the column. Flexible Tygon tubing

Table I. Comparison of Analyses before and after Cation Contaminant Removal with Amberlite IR-120(H) Resin

(All samples except the first contain 5 ml. of fuming nitric acid and 40.00 mg. of fluoride ion)

		Fluoride Ion Recovered, Mg. per 50-Ml. Aliquot					
		Direct Method			After Contaminant Removal		
Cation	Mg. per 250 Ml.			Av. relative error, %			Av. relative error, %
None					No fluoride found		
None		7.98 8.04, 7.96	7.99, Av.	− 0.08	7.98, 8.08, 7.98	8.01, Av.	+0.17
None		8.00 7.98,8.04	8.01	+ 0.08	8.00, 8.06, 7.96	8.01	+0.08
Ferric	1.0	10.14 10.12, 10.08	10.11	+26.42	8.06, 8.02, 8.04	8.04	+0.50
Ferric	5.0	[a]		...	8.04, 7.98, 7.98	8.00	0.00
Ferric	10.0	[a]		...	8.06, 8.04, 8.10	8.07	+0.83
Aluminum	5.0	5.32, 5.16, 5.24	5.24	−34.50	8.04, 7.98, 7.92	7.98	−0.25
Aluminum Ferric	1.0 10.0	[a]		...	8.10, 8.02, 7.96	8.03	+0.33
Aluminum Ferric	5.0 5.0	[a]		...	8.10, 7.98, 8.06	8.05	+0.58
Chromium Nickelous	3.0 3.0	8.12 8.14, 8.22	8.16	+ 2.00	8.00, 8.06, 8.08	8.05	+0.58
Ferric Aluminum Chromium Nickelous	5.0 5.0 3.0 1.0	[a]		...	7.92, 8.00, 8.02	7.98	+0.25
Aluminum Chromium Nickelous	5.0 3.0 1.0	6.60 6.64, 6.62	6.62	−17.33	7.94,8.08, 7.94	7.99	−0.17
Aluminum Chromium Nickelous	1.0 1.0 1.0	7.02 7.08, 7.04	7.05	−11.92	7.90, 8.00, 8.02	7.97	−0.33

[a] Analysis was impossible because alkaline solution was too yellow.

(U. S. Stoneware Co., Akron, Ohio) connects the 500-ml. separatory funnel, which is used as a distilled water reservoir, to the Teflon Y.

Iron contamination in the resin was removed before use by passing successive 50-ml. portions of 3*N* hydrochloric acid through the column until a negative test for iron in the effluent was obtained with sodium thiocyanate. The column was then backwashed with distilled water to remove any trapped air. Finally, the column was rinsed with successive portions of distilled water until the effluent was neutral. After each contaminant removal experiment, the resin was regenerated by this same procedure.

EXPERIMENTAL

Modified ASTM Procedure. The ASTM colorimetric method for fluoride (*1*) was modified as follows to permit quantitative determination of hydrofluoric acid in cation-free nitric-hydrofluoric acid mixtures.

The specific gravity of the mixed acids is determined first by some convenient method (such as weighing a 10-ml. sample in a Kel-F or polyethylene bottle). Then 5.5 grams of sodium hydroxide is dissolved in 20 ml. of water in a 250-ml. volumetric flask, which is chilled in an ice bath. A 5-ml. sample of contaminant-free mixed acid is pipetted directly in the alkaline solution with shaking. The solution is diluted to the mark and mixed thoroughly, making certain that it is still slightly alkaline. A 50-ml. aliquot of this sample solution is transferred to a 100-ml. low-form Nessler tube and capped. To a second Nessler tube, to be used as a blank, is added 50 ml. of distilled water. Then 5 to 7 drops of 0.05% Alizarin Red S is added to each tube, exactly the same amount being added to both the aliquot and the blank.

The pH is adjusted and the aliquot is titrated with 0.1*M* thorium nitrate and the blank with standard sodium fluoride according to steps 4 through 8 in the ASTM method (*1*).

To calculate per cent hydrofluoric acid in the sample:

$$\%\mathrm{HF} = \frac{20}{19} \times \frac{5 \times \text{ml. std. NaF} \times \text{fluoride ion concn. of std. NaF soln.}}{5 \text{ ml.} \times \text{specific gravity of mixed acid} \times 1000} \times 100$$

Ion Exchange Technique. The ion exchange technique was used to remove cation contaminants from the mixed acid system, at the same time effecting a quantitative recovery of fluoride ion in the effluent.

About 75 ml. of distilled water is placed in the polyethylene reservoir at the top of the ion exchange column, then mixed with 5 ml. (from a pipet) of a contaminated nitric-hydrofluoric acid mixture. The diluted acid sample is passed through the column at an elution rate of about 2 drops per second, giving a total elution time of about 15 to 20 minutes. The resin bed must not be allowed to run dry. The effluent is collected in a 250-ml. polyethylene beaker below the surface of a solution containing 5.5 grams of sodium hydroxide in 20 ml. of distilled water. When almost all the sample has passed through, the column is washed with three successive 40-ml. portions of distilled water at the maximum delivery rate of the column. The final washings should be neutral when tested with pH paper, but the effluent solution must be alkaline.

The effluent is transferred to a 250-ml. volumetric flask, diluted to the mark, and mixed thoroughly. Fifty-milliliter aliquots of this solution are analyzed by the modified ASTM procedure.

Evaluation of Resin. To evaluate the Amberlite IR-120(H) resin for removing known amounts of cation contaminants from the mixed acid system, 40 mg. of fluoride ion, 5 ml. of fuming, cation-free nitric acid, and known volumes of the stock iron, aluminum, nickel, and chromium solutions were added to about 75 ml. of distilled water in the polyethylene reservoir. Each cation-contaminated mixed acid sample was then passed through the column and the effluent was collected in an alkaline solution as just described. The column was then washed with three successive 40-ml. portions of distilled water, or until about 225 ml. of effluent was collected. The effluent was diluted to 250 ml. in a volumetric flask. The fluoride ion concentration in 50-ml. aliquots of this solution was determined by the modified ASTM colorimetric procedure.

Concurrently, the effect of known amounts of cation contaminants on the direct colorimetric determination of the fluoride ion concentration in mixed acid samples was determined for comparison. These sample solutions were mixed directly in 250-ml. volumetric flasks, and 50-ml. aliquots were analyzed by the modified ASTM colorimetric procedure.

A comparison of these experimental results is made in Table I.

DISCUSSION

The entire procedure, including contaminant removal by ion exchange, determination of the fluoride ion concentration on duplicate aliquots of the effluent, and calculation of the weight per cent hydrofluoric acid, requires less than 1 hour per mixed acid sample. These procedures have been successfully employed by technicians under field conditions.

Table I shows that up to 10 mg. of ferric ion, 5 mg. of aluminum ion, and 6 mg. of chromium and nickelous ions have been removed by the ion exchange method. A maximum total cation contamination of 14.0 mg. was removed by this method. These ion concentrations represent the upper limit which the exchange capacity of this column will handle. By suitably increasing the

amount of resin and adjusting the flow rate, the exchange capacity might be extended.

The reproducibility of the results for any given sample is shown by the standard deviation,

$$\sigma = \sqrt{\frac{d_1^2 + d_2^2 + d_3^2}{2}}$$

where $d = O - M$, O is the experimental milligrams of fluoride, and M is the arithmetical mean. From the table it is apparent that trace amounts of cation impurities cause a marked inaccuracy in the direct determination of the fluoride ion concentration, although a precision of the same order of magnitude is obtained.

Assuming a true value, T, of 8.00 mg of fluoride ion per 50-ml. aliquot, the relative error in any one determination is $\frac{O - T}{T} \times 100$. Applying this expression for precision to each experiment, an average precision was determined as follows:

$$\text{Average relative error} = \frac{\left(\frac{O_1 - T}{T} + \frac{O_2 - T}{T} + \frac{O_3 - T}{T}\right) \times 100}{3}$$

Hence, the average amount of fluoride found in each experiment was in error by no more than 8 parts in a thousand. The largest deviation from the true value of any one determination was 0.1 mg. of fluoride ion per 8.00 mg. of fluoride sample, or 1 part in 100, or 1.25%.

ACKNOWLEDGMENT

The author is indebted to S. A. Long for suggesting Amberlite IR-120(H) as the specific ion exchange resin being sought for this cation removal application.

LITERATURE CITED

(1) *Am. Soc. Testing Materials, Standards, P. VII*, D 1179-51T (1952).
(2) Boruff, C. S., Abbott, G. B., IND. ENG. CHEM., ANAL. ED. **5**, 236 (1933).
(3) Bumstead, H. E., Wells, J. C., ANAL. CHEM. **24**, 1595 (1952).
(4) Castor, C. R., Saylor, J. H., *Ibid.*, **24**, 1369 (1952).
(5) Shell, H. R., Craig, R. L., *Ibid.*, **26**, 996 (1954).
(6) Willard, H. H., Horton, C. A., *Ibid.*, **22**, 1194 (1950).
(7) Willard, H. H., Winter, O. B., IND. ENG. CHEM., ANAL. ED. **5**, 7 (1933).

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Ion Exchange Separation of Morphine Prior to Its Determination in *Papaver somniferum*

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No rapid, accurate method could be found for the determination of morphine in the poppy plant or extracts from it; a new procedure was developed for isolation and purification of morphine by ion exchange methods. The morphine was finally measured by the color it produces with nitrous acid, by its ultraviolet absorption, or by titration. The method when applied to pure morphine gave an average recovery of 98% with a standard deviation of 1.9 on 18 analyses of samples that contained from 5 to 20 mg. of morphine. The analytical values obtained on opium and various extracts from the poppy plant were lower than those obtained by a colorimetric and a solvent extraction method, but 10 to 40% higher than those obtained by methods in which morphine was isolated by crystallization. Colorimetric methods applied to extracts of the poppy plant without prior separation of the morphine from interferences will give high results.

IN FOLLOWING the processing of morphine from poppy plants, a rapid and accurate method of analysis was desired. The U. S. Pharmacopeia (USP) method (*14*) for morphine in opium involves isolation of crystalline morphine and requires about 600 mg. of the compound. This method, as well as others of similar nature, was not applicable to the problem, because some samples of plant fractions and partially processed material contained no more than 5 mg. of morphine in portions of convenient size for analysis.

Methods sufficiently sensitive (*2, 4, 11*) include the solvent extraction method of Levine and Matchett, which has not been published in detail (*11*), and a colorimetric method (*2*) based on the color of the nitroso compound formed by the reaction of nitrous acid with morphine. When these methods were used on samples which permitted comparison, they gave results much higher than those obtained by the USP method. In the extraction method, alkaloids are extracted from aqueous solutions at pH 8 to 9 by chloroform-ethanol and recovered by evaporation of the solvent. The residue is dissolved in sodium hydroxide solution at pH 10 to 11, and nonmorphine material is removed by extraction with benzene, first from the alkaline solution and later after acidification. Morphine is extracted by chloroform–2-propanol after adjusting the solution to pH 8 to 9. The morphine is recovered by evaporation of the solvent, dissolved in methanol, and then titrated with acid. The colorimetric method gave higher results in the present investigation than the Levine and Matchett method when applied to purified plant extracts and even when applied to the material isolated by the Levine and Matchett procedure.

The ionic character of the opium alkaloids suggested the use of ion exchange resins for the separation of morphine from interfering materials prior to analysis. Exchange resins have been used analytically for the separation of codeine from morphine (*1, 5*) and for the preparation of free morphine from its salts (*6, 10, 19*). Ion exchange has also been used as part of procedures for separating bases, including morphine, from other substances in body fluids (*13, 17*).

In the method presented here, morphine was separated from interferences by the procedure outlined in Figure 1. It was finally estimated by the nitroso colorimetric method, by its ultraviolet absorption, or by titration.

REAGENTS AND EQUIPMENT

Ion Exchange Resins. The cation exchange resin Dowex 50 X 1, 50 to 100 mesh, and the anion exchange resin Dowex 1 X 1, 50 to 100 mesh, were prepared as previously described (*18*). A supply of the cation resin was stored in the hydrogen form and the anion resin in the chloride form.

Reagents for Ion Exchange Separation. Boric acid buffer solutions of pH 8.6 and 9.4 (*9*). These solutions were diluted with distilled water to make them 0.02N in concentration with

respect to the boric acid present. Ammonium hydroxide, 0.5*N*. Sodium hydroxide, 1*N*. Acetic acid, 0.3*N*. Hydrochloric acid, 3*N*.

Reagents for Colorimetric Analysis of Morphine (*2*). Sulfuric acid, 1 volume of concentrated acid to 4 volumes of water. Sodium hydroxide, 40 grams per 100 ml. of water. Sodium nitrite, 1% aqueous solution.

Equipment. Colorimeter, micro ion exchange columns (*19*), steam bath, and usual volumetric ware.

PROCEDURE

Preparation of Samples. In an analysis of opium, a water extract of the sample was prepared as described in the USP method (*14*). For analysis of different parts of the poppy plant, solutions were obtained by 3-hour aqueous extractions of 5 grams of finely ground material in an ASTM (American Society for Testing Materials) rubber extractor (*7*) which had the siphon removed in order to obtain percolation of the water through the sample. These water extracts were filtered free of sediment and diluted to a convenient volume, from which samples were taken for analysis.

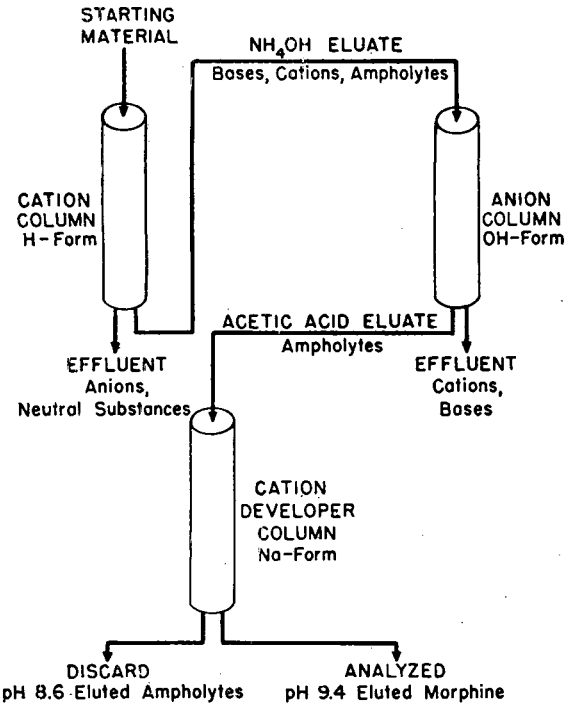

Figure 1. Diagram of ion exchange method of separation of morphine from impurities

In analyzing various concentrates and other samples obtained in processing morphine from the poppy plant, suitable volumes of the solution were taken for analysis without pretreatment. The senior author found morphine can be quantitatively removed by Dowex 50 X 1 from solutions containing large amounts of inorganic salts. However, when solutions of low morphine and high salt content were used, morphine leaked through the column. In samples of this kind, morphine was extracted from the aqueous solution at pH 9 in a continuous extraction apparatus with a chloroform-ethanol (4 to 1 by volume) solution. Organic solvents were evaporated, and the salt-free organic bases were dissolved in water or dilute acid. Samples were taken from these solutions for analysis.

Separation of Morphine from Impurities (Figure 1). A water suspension of Dowex 50 X 1 was poured into a micro ion exchange column to a drained height of about 5 cm. Analytical samples which contained 5 to 20 mg. of morphine in a 5- to 50-ml. volume were passed through the column at a flow rate of 2 to 4 ml. per minute, after which the column was washed with 5 to 10 ml. of water. Samples were eluted with 50 ml. of 0.5*N* ammonium hydroxide.

The anion exchange column of Dowex 1 X 1 Cl was prepared in the same manner as the cation exchange column, but was filled to a drained height of 6 cm. This resin was converted to the hydroxide form in the column by washing with about 15 ml. of

Table I. Recovery of *Pure Morphine*[a] Carried through Ion Exchange Procedure and Measured by *Colorimetric* Nitroso Method

Sample, Mg.	Number of Determinations	Average Recovery, %
5	4	97.2
10	5	97.6
15	4	98.2
20	5	98.6

[a] USP morphine sulfate pentahydrate which gave theoretical carbon and hydrogen analyses and lost moisture equivalent to five molecules of water on drying to constant weight.

Table II. Morphine Content of Various Parts of Poppy Plant Determined by Ion Exchange Separation and Nitroso Color Method

Part of Plant	Number of Determinations	Average, %[a]	Standard Deviation
Capsules	6	0.61	0.01
Septa	2	0.38	..
Nodes	6	0.36	0.01
Stems	3	0.12	..

[a] Corrected for approximate moisture content.

1*N* sodium hydroxide. This was followed by a distilled water wash until the effluent had a pH of about 7. Contents of the column were stirred once or twice with a small stirring rod during the washing. After passage of the cation eluate through this column, three 5-ml. washes followed. Then ampholytes were eluted with 50 ml. of 0.3*N* acetic acid. The eluate, collected in a 100-ml. evaporating dish containing 3 to 5 ml. of 3*N* hydrochloric acid, was evaporated to dryness on a steam bath with the aid of an air current.

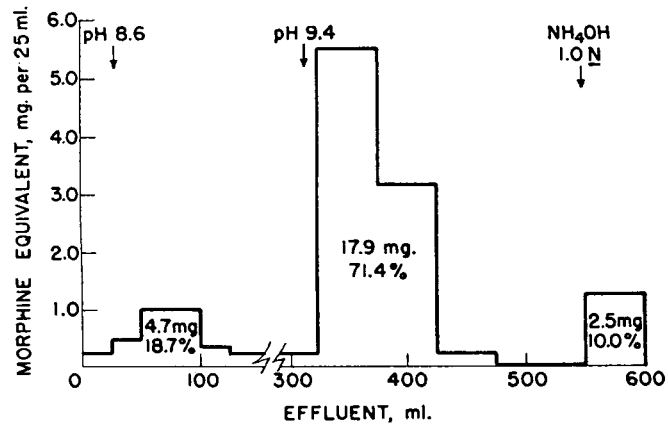

Figure 2. Elution from Dowex 50 X 1 Na by 0.02*N* buffers in final purification step of procedure

Sample, water extract of poppy capsules

After the residue had been dissolved in 5 to 10 ml. of water, it was put on a 5-cm. developer column of Dowex 50 X 1 Na. The column was elutriated with pH 8.6 buffer until the effluent had reached a pH of about 8.6. Then 30 ml. more buffer was passed through. Total volume required was 50 to 100 ml. The morphine-containing fraction then was eluted with 225 ml. of the pH 9.4 buffer. This eluate, after acidification with 3*N* hydrochloric acid, was concentrated on the steam bath, then diluted to 50 or 100 ml. From this volume, samples containing 0.1 to 2 mg. of morphine were taken for analysis.

Nitroso Colorimetric Method of Analysis (*2*). Two samples, each containing 2 mg. of morphine or less, were pipetted into 25-ml. volumetric flasks, and the total volume of each was brought to approximately 5 ml. To each of these solutions, 3 ml. of the sulfuric acid solution was added. Two milliliters of the nitrite solution was added to one flask, the second flask serving as a blank. After the flasks stood for 10 minutes at room temperature, 4 ml. of sodium hydroxide solution was added to each flask with mixing, following which the flasks were cooled in an ice

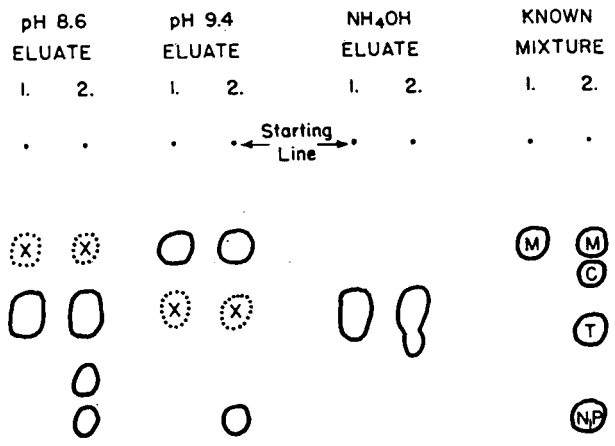

Figure 3. Paired chromatograms of alkaloids separated in Figure 2 with known alkaloids for comparison

1. Color developed with nitroso reagent
2. Color developed with Munier's reagent

M. Morphine
C. Codeine
T. Thebaine
N. Narcotine
P. Papaverine
X. Trace amounts

bath for 5 minutes. The cooled solutions were diluted to volume and the color was read in a colorimeter using a Klett No. 42 filter. The instrument was adjusted to zero against each blank before the reading was made on the sample.

RESULTS

Precision and accuracy of the method when applied to pure morphine are shown in Table I. Average value for recovery of morphine was 98%, with a standard deviation of 1.9. Recovery of known amounts of morphine added to opium was equally good.

To determine whether excessive amounts of narcotine, papaverine, codeine, and thebaine would interfere in the method, additional experiments were run. In these mixtures of two alkaloids were analyzed: 10 mg. of morphine and 10 mg. of one of the associated alkaloids. In these mixtures narcotine, papaverine, and thebaine reduced the recovery of morphine to as low as 91%. On elution with ammonium hydroxide, the first cation exchange column turned light gray in the presence of these alkaloids. Further elution of this column with methanolic ammonium hydroxide yielded large amounts of the associated alkaloids. These observations indicated precipitation of the associated alkaloids by the aqueous ammonium hydroxide and occlusion of small amounts of morphine. Codeine did not interfere and was recovered quantitatively in the anion column effluent.

Analyses of parts of the poppy plant, *Papaver somniferum*, are given in Table II. Values are in the range reported by other investigators for the morphine content of different parts of the plant (*15, 16*).

Table III compares values obtained by this method with values obtained by the Levine and Matchett method (*11*) and modified USP methods on different morphine-containing natural products.

Application of paper strip chromatography (as described later) to the end product of the Levine and Matchett method showed that nitroso-color–producing substances other than morphine were present. These other substances probably account for at least part of the positive error in the Levine and Matchett method when applied to a poppy plant extract.

Examination of Developer Cation Column Eluate. Nitroso-color–producing materials, selectively eluted by different buffers from the developer cation column used in the analytical procedure, are shown in Figure 2. These results show that the mixture of ampholytes obtained from the poppy plant contained bases both stronger and weaker than morphine which develop a color with nitrite.

The material eluted with the pH 8.6 buffer gave an ultraviolet absorption peak in acid from 273 to 277 mμ. The absorption curve did not resemble that of morphine, and it varied considerably from sample to sample.

The material eluted with the pH 9.4 buffer gave an ultraviolet absorption typical of morphine. It had a peak of 285 mμ in acid and 297 mμ in alkali. Of the solutions eluted at pH 9.4, morphine content for a large number of samples was measured by ultraviolet absorption. A small background correction was required. Some of the absorption curves indicated a trace of narcotine or papaverine. Evidence for the presence of these alkaloids in the pH 9.4 eluted fraction also was obtained by paper chromatography (Figure 3).[5] The values calculated from the ultraviolet absorption of this fraction agreed closely with those obtained by the nitroso colorimetric method of analysis and by titration on the same samples (see Table IV).

Following removal of the morphine, the column was elutriated with ammonium hydroxide, giving an additional amount of nitroso-color–producing material. The ultraviolet absorption curve of this eluate was the same shape as that of morphine, but it differed in that the maximum was at 280 mμ in acid and 294 mμ in alkali. Paper strip chromatography (Figure 3) showed, however, that no morphine was present in this eluate.

The composition of the eluted fractions was examined further by paper chromatography according to the procedure of Munier (*12*) as described by Block (*3*). The alkaloids from each fraction were extracted from inorganic salts at pH 8.9 by means of chloroform and ethanol. After removal of the solvent the residue was dissolved in aqueous ethanol. Duplicate chromatograms from each sample and a known mixture of alkaloids were run, after which the chromatographs were separated. One strip was developed with the nitroso reagent used in the colorimetric determination. This reagent gives no color with thebaine, codeine, narcotine, and papaverine, and it gives a yellow color with most compounds containing a phenolic hydroxyl. The companion strip was developed with Munier's reagent which gives a positive test for both phenolic and nonphenolic opium alkaloids. Typical chromatograms of the alkaloids in various eluates detected by the two reagents are shown in Figure 3.

As shown by the recovery of pure morphine (Table I), a small loss of morphine occurs in its separation from aqueous solutions on ion exchange resins. This loss may result from some leakage of morphine with the pH 8.6 buffer. Evidence for such a loss

Table III. Comparison of Methods of Analyses

Sample	Methods, % Levine and Matchett	Ion exchange	Modified USP method
Feed solids (poppy capsules)	0.68	0.59	0.45
Concentrate from ion exchange process	2.16	1.82	1.62
Concentrate from distillation process	3.72	3.25	2.28
USP opium[a]	12.7	11.25	10.3

[a] Specifications for USP XIV opium permit a range of 10.0 to 10.5% morphine. Sample contained 4.7% moisture.

Table IV. Agreement of Analyses by Different Methods of Measuring Product of Ion Exchange Separation

Sample	Morphine, % Titration[a]	Nitroso color	Ultraviolet absorption[b]
Water extract of straw	0.063	0.059	0.062
Straw sample	0.51	0.48	0.51
Aqueous concentrate	0.38	0.38	0.39

[a] Free base was extracted from the final eluate by chloroform-ethanol at pH 8 to 9. After evaporation of solvent, the base was dissolved in methanol and titrated with 0.01*N* acid.
[b] Small background correction required for these estimations.

is indicated in Figure 2, in that the pH 8.6 buffer continuously elutes a small amount of nitroso-color-producing material after the bulk of the nitroso-color-producing material was removed with this buffer. Additional evidence for the elution of morphine with the pH 8.6 buffer is shown in Figure 3. The chromatograms in Figure 3 also show the elution of a small amount of nitroso-color-producing impurity with the morphine at pH 9.4. These small positive and negative errors of from 2 to 5% are not of great enough magnitude to account for the difference between the values by the ion exchange separation and those obtained by the modified USP method or the Levine and Matchett method given in Table III. Therefore, if the ion exchange separation method gives incorrect high results because of nonmorphine color-producing substances, these unknowns cannot be differentiated from morphine by ion exchange resins, paper chromatography, or ultraviolet spectrophotometry as applied here. The possibility of such substances being present, however, must not be ignored.

Evidence from these ion exchange separations shows the presence of color-producing substances other than morphine in the poppy plant. If these other substances are not removed, they interfere in colorimetric methods and cause high results. This may account in part for the high values obtained by previously reported colorimetric methods (*8, 15, 16*).

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LITERATURE CITED

(1) Achor, L. B., Geiling, E. M. K., ANAL. CHEM. **26**, 1061 (1954).
(2) Adamson, D. C. M., Handisyde, F. P., *Analyst* **70**, 305 (1945).
(3) Block, R. J., "Paper Chromatography," p. 137, Academic Press, New York, 1952.
(4) Cramer, J. W. W., Voerman, J. G., *Acta Pharm. Intern.* **1**, 219 (1950).
(5) Grant, E. W., Hilty, W. W., *J. Am. Pharm. Assoc.* **42**, 146 (1953).
(6) Jindra, A., *J. Pharm. Pharmacol.* **1**, 87 (1949).
(7) Joint Rubber Insulation Committee, *J. Ind. Eng. Chem.* **9**, 314 (1917).
(8) Klee, F. C., Kirch, E. R., *J. Am. Pharm. Assoc. Sci. Ed.* **42**, 146 (1953).
(9) Lange, N. A., "Handbook of Chemistry," 7th ed., p. 1127, Handbook Publishers, Sandusky, Ohio, 1949.
(10) Levi, L., Farmilo, C. G., *Can. J. Chem.* **30**, 793 (1952).
(11) Matchett, J. R., Levine, J., IND. ENG. CHEM., ANAL. ED. **13**, 264 (1941).
(12) Munier, R., *Bull. soc. chim. biol.* **33**, 857, 862 (1951).
(13) Oberst, F. W., *J. Lab. Clin. Med.* **24**, 318 (1938).
(14) "Pharmacopeia of the United States XIV," 14th rev., p. 400, Mack, Easton, Pa., 1953.
(15) Poethke, W. von, Arnold, E., *Pharm. Zentralhalle* **88**, 1 (1949).
(16) Reith, J. F., Indexmans, A. W. M., *Pharm. Weekblad* **85**, 309 (1950).
(17) Stolman, A., Stewart, C. P., *Analyst* **74**, 536, 543 (1949).
(18) Van Etten, C. H., ANAL. CHEM. **27**, 954 (1955).
(19) Van Etten, C. H., Wiele, M., *Ibid.*, **25**, 1109 (1953).

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Use of Ionic Dyes in the Analysis of Ionic Surfactants and Other Ionic Organic Compounds

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On the basis of previous investigations on the interaction between ionic dyes and ionic surfactants of opposite charge, a partition technique for the analysis of all classes of ionic surfactants and similar organic compounds has been developed. The theory of this new technique is presented. Experimental results with cationic and anionic surfactants of various kinds support the theoretical expectations; sensitivity of the new method is high. The qualitative detection limit in favorable cases is of the order of 1 to 2 p.p.b., while quantitative estimations of concentrations of the order of 0.1 to 1 p.p.m. and amounts of the order of 0.001 to 0.01 mg. are found to be possible. Hydrolyzable and nonhydrolyzable surfactants in mixtures can be determined.

IN THE analytical chemistry of detergents and surfactants in general the colorimetric methods involving the use of ionic dyes of various kinds have been very popular (*1, 2, 6, 7, 10, 11*). The interaction between ionic dyes and ionic surfactants which forms the basis of these methods is also involved in the popular spectral-change method for the determination of critical micelle concentrations of surfactants. The precise nature of the interaction between dyes and surfactants involved was recently studied in some detail and the findings applied to the understanding of the detailed nature of the spectral-change method (*9*). In the present paper further application is made to the development of a new analytical technique for the detection, estimation, and separation of small quantities of various classes of ionic surfactants and related compounds.

Of the various kinds of possible interaction between dyes and surfactants, the one that is of interest here is the interaction between ionic dyes and surfactants of opposite charge. Various previous investigators have explained this interaction in terms of the formation of a "complex" between the dye and the surfactant (*11*). The investigations in this laboratory (*9*) showed that the reaction involved is a simple metathetical one between the large surfactant ion and the large dye ion of opposite charge to produce compounds which usually have only slight solubility in water. These compounds are stoichiometric simple 1-1 salts in which electroneutrality is maintained by the dye ions and the surfactant ions only (except when acid or basic salts are formed by divalent dyes). The isolation, purification, and analysis of some of these compounds have been reported (*9*).

Most of the existing analytical methods for surfactants involving the use of dyes utilize this compound formation between a dye and an oppositely charged surfactant. In the titration methods of Hartley and Runnicles (*7*) and Salton and Alexander (*10*) the color change accompanying the formation of some of these compounds is utilized as an indication of the end point. This is made possible by the fact that the salt formed by two oppositely charged surfactants usually has a lower solubility in water and higher solubility in organic solvents than a dye-sur-

factant salt. The situation here is analogous to Mohr's method for analyzing halides, in which the formation of an insoluble colored precipitate, silver chromate, indicates the end point of a reaction forming a less soluble white or yellow precipitate of a silver halide. In the two-phase titration method of Epton (*6*) and its many modifications the initially formed dye-surfactant salt is extracted almost completely by the organic solvent. When the titrant, consisting of an oppositely charged surfactant, is added, surfactant-surfactant salt formation takes place. The end point is indicated when enough titrant is added so that the small amount of the indicating dye present is displaced from the dye-surfactant salt and comes back to the aqueous phase.

A partition method similar to the one described here has been used for colorimetric assay of quaternary ammonium salts by Auerbach (*1*) and Colichman (*2*). Because of insufficient understanding of the underlying phenomenon, however, its generalization for long-chain amine salts, anionic surface active compounds of various kinds, and similar compounds has not been apparent. For long-chain sulfates and sulfonates, an extraction method depending upon complete isolation and colorimetric estimation of a dye-surfactant salt has been suggested (*5*, *8*). For most of these methods, however, the precise nature of the interaction of dyes and surfactants of opposite charge has not been properly appreciated. Colichman, for example, in discussing the phenomenon as used in the partitional assay method of Auerbach (*1*) and himself (*2*), has attributed the color changes involved to the formation of ion pairs (*3*) and of micelles (*4*).

PARTITION TECHNIQUE THEORY

Organic liquids such as chloroform or bromobenzene are not good solvents for most ionic dyes such as methylene blue (cationic) and bromophenol blue (anionic). As a result, in a two-phase system, when one such immiscible solvent is present with a solution of a dye in water, very little color is seen in the organic phase. If some surfactant is present, however, along with an excess of a suitable dye of opposite charge, the dye-surfactant salt formed, usually being considerably more soluble in organic solvents than in water, is partitioned preferentially in the organic layer. From previous calibration or from a knowledge of the absorptivity of the dye in the organic solvent, the concentration of the dye in such a solvent can be found by measuring the absorbance. Since the dye extracts as a simple dye-surfactant salt, the concentration of the surfactant in the aqueous phase can thus be estimated from such equilibrium partition experiments. Any contribution due to the dye itself can be subtracted as a blank correction. The sensitivity can be increased by a large factor by increasing the ratio of the water phase to the organic phase.

Assuming complete extraction and no blank correction, the order of the possible sensitivity can now be easily calculated. A dye such as pinacyanol or methylene blue has a molar absorptivity of the order of 10^5. A $10^{-5}M$ solution should therefore give an absorbance of about 1 in a 1-cm. cell in a spectrophotometer. Since qualitative detection of a difference in absorbance of about 0.005 is not difficult, the detectable concentration of the dye in the organic layer is as low as $5 \times 10^{-8}M$. Since a factor of 10 can be easily achieved in the partition between the aqueous and the nonaqueous phase, the limit of detection is of the order of 5×10^{-9} mole per liter of surfactant in water. For a surfactant having a molecular weight of 300 this concentration corresponds to about 0.0015 p.p.m. In such a favorable case quantitative estimations of concentrations of the order of 0.1 p.p.m. should be feasible.

The attainment of a qualitative sensitivity of the order of 0.001 p.p.m. or higher depends on a large number of factors, such as ideality of partitional extraction and sensitivity of determination. Adsorption on the walls of the containing vessels may cause considerable difficulty also. It is easy to determine quantitatively concentrations of the order of $1 \times 10^{-6}M$ in the aqueous phase. In these experiments the aqueous phase was 80 ml. in volume and, in a $1 \times 10^{-6}M$ solution, contained 8×10^{-8} mole. If each molecule is supposed to occupy an area of 30 sq. A. in a monolayer, this amount of surfactant corresponds to a monolayer area of only 145 sq. cm., which is about the gross area of the 100-ml. flask containing the solution. While this indicates the possibility of using the method in the measurement of small amounts of adsorption, it also points out the difficulty that adsorption may cause in analyses.

For the understanding of the application of this partition method to various surfactants a discussion of the various equilibria involved is useful.

Let us consider an anionic detergent, NaD, and a cationic dye, CCl, where D stands for a detergent ion and C for a colored dye ion. Using suffixes "aq" for the aqueous phase and "org" for the organic phase, we can envisage the various equilibria involved in the schematic representation below:

$$\begin{array}{ccccccccc} COH_{(aq)} & \rightleftharpoons & CCl_{(aq)} & \rightleftharpoons & CD_{(aq)} & \rightleftharpoons & NaD_{(aq)} & \rightleftharpoons & HD_{(aq)} \\ \Big\| \uparrow\downarrow K_1 & & \uparrow\downarrow K_2 & & \uparrow\downarrow K_3 & & \uparrow\downarrow K_4 & & \uparrow\downarrow K_5 \\ COH_{(aq)} & \rightleftharpoons & CCl_{(org)} & \rightleftharpoons & CD_{(org)} & \rightleftharpoons & NaD_{(org)} & \rightleftharpoons & HD_{(org)} \end{array}$$

The equilibria K_2 and K_4 involve the partitions of the dye and the detergent themselves. Choice of a suitable solvent can minimize these, the partition being favorable in most cases to the aqueous phase. Any contribution can be accounted for as a blank correction. The equilibrium K_1, involving the extraction of the basic form of the dye, becomes important only in basic solutions. The equilibrium K_3 involves the dye-surfactant salt. As such this is the key equilibrium. On its nature and relation to the other equilibria depends the success of the method. Equilibrium K_5 is of importance in the case where HD is a weak acid and the solution itself is acidic. The partition of the free acid is usually favorable to the organic phase. A corresponding scheme can be set up for a cationic surfactant and an anionic dye.

From these various equilibria it should be apparent that the results of the method depend on the concentration of the dye, pH, the physicochemical nature of the dyes and the surfactants, the nature of the organic solvent, and the ratio of the volumes of the two phases. In any particular analytical procedure it is necessary, therefore, to keep these factors constant.

EXPERIMENTAL

Materials. The sodium lauryl sulfate, cetyltrimethylammonium bromide, and the dyes methylene blue, pinacyanol, and bromophenol blue have been described (*9*). The sodium laurate used was prepared from lauric acid (*9*), by neutralization with a slight excess of sodium hydroxide. The other indicator dyes used were commercial samples. The dodecylammonium chloride used was prepared from a sample of pure dodecylamine (obtained from the Armour Research Foundation). Santomerse-3 (obtained from the Monsanto Chemical Co.) was used as such.

The experiments were all carried out in commercial distilled water, which was used as received in most cases. As this water is usually supersaturated with respect to carbon dioxide, it was equilibrated against air before use for hydrolyzable surfactants such as fatty acid soaps.

Procedure. An aqueous solution, 80 ml. in volume, containing the surfactant, dye, and acid or alkali, was extracted with 10 ml. of an immiscible organic solvent by shaking in a 100-ml. volumetric flask. The dye concentration used in each series of experiments was constant and was approximately five times the maximum concentration of surfactant used. The absorbances of the organic layer were measured in 1-cm. cells in a Beckman Model DU spectrophotometer against the pure organic liquid as the standard. In the absence of any detergent, the absorbance reading was below 0.05 in all cases except for pinacyanol in basic solution. The absorbance determinations were carried out at the respective band maxima.

Precision. For the attainment of the ultimate in precision and accuracy, precise calibrations are necessary. Fortunately, in all the quantitative series of experiments, agreement with Beer's law was obtained. In each series of experiments only

single determinations were made at six to nine concentrations of surfactants. In spite of this, disregarding not more than one of these determinations in any series, the agreement with simple Beer's law in all cases was within 1 to 3%. The deviations were random, and systematic trends were not observed in any of the series examined. Therefore, precision and accuracy can be improved considerably where such improvements are desirable. The order of sensitivity varies with different surfactants of different classes. The optimum concentration for quantitative estimation varies from 0.5 to 5 $\times$ $10^{-6}M$. It is possible to extend the method to sample volumes of the order of 10 ml. Quantitative estimations of 0.5 to 5 $\times$ 10^{-8} mole of surfactant can thus be carried out. For a surfactant of molecular weight 300 this corresponds to 0.0015 to 0.015 mg.

Exhaustive studies of all the possible dyes and solvents have not been undertaken. For applying this method to any particular situation, the best combination of the dye and the solvent may, however, be easily found out by actual analyses or by isolating different dye salts of the organic ions and studying their relative solubilities in water and various organic solvents. The sensitivity may be materially enhanced also by increasing the ratio of the volumes of the aqueous and the nonaqueous phase.

Rapidity of Operation. With dyes and surfactant solutions prepared beforehand it has been possible to carry out as many as 20 analyses successfully in 3 hours with conventional laboratory ware. The speed of the analysis is thus satisfactory. It may be further improved by performing absorbance measurements in a suitably designed partitioning vessel.

ESTIMATION OF LONG-CHAIN ALKYL SULFATES AND SULFONATES

The example of the alkyl sulfates chosen was sodium lauryl sulfate. Methylene blue was used as the cationic dye. The aqueous layer was made 0.01M in hydrochloric acid. The dye concentration used was 8 mg. per liter, and chloroform was used as the extracting organic liquid. The absorbances were measured at 655 mμ. The sensitivity was close to theoretical. A 1 $\times$ $10^{-6}M$ concentration of sodium lauryl sulfate (0.288 p.p.m.) corresponded to an absorbance of 0.712 in a 1-cm. cell. The qualitative detection limit of 0.005, therefore, corresponds to approximately 0.002 p.p.m. as outlined in the theoretical section. A 20% higher sensitivity was obtained using pinacyanol, another cationic dye. In this case, however, chloroform could not be used because it extracts the dye itself appreciably. Bromobenzene was employed as the organic solvent, the dye concentration used being 8 mg. per liter. In acid solution, this dye itself appears almost colorless in the aqueous layer, although the organic layer is rendered blue by the dye-surfactant salt.

For sulfonates, experiments with Santomerse-3 showed sensitivities of the same order.

For estimations in acid solutions, possibilities of hydrolytic decompositions must not be overlooked, even though alkyl sulfates and sulfonates show considerable stability towards acids. A 6 $\times$ $10^{-6}M$ solution of sodium lauryl sulfate, kept in 0.08M hydrochloric acid, showed no change in concentration, as measured by this method, over a period of 3 days. However, when hydrolytic decomposition is considered likely or possible the acidification should preferably be made just before the actual analysis.

ESTIMATION OF FATTY ACIDS AND FATTY ACID SOAPS

Lauric acid was chosen as a typical fatty acid. For the estimation of fatty acid soaps, basic solutions have to be used; otherwise the free acid extracts too strongly (equilibrium K_5). Methylene blue was first tried as the cationic dye. Although this dye extracts considerably in the basic red-violet form in the organic layer, the contribution of this color to the peak for the dye-detergent salt at 655 mμ is small and can easily be corrected for. No satisfactory organic solvent, however, could be found. With solvents of low dielectric constant (ϵ), such as chloroform (ϵ = 5.1), bromobenzene (ϵ = 5.4), tetrachloroethylene (ϵ = 2.4), or trichloroethylene (ϵ = 3.43), equilibrium K_2 was satisfactory (very little dye extracting in the blue form), but equilibrium K_3 and probably K_5 were unsatisfactory, so that the sensitivity was low. With solvents of high dielectric constant such as nitromethane (ϵ = 39.4) and nitroethane (ϵ = 30.0), the equilibrium K_2 was unfavorable, too much dye being extracted as such. With solvents of intermediate dielectric constant, also, such as ethylene dichloride (ϵ = 10.5), the blank correction was still too high.

Better results were, however, obtained with pinacyanol. The dye concentration used in this case was 8 mg. per liter, bromobenzene was used as the extracting solvent, and the absorbance was measured at 621 mμ. Quantitative experiments using boric acid–sodium borate buffers showed that the blank reading and the sensitivity were dependent on pH. At pH 9.0 the blank was 0.363 and the sensitivity 0.277 in absorbance units for a 1 $\times$ $10^{-6}M$ solution (0.224 p.p.m.). At pH 8.2 the blank was considerably less (0.168), but the sensitivity was lowered to 0.172 for a 1 $\times$ $10^{-6}M$ solution. At high pH, the equilibrium K_1 is unfavorable, more dye extracting in the basic form, while K_3 is favorable, less free acid being left to extract. At low pH, the blank is less because K_1 is favorable but K_5 competes with K_3—i.e., the free acid extracts appreciably. This results in lower sensitivity of the extraction of the surfactant as the dye salt.

ESTIMATION OF FATTY ACID SOAPS AND NONHYDROLYZABLE ANIONIC SURFACTANTS IN PRESENCE OF EACH OTHER

In acid solutions the contribution of fatty acid soaps is almost negligible. This is due to the essentially complete hydrolysis of the fatty acid soap into the acid. In the presence of $10^{-2}M$ hydrochloric acid, for example, a 1.3 $\times$ $10^{-4}M$ solution of sodium laurate gave an absorbance of the order of 0.001. As this concentration of the fatty acid soap is 50 to 100 times the maximum concentration of less hydrolyzable anionic surfactants, such as the alkyl sulfates and sulfonates likely to be used in this method, considerable excess of fatty acids does not interfere with the estimation of the alkyl sulfates and sulfonates.

For the estimation of the fatty acids in the presence of the sulfates of sulfonates it is necessary to apply a differential method. In basic solution (pH approximately 8.5) the fatty acids and the sulfates and sulfonates can be determined together. If now the sulfates and the sulfonates are determined separately in acid solution, the fatty acids can be estimated by difference. Unfortunately, as has been discussed in the two previous sections, the sensitivity of the determination of the fatty acids is less than that for the others by a factor of 3 or so in the systems that have been tried so far. In the presence of comparable amounts of fatty acids and the sulfates or sulfonates, therefore, the accuracy of determination of the former is somewhat less.

ESTIMATION OF CATIONIC SURFACTANTS

Estimation of Quaternary Ammonium Salts. For the estimation of the cationic surfactants one has to use suitable anionic dyes. Qualitative experiments showed that indicator dyes such as thymol blue and bromophenol green are useful. Bromophenol blue was chosen for this detailed study.

Cationic surfactants and bromophenol blue undergo two kinds of reactions. With cetyl trimethylammonium bromide, which has been used in the work as an example of a long-chain quaternary ammonium compound, bromophenol blue forms two different compounds. In alkaline solutions a blue dicetyl trimethylammonium salt of the indicator dye is formed, whereas in acid solution a yellow monocetyl trimethylammonium salt is obtained. The isolation and analysis of these salts have been reported (*9*).

Table I. Comparative Sensitivities for Different Classes of Surfactants

Class	Substance	Dye	Solvent	Sensitivity, Absorbance Units in 1-Cm. Cells: Per $10^{-6}M$	Per p.p.m.
Anionic nonhydrolyzable	Sodium lauryl sulfate	Methylene blue in 0.01M HCl	Chloroform	0.712	2.472
Anionic hydrolyzable	Sodium laurate	Pinacyanol in boric acid–sodium borate buffer (pH 9.0)	Bromobenzene	0.277	1.237
		Pinacyanol in same buffer (pH 8.2)	Bromobenzene	0.172	0.768
Cationic nonhydrolyzable	Cetyl trimethyl-ammonium bromide	Bromophenol blue in $10^{-2}M$ HCl	Chloroform	0.232	0.636
		Bromophenol blue in $10^{-2}M$ NaOH	Chloroform	0.116	0.318
Cationic hydrolyzable	Dodecylammonium chloride	Bromophenol blue in $10^{-2}M$ HCl	Chloroform	0.133	0.599

It can be expected, therefore, that cetyl trimethylammonium bromide and similar compounds can be estimated in two different ways—as the blue di- salt in alkaline solutions and the yellow mono- salt in acid solutions. This was found to be the case. In the presence of 0.01M sodium hydroxide, cetyl trimethylammonium bromide was estimated using bromophenol blue (concentration 16 mg. per liter) and chloroform at 606 mμ. The sensitivity was somewhat less than expected, mainly because one dye ion carries two surfactant ions. A $1 \times 10^{-6}M$ solution (0.365 p.p.m.) corresponded to an absorbance of 0.232 in 1-cm. cells. The estimation as the yellow form was carried out in 0.01M hydrochloric acid solution and a dye concentration of 6 mg. per liter using chloroform. The sensitivity was somewhat less than that obtained in the alkaline solution. A concentration of $1 \times 10^{-6}M$ (0.365 p.p.m.) corresponded to an absorbance of 0.116 in 1-cm. cells. The band maximum in this case was at 416 mμ.

Estimation of Long-Chain Amines. Dodecylamine in its hydrochloride form was chosen as the example. In 0.01M hydrochloric acid and 16 mg. per liter dye concentration, the estimation proved successful. A $1 \times 10^{-6}M$ solution (0.222 p.p.m. of the hydrochloride salt) give an absorbance of 0.133 in a 1-cm. cell.

Estimation of Long-Chain Quaternary Ammonium Compounds and Long-Chain Amines in Presence of Each Other. Separate estimations of the quaternaries (which do not hydrolyze) and the amine salts (which can be made to hydrolyze easily in alkaline solutions) can be carried by conducting the experiments at different pH values. In the presence of 0.01M alkali a $2.5 \times 10^{-4}M$ solution of dodecylammonium hydrochloride gave a contribution of only 0.06 in the absorbance value. As this concentration is about 50 times the maximum concentration of the quaternary ammonium compound likely to be used in this method, the quaternary compounds can be estimated in the presence of considerable excess of amine salts. A higher pH would decrease the contribution of the amine even more because the higher the pH the greater is the hydrolysis of the amine salt into the amine and removal by the organic liquid.

The estimation of the amine salts in the presence of the quaternaries can be carried out by estimating the total cationics in acid solution and the quaternaries only in alkaline solutions. The amine is obtained by difference. As discussed before, the sensitivity of the method for the quaternaries and the amines in the acid process are comparable, and the sensitivity of the quaternary in the alkaline region is higher. The accuracies of the estimations of each of these when present in comparable amounts should thus be of the same order.

APPLICATION TO OTHER SYSTEMS

Application of this new partition method to the analysis of surfactants of various kinds is summarized in Table I. Qualitative and semiquantitative experiments have shown the method to be applicable to various other organic compounds with acidic and basic functions, such as acids and amines of moderately high molecular weights, alkaloids, and quaternary ammonium compounds as small as tetra-*n*-propylammonium bromide. Very dilute solutions of ionic dyes can be estimated by using dyes of opposite charge. The method should thus find considerable application in the analysis of many classes of ionic organic compounds. The only requisite for applicability of the method is that there be a dye carrying a charge opposite to the organic ion and forming a stoichiometric compound with it whose solubility in an immiscible organic solvent is appreciably higher than its solubility in water. As the dyes themselves can be estimated at low concentrations (about $10^{-5}M$) the sensitivity of determination of these other organic ionic compounds, aided by a large factor (about 10) in the partition, is thus correspondingly high.

One final point: The specificity of this method is rather low. This is not inconvenient where single species are to be determined, but when several species are determined simultaneously, the method may give a rather complicated average, as the solubility characteristics of the different species may not be the same. However, because of the different solubilities and equilibria involved, it may be possible to utilize individual differences in similar compounds, and qualitative separations of homologous compounds may be effected.

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LITERATURE CITED

(1) Auerbach, M. E., Ind. Eng. Chem., Anal. Ed. **15**, 492 (1943).
(2) Colichman, E. L., Anal. Chem. **19**, 430 (1947).
(3) Colichman, E. L., *J. Am. Chem. Soc.* **72**, 1834 (1950).
(4) *Ibid.*, **73**, 1795 (1951).
(5) Edwards, G. R., Ewers, W. E., Mansfield, W. W., *Analyst* **77**, 205 (1952).
(6) Epton, S. R., *Nature* **160**, 795 (1947); *Trans. Faraday Soc.* **44**, 226 (1948).
(7) Hartley, G. S., Runnicles, D. F., *Proc. Roy. Soc. (London)* **A168**, 424 (1938).
(8) Jones, J. H., *J. Assoc. Offic. Agr. Chemists* **28**, 398 (1945).
(9) Mukerjee, Pasupati, Mysels, K. J., *J. Am. Chem. Soc.* **77**, 2937 (1955).
(10) Salton, M. R. J., Alexander, A. E., *Research (London)* **2**, 247 (1949).
(11) Tschoegl, N. W., *Revs. Pure and Appl. Chem. (Australia)* **4**, 171 (1954).

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Slope-Ratio Liver-Storage Bioassay for Vitamin A

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A slope-ratio liver-storage bioassay for vitamin A has been developed employing a "5-point common-zero" statistical design. Five groups of depleted rats are supplemented as follows: two levels of the reference standard (1000 and 2000 units), two similar levels of the test material, and a negative-control group. The relative potency is determined by the ratio of the slopes of the two linear dose-response lines. The liver-storage of vitamin A showed an essentially linear response over a dose range from 500 to 10,000 units. When the USP vitamin A reference standard was fed, 67% of the ingested dose was found stored in the liver. Direct comparison of the results obtained by either the slope-ratio liver-storage or growth procedure showed no significant difference. The slope-ratio liver-storage bioassay for vitamin A is both rapid and precise and is recommended for general application.

THE liver-storage bioassay for vitamin A originally devised by Guggenheim and Koch (*12*) has been finding increasing application in recent years (*6, 10, 14*). It is based on the fact that substantial amounts of large doses of vitamin A are deposited proportionally in the liver under suitably controlled conditions. The original procedure (*12*) was subsequently modified by Lemley and coworkers (*14*) and Foy and Morgareidge (*10*). Subsequent modifications have been summarized in some detail by Bliss and György (*6*). With the development of a better procedure for the determination of vitamin A in liver (*1*) and the availability of new methods of statistical design (*18, 19*), further development of the basic liver-storage procedure became possible. The following slope-ratio liver-storage bioassay will supplement and, except for low-potency oils, will replace the more laborious and less precise rat-growth procedure recommended by the United States Pharmacopeia (*16*).

ANALYTICAL PROCEDURE

Depletion. The purpose of the depletion period is to provide normal growing rats which have been depleted of measurable liver vitamin A reserves. Male albino rats are placed on the USP vitamin A test diet at weaning. Following a depletion period of from 9 to 18 days, the animals have no detectable vitamin A (less than 10 units by the Carr-Price reaction) in lipide extracts of their livers and are ready for supplementation.

Design of Assay. An example of a slope-ratio liver-storage bioassay designed to test an assay oil—for example, a concentrate expected to contain about 300,000 units per gram—against the United States Pharmacopeia vitamin A reference standard is given in Table I. The animals are divided into equal groups assigned to each of the five dosage schedules. In addition to a negative-control group (R_0), the standard is fed at two levels (R_1 and R_2) and the assay oil at two similar levels (A_1 and A_2).

Supplementation. Supplements of both reference standard and assay materials are prepared by dissolving a calculated quantity of the vitamin A–containing material in vitamin A–free cottonseed oil (Wesson oil) fortified with 0.1% hydroquinone (*4*). The supplements are administered orally by calibrated dropper (about 25 mg. per drop). Two drops or 4 drops are given per day on each of 3 consecutive days, resulting in a total dose of 6 drops (about 150 mg.) or 12 drops (about 300 mg.). The feeding solution of the standard is prepared so that 100 mg. of the USP reference standard is contained in the total dose of 6 drops (or 200 mg. of reference standard in 12 drops). One hundred milligrams of the USP reference standard contains 0.344 mg. of crystalline vitamin A acetate (equivalent on a molar basis to 0.300 mg. of the alcohol) and by definition is equal to 1000 USP units (*15*) or 1000 International units (*20*). If the material to be assayed is a vitamin A compound of known composition, 6 drops of the feeding solution should be made to contain an amount of sample equivalent on a molar basis to approximately 0.300 mg. of vitamin A alcohol—i.e., 0.344 mg. of vitamin A acetate or 0.630 mg. of vitamin A palmitate. If the material to be assayed is a vitamin A–containing oil of unknown composition, its specific absorbance at 326 mμ is divided by 1835 (the specific absorbance of crystalline vitamin A alcohol) to obtain an estimate of the vitamin A content in grams of vitamin A alcohol per gram of sample. Six drops of the feeding solution should again contain an amount of sample equivalent on a molar basis to approximately 0.300 mg. of vitamin A alcohol. The supplements are administered on each of 3 consecutive days and the animals are sacrified 48 hours later. The entire liver is carefully removed, blotted, weighed, and stored at −15° C. until analyzed.

Table I. Example of Plan of Slope-Ratio Liver-Storage Bioassay

(Ten animals used in all tests)

Compound Group	Control R_0	U.S.P. Ref. Std. R_1	U.S.P. Ref. Std. R_2	Assay Oil A_1	Assay Oil A_2
Total weight of material fed, mg.	0	100	200	3.0	6.0
Total equivalent weight of vitamin A alcohol fed, mg.	0	0.3 (or 1000 USP units)	0.6 (or 2000 USP units)	0.3 (approx.)	0.6 (approx.)
No. of drops per day	0	2	4	2	4
Total no. of drops	0	6	12	6	12
		Example			
Vitamin A content of individual rat livers (details of calculation in Table II)	0	613	1322	569	1087
	0	613	1417	613	1138
	0	659	1453	639	1197
	0	646	1446	565	1285
	0	654	1461	690	1241
	0	571	1285	567	1057
	0	529	1277	609	1123
	0	690	1307	602	1372
	0	650	1329	584	1380
	0	683	1270	555	1248

Chemical Analysis. The simplified analytical procedure (*1*) for the determination of liver vitamin A can be briefly summarized as follows: The entire rat liver is ground in a mortar with anhydrous sodium sulfate until completely dry. The dry powder is transferred quantitatively to a 250-ml. Perma-red glass-stoppered Erlenmeyer flask. One hundred milliliters of peroxide-free anhydrous ethyl ether is added, and the flask is shaken for 2 minutes. It is then set aside for about 1 hour until the solids have separated from the ether layer. An aliquot of the ether layer is transferred quantitatively to a colorimeter tube, and the solvent is evaporated under nitrogen. The intensity of the blue color formed on the addition of antimony trichloride is measured in an Evelyn colorimeter using a 620 mμ filter. The amount of vitamin A present is determined by reference to a standard curve obtained using the United States Pharmacopeia vitamin A reference standard. The results are recorded as total units of vitamin A per liver based on the blue-color value.

Statistical Analysis. The slope-ratio bioassay employs a "5-point common-zero" experimental design developed by Wood (*18*) and Wood and Finney (*19*). By this procedure the relative potency of the assay oil in terms of the reference standard and the biopotency in liver storage units per gram can be determined.

The bioassay is designed so that five equal groups are supplemented as follows: Two levels of the reference standard with a dose ratio of 1 to 2 (R_1 and R_2), two levels of the assay oil with a dose ratio of 1 to 2 (A_1 and A_2), and a common-zero group receiving no supplement (R_0). The vitamin A contents of the individual rat livers are tabulated and the mean (M) and the sum of the squares of the deviations from the mean are determined for each group. When the mean liver stores are below 50 units, the standard deviation of the combined analytical procedure and bioassay variation is about ±10 units. Thus, the variance of the R_0 group is estimated to be $n \times 10^2$. When the mean liver storage

of the R_0 group is greater than 50 units, the variance calculated in the usual manner is employed.

Since the design includes five groups,

$$\text{Error variance } (s^2) = \frac{\text{total within group sum of squares}}{N - 5}$$

The departures of the two dose-response lines from linearity are measured by the two quantities L_R and L_A which are calculated and tested for significance as follows:

$$L_R = M_0 + M_{R_2} - 2M_{R_1}$$

$$L_A = M_0 + M_{A_2} - 2M_{A_1}$$

$$\text{Variance}_L = \frac{30\ s^2}{N} \qquad t = \frac{L_R \text{ or } L_A \text{ (whichever is greater)}}{SE_L}$$

$$\text{Standard error}_L = \sqrt{\text{variance}_L} \qquad df = N - 5$$

If the deviation from linearity (L_R or L_A) is significant as determined by the t test, the assay should be discarded.

The constants of the two regression lines of slopes b_R and b_A which must share a common y-intercept (a) are calculated as follows:

$$a = M_0 - \frac{(L_R + L_A)}{7}$$

$$b_R = \frac{M_{R_2} - M_0}{2} + \frac{(6L_A - L_R)}{70}$$

$$b_A = \frac{M_{A_2} - M_0}{2} + \frac{(6L_R - L_A)}{70}$$

The relative potency, P_*, of the assay oil in terms of the reference standard, its variance, V_{P_*}, and standard error, SE_{P_*}, are calculated according to the following equations:

$$P_* = b_A/b_R$$

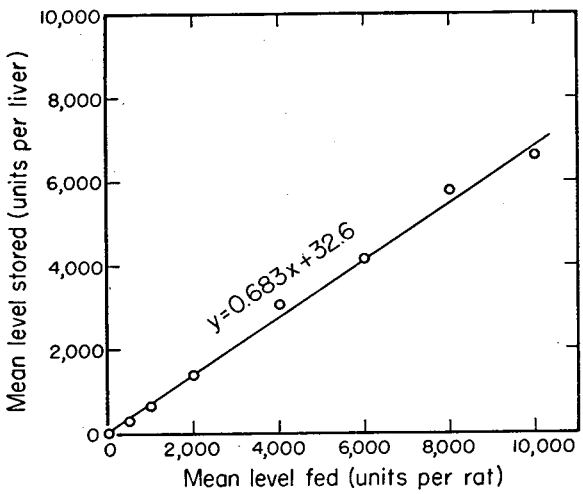

Figure 1. Dose response curve for vitamin A liver storage

Crystalline vitamin A acetate was fed to groups consisting of 10 male rats each. The slope-ratio liver-storage bioassay was followed in other details. Regression line was calculated by least squares. The y-intercept is not significantly different from the origin; $t = 0.74$

Table II. Example of Calculations[a]

Dose	Total Weight of Material Fed, Mg.	No. of Animals	Means	Σx	Sum of Squares
R_0	0	10	0	–	1,000
R_1	100	10	630.8	6,308	21,936
R_2	200	10	1,356.7	13,567	55,215
A_1	3	10	599.2	5,992	15,516
A_2	6	10	1,212.8	12,128	114,556
Totals		50		37,995	208,223

$$s^2 = \frac{208,223}{45} = 4649.40$$

Linearity

$$L_R = 0 + 1356.7 - 2 \times 630.8 = 104.1$$

$$L_A = 0 + 1212.8 - 2 \times 599.2 = 14.4$$

$$\text{Variance}_L = 30 \times 4649.40/50 = 2789.64$$

$$SE_L = \sqrt{2789.64} = 52.82 \qquad t = \frac{104.1}{52.82} = 1.971$$

Regression

$$a = 0 - \frac{104.1 + 14.4}{7} = -16.93$$

$$b_R = \frac{1356.7 - 0}{2} + \frac{(6 \times 14.4 - 104.1)}{70} = 678.6$$

$$b_A = \frac{1212.8 - 0}{2} + \frac{(6 \times 104.1 - 14.4)}{70} = 615.1$$

Relative potency

$$P_* = \frac{615.1}{678.6} = 0.9065 \text{ or } 90.65\% \pm 1.92\%\ SE$$

$$V_{P_*} = \frac{2 \times 4649.40}{7 \times 50 \times 678.6 \times 678.6}\,[8(0.9065)^2 - 9(0.9065) + 8] = 0.0003698$$

$$SE_{P_*} = 0.0192 \text{ or } 1.92\%$$

Absolute biopotency

3.0 mg. of assay oil are 90.65% as potent as 100 mg. of U.S.P. reference standard (1000 U.S.P. units). Therefore, 3.0 mg. of assay oil are equivalent to 906.5 liver storage units. Assay oil = 302,200 liver storage units per gram.

[a] Details of dosage in Table I.

$$V_{P_*} = \frac{2s^2}{7N(b_R)^2}(8P_*^2 - 9P_* + 8) \qquad SE_{P_*} = \sqrt{V_{P_*}}$$

The results are usually expressed in terms of per cent P_* as follows:

$$\text{Relative potency} = (100\ P_*)\% \pm (100\ SE_{P_*})\%$$

The calculations of the relative potency and its accompanying statistics and the absolute biopotency in liver storage units per gram are given in detail in Table II. Derivations of the formulas and details of computations are available in the original presentation of this procedure by Wood and Finney (*18*, *19*) or in two recent reviews (*5*, *9*).

ANALYTICAL DETAILS

Animals. Male weanling rats of low vitamin A status can best be obtained by maintaining the mothers on a vitamin A–low diet. As soon as pregnancy is observed, the females are segregated and placed on the United States Pharmacopeia vitamin A test diet. After parturition, the mothers are placed on a stock diet for 7 to 14 days and then again placed on the vitamin A test diet. Weanling males obtained in this manner have been found very suitable for routine bioassay. With such a breeding program, litter mates are completely randomized between the experimental groups. Weanling male rats supplied by the Holtzman Rat Co. and designated as being suitable for vitamin A bioassay have proved to be satisfactory.

The length of the depletion period will, of course, vary with the

Table III. Statistical Relationships of the Slope-Ratio Liver-Storage Bioassay[a]

	No. of Determinations	Total	Mean	$\hat{\sigma}$ [b]
y- intercept, a	124	−1983.0	−16.0	±13.0
Linearity R	24	+1710.2	+71.2	±50.7
Linearity A	124	+5644.4	+45.5	±74.9
Standard error (corrected to $P_* = 1$)[c]	124	± 358.18	± 2.89	± 0.77

[a] Summarized from a series of 124 consecutive slope-ratio liver-storage bioassays involving about 3000 rats.
[b] Estimate of standard deviation.
[c] VP_* is a complex function of s^2, P_*^2, and P_*: It has been experimentally determined that at dose ratios of 1 and 2, the group variances vary as 1 and 4. Thus, if $\hat{v}$ is the estimated mean variance of the R_1 group,

$$s^2 = \frac{0\hat{v} + \hat{v} + 4\hat{v} + P_*\hat{v} + 4P_*\hat{v}}{N - 5} = \frac{5\hat{v}}{N - 5}(1 + P_*)$$

$$\frac{s^2P_* = 1.0}{s^2P_* \neq 1.0} = \left(\frac{2}{1 + P_*}\right)$$

Furthermore, $VP_* = \frac{2}{7N(bP_*)^2} s^2(8P_*^2 - 9P_* + 8)$

$$VP_* = 1.0 = VP_* \neq 1.0 \left(\frac{s^2P_* = 1.0}{s^2P_* \neq 1.0}\right)\left(\frac{8 - 9 + 8}{8P_*^2 - 9P_* + 8}\right)$$

$$= VP_* \neq 1.0 \left(\frac{2}{1 + P_*}\right)\left(\frac{7}{8P_*^2 - 9P_* + 8}\right)$$

Therefore, in order to obtain the best approximation of the within-bioassay variance, VP_* and SEP_* have been computed to a uniform basis where $P_* = 1$ using the above equation.

vitamin A status of the weanling rats. This was studied by starting supplementation of the animals on either the ninth or the 18th day after weaning. No significant difference in the average storage response was observed, but the within-group variation was less with the longer period of depletion. Use of animals in a state of vitamin A deficiency should be avoided both because of the lack of uniformity and because of pathologic changes.

There appears to be a sex difference in the ability of the rat to store vitamin A, females storing a significantly greater amount (*7*, *8*). The inclusion of both males and females in a single bioassay would result in either an undesirable increase in the experimental error or unnecessary complications in the calculations.

Supplementation. The magnitude of the supplement is not a critical factor as long as it is within the range of 500 to 5000 units. As shown in Figure 1, the liver storage of vitamin A varies linearly as the dose over the range from 500 to 10,000 units.

The procedure of feeding on 3 successive days and killing 48 hours after administration of the final dose was adopted after determining the time and duration of maximum liver storage, as shown in Figure 2. The liver storage of vitamin A increases to a maximum at about 2 days. This level is maintained for several days before a significant decrease is evident. Thus the recommended supplementation procedure ensures maximum storage of the last dose and near maximum storage of the two preceding doses while retaining the advantages of a multiple dose schedule.

Evaluation of Statistical Procedure. Under controlled conditions, the liver storage of vitamin A is essentially a linear function of the ingested dose. The data of Figure 1 show this straight-line relationship over the dose range of 500 to 10,000 units. Nonlinear dose-response curves in the dose range of 10 to 40 units have been reported (*11*, *12*). In Table III the mean y-intercepts and deviations from linearity of a series of 124 consecutive slope-ratio liver-storage bioassays have been summarized. These results show a small positive but statistically significant deviation from true linearity which results in a small negative y- intercept. The data in Table IV summarizing the responses of the USP reference standard in 16 bioassays also show this trend. In contrast the data in Figure 1 show a positive y- intercept resulting from a small negative deviation from linearity. These small deviations from linearity have a negligible effect on the determination of the relative potency and are of no consequence when dose levels of higher than 500 units are fed.

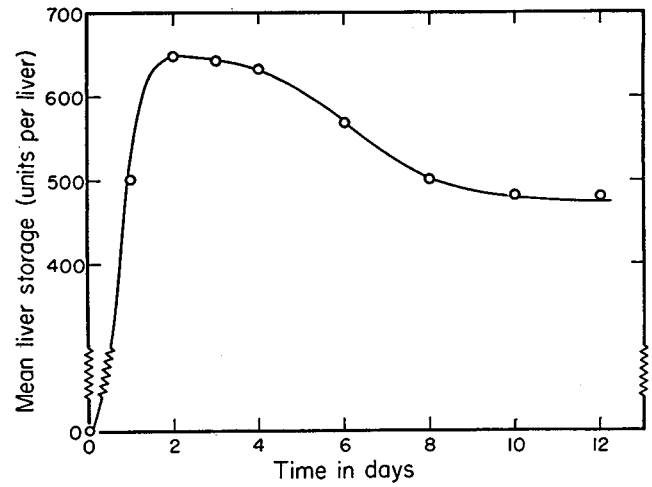

Figure 2. Liver storage of a single dose of vitamin A

Single doses of 1000.8 units of the USP vitamin A reference standard were fed to groups consisting of five male rats each. Slope-ratio liver-storage bioassay was followed in other details

Table IV. Response of USP Reference Standard in Slope-Ratio Liver-Storage Bioassay

No. of Rats/ Level	Mean Vitamin A Liver Stores/Rat: Zero dose fed	1000 units fed	2000 units fed	Slope b	y- Intercept a (Units)
10	0	621.3	1292.9	0.6465	− 8.4
9	0	702.7	1553.3	0.7652	−13.2
10	0	630.8	1356.7	0.6784	−15.9
9	0	639.9	1359.4	0.6715	− 5.1
12	0	653.3	1478.8	0.7228	−12.1
9	0	649.9	1297.3	0.6489	+ 0.2
9	0	677.8	1362.6	0.6806	− 0.5
8	0	633.8	1456.3	0.7103	−13.6
8	0	656.4	1334.1	0.6646	− 1.9
9	0	658.8	1380.3	0.6834	− 3.7
7	0	592.0	1251.6	0.6194	− 4.9
10	0	608.4	1271.3	0.6360	− 9.4
10	0	629.4	1313.9	0.6570	− 9.2
10	0	640.0	1383.7	0.6919	−17.3
10	0	643.1	1328.3	0.6642	− 7.0
9	0	560.3	1162.3	0.5772	− 3.0

Total animals = 447
Mean slope = 0.6699 $\hat{\sigma}$ = ±0.0431 SE = ±0.0111
Mean y-intercept = −7.81 $\hat{\sigma}$ = ±5.47 SE = ±1.41
Vitamin A stored = 0.6699 × vitamin A fed − 7.81

The total error variance, s^2, is calculated from the individual variances of each of the five experimental groups. These individual variances are not the same for each experimental group but are approximately four times greater when the dose level is doubled. Thus, the total error variance is determined primarily by the magnitude of the variances of the R_2 and A_2 groups. This is contrary to the assumptions upon which the statistical analysis is based. Inclusion of the low variance of the common-zero group effectively averages the within-group variances and leads, the authors feel, to a realistic estimate of the standard error of the estimated potency.

In discussing the liver-storage bioassay, Bliss and György (*6*) suggested the use of a log dose-log response statistical analysis. Since the liver storage of vitamin A is essentially a linear function of the ingested dose, calculation as a log-log function would likewise yield a straight line. The log-log transformation will effectively reduce heterogeneity in the variance. However, if the mean liver storage of the unsupplemented group

is not equal to zero, the log-log transformation of the two straight lines determined by the responses of the standard and test materials will result in two curved nonparallel lines. In fact, the greater the liver storage of vitamin A in the negative control group, the greater the nonlinearity and deviation from parallelism. Nonlinearity and deviation from parallelism are contrary to the assumptions upon which the log-log statistical analysis is based. In addition, the log dose-log response procedure cannot utilize the advantages of the negative control group and the statistical restriction of the intersection at the y-intercept.

Bioassay Precision. An estimate of the within-bioassay variation can be obtained from the mean standard error of ±2.9% observed in the series of routine bioassays given in Table III. This low standard error results from the combination of standardized treatment of animals, improved analytical techniques, and the more restrictive statistical design.

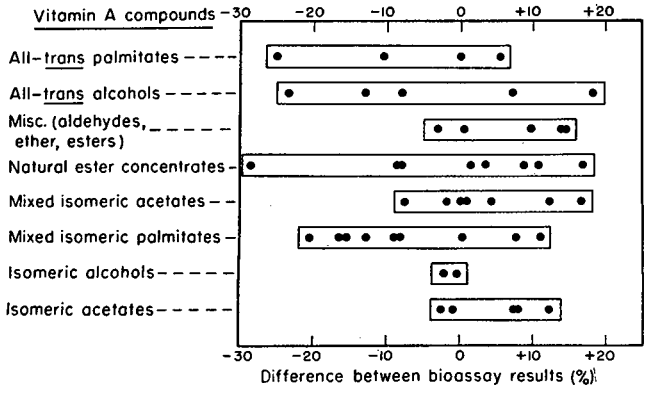

Figure 3. Comparison of growth and slope-ratio liver-storage bioassays of various vitamin A compounds

Abscissa values calculated by subtracting relative biopotency (%) as determined by slope-ratio liver-storage bioassay from corresponding relative biopotency (%) determined by growth bioassay. Statistical analysis of differences yields the following data: mean difference = − 0.72; $\hat{\sigma}$ diff. = ± 11.67; SE diff. = ± 1.74 (t = 0.41, P = 0.6 to 0.7)

The between-bioassay variation can be estimated from the data of Table IV. This summary of the data on the United States Pharmacopeia reference standard is taken from a series of routine slope-ratio liver-storage bioassays. The standard deviation of the slopes in this series is $\hat{\sigma} = \pm 0.043$. Since the relative potency is the ratio of two slopes, and assuming that the variance of the response of the test material is the same as that of the reference standard, the standard deviation of this ratio is calculated to be ±9.1%. Nine determinations of the relative potency of a number of vitamin A palmitates ($\hat{\sigma} = \pm 8.6\%$) and nine determinations of the relative potency of a number of vitamin A alcohols ($\hat{\sigma} = \pm 6.5\%$) furnish comparable data. These direct estimates of the between-bioassay variation include seasonal fluctuations and minor changes in environment and animals as well as preparations of varying purity in the latter two cases. Thus the authors feel that the agreement between the internal estimate of variability and the direct estimates is satisfactory.

Correspondence with Other Bioassays. The slope-ratio liver-storage bioassay possesses distinct advantages over the conventional growth type of bioassay. However, the growth bioassay is the currently accepted method for the biological evaluation of vitamin A preparations. The results of the comparative bioassays by both growth and slope-ratio liver-storage procedures of a number of different preparations of vitamin A, including alcohols, esters, and isomers, are given in Figure 3. The slope-ratio liver-storage bioassay yielded results which averaged 0.7% higher than the corresponding growth bioassays. Statistical analysis of these data by Student's method for paired values indicates the difference was not statistically significant (P = 0.6 to 0.7). Additional bioassay data on the geometric isomers of vitamin A acetate and aldehyde support this observation (*2, 3*). It appears that the observed differences between slope-ratio liver-storage and growth bioassays of a single material may be attributed to the experimental error of the bioassays rather than to a significant difference in response between the two procedures.

DISCUSSION

This slope-ratio liver-storage bioassay for vitamin A should be of general application, both for vitamin A research and for the evaluation of commercial preparations. As it is both rapid and precise, application to a routine bioassay program is obvious. The slope-ratio procedure appears to be applicable to a wide variety of vitamin A preparations and derivatives. A limitation is that the potency of the test material should be more than 5000 units per gram to avoid excessive administration of accompanying oil. However, the bioassay of low potency materials such as margarine (15,000 I.U. per pound) could readily be accomplished by saponification of the sample and bioassay of the nonsaponifiable fraction by the slope-ratio procedure.

With this procedure about 67% of an orally ingested dose of vitamin A acetate is found stored in the liver. This is much greater than the 25 to 35% relative storage in the liver-storage bioassays reported by most investigators (*10, 12, 14*). The high relative storage observed in the slope-ratio liver-storage bioassay will necessitate re-evaluation of some previous investigations. For example, it has been stated (*17*) that the administration of aqueous emulsions of vitamin A results in two to three times the liver stores compared with feeding vitamin A in oil. Differences of this magnitude are obviously impossible when 67% of the vitamin A in oil solution is stored.

Because of the use of a linear statistical design, several modifications of the basic procedure are possible which lend themselves to unique applications. Once the linearity of the response of a particular type of vitamin A preparation has been established, the design can be simplified to a three-point common-zero type as described by Wood (*18*) and Wood and Finney (*19*). In this design the test material and standard are each fed at a single dose level with the negative-control group determining the y-intercept. The ratio of the slopes determines the relative activity of the two preparations. While some precision is lost, the reduction in number of rats and concomitant gain in speed may make this modification attractive for a routine testing program.

Rats that have not been depleted of their vitamin A liver stores can be employed in this procedure involving a linear experimental design. Thus the response of supplementary vitamin A can be investigated in animals whose nutritional status is normal. The vitamin A liver stores in an unsupplemented group of rats determines the fifth or common-zero point (y-intercept). The liver-storage bioassay of vitamin A using undepleted animals is a unique modification of the slope-ratio procedure.

The use of the slope-ratio liver-storage procedure for routine vitamin A bioassays appears justified in view of the good correspondence with the conventional growth technique. From the standpoints of simplicity, speed, and accuracy it can be recommended for the evaluation of vitamin A and its derivatives. The liver-storage response under these conditions has not been investigated for provitamin A. However, Foy and Morgareidge (*10*) have reported good correspondence between growth and liver-storage procedures using β-carotene. Johnson and Bauman (*13*) have reported that the growth response paralleled liver storage for all-*trans*-β-carotene, neo-β-carotene B, neo-β-carotene U, and all-*trans*-α-carotene, but not with α-carotene. For most of the provitamins A, the correspondence of liver-storage and growth bioassays appears good. The slope-ratio liver-storage

procedure as outlined above would appear very satisfactory for application to both routine and research vitamin A bioassays.

ACKNOWLEDGMENT

Appreciation is expressed to W. J. Swanson and H. A. Risley for their expert technical assistance in this investigation.

LITERATURE CITED

(1) Ames, S. R., Risley, H. A., Harris, P. L., ANAL. CHEM. 26, 1378 (1954).
(2) Ames, S. R., Swanson, W. J., Harris, P. L., *J. Am. Chem. Soc.* 77, 4134 (1955).
(3) *Ibid.*, p. 4136.
(4) Bliss, C. I., "Suggested Revision of the USP Biological Assays for Vitamins A and D," Animal Nutrition Research Council, Nov. 15, 1948.
(5) Bliss, C. I., in "Vitamin Methods," P. György, ed., vol. II, pp. 560–75, Academic Press, New York, 1951.
(6) *Ibid.*, pp. 93–7.
(7) Booth, V. H., *J. Nutrition* 48, 13 (1952).
(8) Brenner, S., Brookes, M. C. H., Roberts, L. J., *Ibid.* 23, 459 (1942).
(9) Finney, D. J., "Statistical Method in Biological Assay," pp. 187–211, Hafner Publishing Co., New York, 1952.
(10) Foy, J. R., Morgareidge, K., ANAL. CHEM. 20, 304 (1948).
(11) Guerrant, N. B., *J. Nutrition* 37, 37 (1949).
(12) Guggenheim, K., Koch, W., *Biochem. J. (London)*, 38, 256 (1944).
(13) Johnson, R. M., Bauman, C. A., *Arch. Biochem.* 14, 361 (1947).
(14) Lemley, J. M., Brown, R. A., Bird, O. D., Emmett, A. D., *J Nutrition* 33, 53 (1947).
(15) Pharmacopeia of the United States, "USP Vitamin A Reference Standard, Instructions for Use," May 18, 1948.
(16) Pharmacopeia of the United States, vol. XIV, Mack, Easton, Pa., 1950.
(17) Sobel, A. E., Sherman, M., Lichtblau, J., Snow, S., Kramer, B., *J. Nutrition* 35, 225 (1948).
(18) Wood, E. C., *Analyst* 71, 1 (1946).
(19) Wood, E. C., Finney, D. J., *Quart. J. Pharm. and Pharmacol.* 19, 112 (1946).
(20) World Health Organization, *Tech. Rept. Ser.* 3, 4 (1950).

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Use of Periodic Acid for Detecting and Locating Ethylenic Unsaturation

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Periodic acid has proved to be a promising reagent in the detection and location of both terminal and exocyclic double bonds in organic molecules. Like ozone, this per acid oxidizes unsaturated compounds by splitting the —C=C— grouping. The resulting products are carbonyl derivatives which can be readily characterized and estimated as their 2,4-dinitrophenylhydrazones or dimethones. The yield of the aldehydic or ketonic fragments thus obtained corresponds to that calculated from the number of double bonds present.

THE first important application of the use of periodic acid as a selective oxidizing agent for α-glycols was made by Malaprade (*8*, *9*) in 1928. It is now being extensively employed in the field of organic reactions to detect various functional groups—viz.,

—CHOH—CH(NH_2)—	—CO—CO_2H
—CHOH—CHO	CO_2H—CH_2—CO_2H
CHO—CO—	CO_2H—CH_2—CHO
—CO—CO—	CH_2OH—CH(NH_2)—CO_2H
CHO—CH_2OH	H_2N—CH_2—CH_2—CH_2OH

as well as the active methylene linkage in citric and malic acids and the like. The use of periodic acid in the analysis of organic compounds and in determination of their structure has become of increasing importance. Several review articles (*3*, *5–7*, *10*) on the reagent have been published. However, a careful survey of the relevant literature reveals that the oxidizing action of periodic acid on unsaturation in organic molecules has not yet been studied. The present investigation was undertaken with a view to developing, if possible, a suitable method for identification and location of double bonds in unsaturated molecules. Oxidation experiments with periodic acid have been studied in various unsaturated systems when the carbon to carbon double bond is split, resulting in carbonyl compounds (Table I). Volatile carbonyl fragments are then isolated by steam distillation and subsequently identified as their 2,4-dinitrophenylhydrazones or dimethones. Both iodine and iodic acid are produced in this reaction.

The presence of a large amount of iodic acid in the reaction products indicates that the Malaprade reaction has been operative after the formation of α-glycol. The mechanism by which iodine is produced is not fully understood. It appears to come from the direct reduction of periodic acid, because no iodine is produced when iodic acid is refluxed with unsaturated compounds. Further work on this is in progress.

MECHANISM OF REACTION

When periodic acid is used in a large excess to oxidize double bonds, it is converted mainly to iodic acid (about 84%) and in a secondary measure to iodine (Table II). The yield of iodic acid has been found to increase with an increasing amount of periodic acid, but the yield of iodine increases with decreasing amounts of the per acid. Thus, it is not possible to account for the double bond reaction by the production of either iodic acid or iodine.

The oxidation of the double bond by periodic acid appears to proceed with the formation of an α-oxirane compound (*12*) (II) as observed in the oxidation of double bonds by perbenzoic and perphthalic acids. The epoxy compound thus formed (II) is transformed into α-glycol (*12*). Excess periodic acid present in the medium then initiates the Malaprade reaction producing carbonyl derivatives (IV). (See structural formula.)

The epoxide (II) may also be produced by the ozonization effect of periodic acid according to Smith and Duke (*11*):

$$2\,H{:}\ddot{O}{:}H \longrightarrow \left[\ddot{O}{:}H\right]^{+} + \left[{:}\ddot{O}{:}H\right]^{-} + 2H^{+} + 2e^{-}$$

$$H_5IO_6 + 2H^{+} + 2e^{-} \longrightarrow HIO_3 + 3H_2O$$

$$6(OH^{+}) \longrightarrow O_3 + 3H_2O$$

Table I. Periodic Acid Oxidation of Unsaturated Compounds

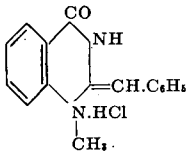
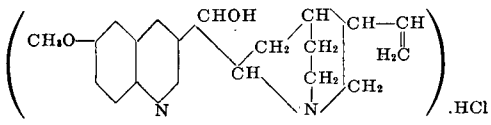
Compound	Oxidation Product	Yield, %	Identification
$C_6H_5CH{=}CH.CO_2H$ Cinnamic acid	C_6H_5CHO	98	2,4-Dinitrophenylhydrazone, m.p. 234° C.
 Glycosine hydrochloride (*1, 2*)	C_6H_5CHO	98	2,4-Dinitrophenylhydrazone, m.p. 234° C.
 Quinine hydrochloride	H—CHO	95	(*a*) Dimethone, m.p. 188–189° C. (*b*) 2,4-Dinitrophenylhydrazone, m.p. 155° C.
Cinchonine hydrochloride	H—CHO	95	Dimethone, m.p. 188–189° C.
Cinchonidine hydrochloride	H—CHO	95	Dimethone, m.p. 188–189° C.
Quinidine hydrochloride	H—CHO	95	Dimethone, m.p. 188–189° C.
Cupreine hydrochloride (demethylquinine hydrochloride)	H—CHO	95	Dimethone, m.p. 188–189° C.

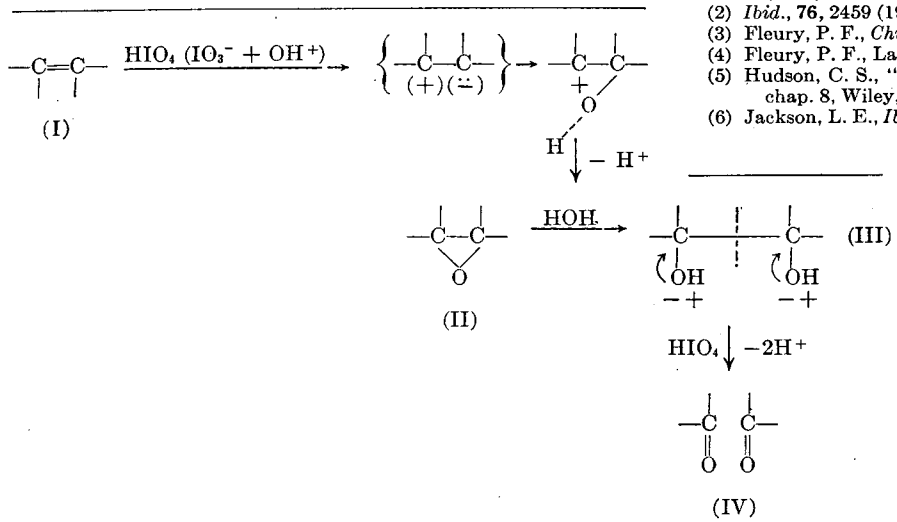
Table II. Oxidation Products of Periodic Acid

Quinine Hydrochloride, Gram	Periodic Acid Added, Grams	Iodic Acid Produced, Grams	Iodine Liberated, Gram	Unreacted Periodic Acid, Gram[a]
0.6112	3.9790	3.0756	0.3177	0.1137
0.4662	1.2750	0.4552	0.4998	...
0.4662	1.2750	0.4541	0.5189	...
0.4858	3.9790	2.9625	0.20	0.45
0.2665	2.3880	1.6220	0.1210	0.64

[a] Excess periodic acid was estimated in presence of iodic acid according to the method of Fleury and Lange (*4*).

EXPERIMENTAL

Oxidation of Cinnamic Acid. One gram of cinnamic acid was dissolved in 250 ml. of water, to which 6.0 grams of periodic acid was added. The reaction mixture was left at room temperature for 4 hours, then steam distilled. Benzaldehyde was produced in 98% yield, along with a copious amount of iodine. The benzaldehyde was extracted from the steam distillate with 100 ml. of ether, which was then washed with an aqueous solution of sodium sulfite and water to remove iodine. Benzaldehyde was obtained from the ether extract by concentrating the extract in an atmosphere of nitrogen. The benzaldehyde gave 1.899 grams (98% of theory, melting point 234° C.) of orange-red crystals of 2,4-dinitrophenylhydrazone when reacted with 2,4-dinitrophenylhydrazine.



Oxidation of Quinine. One gram of quinine hydrochloride was added to 6.09 grams of periodic acid in 250 ml. of water. The mixture was kept for 4 hours at room temperature, then steam distilled. The distillate containing formaldehyde was treated as above. The ether extract of the distillate was concentrated and treated with an alcoholic solution of dimedone until dimethone separated (yield, 0.76 gram). The dimethone crystallizes from alcohol in colorless needles (melting point 188–189° C.). Formaldehyde liberated from quinine was identified as its 2,4-dinitrophenylhydrazone (melting point 155° C.).

Oxidation of Other Unsaturated Compounds. When oxidized in a similar manner, the hydrochlorides of quinidine, cinchonine, cinchonidine, and cupreine produced formaldehyde in 94 to 95% yield. Glycosine hydrochloride, under the same experimental conditions, liberated benzaldehyde (*1, 2*).

DISCUSSION

The results indicate that periodic acid, like ozone, can be used for the detection and location of a carbon to carbon double bond (terminal as well as exocyclic). The reaction involves cleavage of the double bond, resulting in carbonyl derivatives. During this oxidation of the double bond, periodic acid is reduced, the end products always being iodine and iodic acid. Iodine does not originate from iodic acid, but from the direct reduction of periodic acid, the reaction mechanism of which is not yet clearly understood. The production of iodine and iodic acid is not quantitative to account for the double bond reaction. The oxidation reaction at the —C=C— grouping with periodic acid appears to proceed with the formation of an α-oxirane derivative. It is then converted into α-glycol, which undergoes the Malaprade reaction with an excess of periodic acid present in the medium. However, this per acid does not react with an aromatic unsaturation. Cleavage of the double bond with periodic acid proceeds smoothly with water-soluble substances, but may present some difficulties with water-insoluble compounds.

LITERATURE CITED

(1) Chatterjee, A., Majumdar, S. G., *J. Am. Chem. Soc.* **75**, 4365 (1953).
(2) *Ibid.*, **76**, 2459 (1954).
(3) Fleury, P. F., *Chim. anal.* **35**, 197 (1953).
(4) Fleury, P. F., Lange, J., *J. pharm. chim.*, [8] **17**, 107 (1953).
(5) Hudson, C. S., "Organic Reactions," Roger Adams, ed., vol. II, chap. 8, Wiley, New York, 1947.
(6) Jackson, L. E., *Ibid.*, p. 341.
(7) Lange, J., "Action de l'Acide Periodique sur les Polyalcools," Les Editions Vega, 43 Rue Madame, Paris, 1933.
(8) Malaprade, L., *Bull. soc. chim.* [4] **43**, 683 (1928).
(9) Malaprade, L., *Compt. rend.* **186**, 382 (1928).
(10) Smith, G. F., "Analytical Applications of Periodic Acid and Iodic Acid and Their Salts," 5th ed., G. Frederick Smith Chemical Co., Columbus, Ohio.
(11) Smith, G. F., Duke, F. R., IND. ENG. CHEM., ANAL. ED. **15**, 120 (1943).
(12) Swern, D., *Chem. Revs.* **45**, 26 (1949).

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Recording Nickel Carbonyl Detector

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To meet the need for detecting nickel carbonyl in the atmosphere of a pilot plant area of the Hercules Experiment Station, a very sensitive, continuously recording nickel carbonyl detector was developed. When air, contaminated with nickel carbonyl, impinges upon a hot borosilicate glass plate, solid nickel compounds are deposited on the surface. A novel optical arrangement using polarized light incident at the Brewsterian angle for borosilicate glass measures the quantity of deposit. Concentrations in the range of 0.05 to 4 p.p.m. by volume can be measured. At a concentration of 1 p.p.m. the accuracy is ±0.2 p.p.m. or ±20% of the amount present. The detector is also sensitive to iron carbonyl and can presumably be used for other metallo-organic vapors and gases, such as tetraethyllead.

IN OXO and carbonylation reactions using nickel as a catalyst, nickel carbonyl [$Ni(CO)_4$] is generated in the system. Because of the extreme toxicity of this compound, its presence in plant atmospheres constitutes a potential health hazard to personnel. In order to detect such hazardous conditions, an instrument is needed to continuously record nickel carbonyl concentration in the atmosphere.

The threshold limit for nickel carbonyl given by the American Conference of Governmental Industrial Hygienists (*2*) at the time this work was being done was 1 p.p.m.; this value was superseded in 1954 by a recommended limit of 0.001 p.p.m. (*1*). A very sensitive instrument is required to measure such small concentrations with any degree of accuracy. Chemical methods of analysis are sufficiently sensitive, but they are time-consuming and give only spot checks of concentration.

With the hope of finding an adaptable commercial instrument, several methods of instrumentation, including infrared spectroscopy, spark spectroscopy, and flame photometry, were evaluated for nickel carbonyl detection. To evaluate the infrared spectroscopy method, a commercial infrared gas analyzer was tested. Its limit of detection was approximately 4 p.p.m. by volume. The limit of detection of a laboratory-type spark spectrograph was established as 0.1 p.p.m., but to obtain this sensitivity, a 1-hour exposure of the photographic plate was required. A flame photometer equipped with a multiplier phototube as a radiation detector also had a detection limit of 0.1 p.p.m. The flame photometer, however, has two distinct disadvantages as a plant instrument. The rate of oxygen consumption (8 cubic feet per hour) makes it expensive to operate continuously, and the open flame makes it dangerous to operate in many plants. It was felt that none of the instruments tested would operate satisfactorily without extensive modification.

A review of some of the basic properties of nickel carbonyl led to the development of a sensitive, stable instrument based on the following three basic principles.

1. When a stream of air containing nickel carbonyl impinges on a hot surface, the nickel carbonyl decomposes, yielding metallic nickel and carbon monoxide. The nickel then reacts with components of the atmosphere to give compounds of nickel which deposit on the hot surface. In the absence of carbon monoxide, the rate of decomposition is proportional to the concentration of nickel carbonyl in the air stream (*3*).

2. For small quantities of deposit, the reflectance of light from the deposit is a measure of the amount of material deposited. Although the reflectance is not a linear function of the quantity of deposit, it varies directly with the quantity of deposit as long as the deposit remains thin.

3. When a collimated beam of light is incident upon the surface of a dielectric at an angle whose tangent is equal to the refractive index of the dielectric (Brewster's angle), the light is completely polarized by reflection, but at any angle of incidence other than Brewster's angle, the reflected light is not completely polarized.

These three principles are employed in the following manner in the instrument.

A stream of air from the plant atmosphere flows through a nozzle and impinges upon a hot borosilicate glass disk. If nickel carbonyl is present in the stream, a deposit forms on the disk directly below the nozzle. A collimated beam of plane-polarized light is incident upon the disk at the point where the deposit, if any, is formed. The angle of incidence of this beam is the Brewsterian angle for borosilicate glass, and its plane of polarization is perpendicular to the plane of incidence. This arrangement, which corresponds to the crossing of Nicol prisms, results in extinction, so that no light is reflected from the disk. The refractive index μ, and, therefore, the Brewsterian angle (arc tan μ), for the deposit is different from that for glass. Thus, the condition of extinction obtains only for the glass, and any light reflected from the surface is due entirely to the deposit.

The intensity of the reflected light is measured by a recording photomultiplier photometer which is calibrated in parts per million of nickel carbonyl.

DESCRIPTION OF INSTRUMENT

The instrument consists of three principal parts: a mechanical unit, an optical system, and a detector. A photograph of the instrument is shown in Figure 1. Because the instrument described here is an experimental model, no attempt was made to refine it. Wherever possible, components common to most optical laboratories were used.

Mechanical Unit. In the mechanical unit (Figure 2) a borosilicate glass disk, 3.75 inches in diameter and 0.2 inch thick, is sandwiched between two 200-watt Chromolox ring-type electrical heaters which maintain the temperature of the disk at 350° C. These heaters are enclosed in stainless steel housings and are connected to the line through a Variac autotransformer. The lower heater housing is connected through a Transite insulator to a shaft which is geared to a clock mechanism. The clock rotates the disk one complete revolution in 24 hours.

The components of the mechanical unit are mounted in a water-cooled aluminum housing. The upper compartment of the housing, which encloses the disk, is both light-tight and air-tight. Also enclosed in this compartment is a glass nozzle from which the sample stream flows and impinges on the periphery of the disk protruding from between the heater housings.

The pressure within the compartment is maintained below atmospheric by means of a water aspirator. The difference in pressure between the compartment and the atmosphere causes the sample stream to flow through the nozzle. The sample flow rate is about 500 cc. per minute.

If the sample stream contains any nickel carbonyl, it decomposes upon striking the surface of the hot disk and leaves a solid deposit of nickel compounds on the surface of the disk directly below the nozzle. Because the disk is constantly rotating, the quantity of deposit at any point on the periphery of the disk is a measure of the concentration of nickel carbonyl in the sample stream at the time that point was directly below the nozzle.

The disks can be cleaned for re-use by washing them with warm 6*N* hydrochloric acid.

Optical System. The physical arrangement of optical com-

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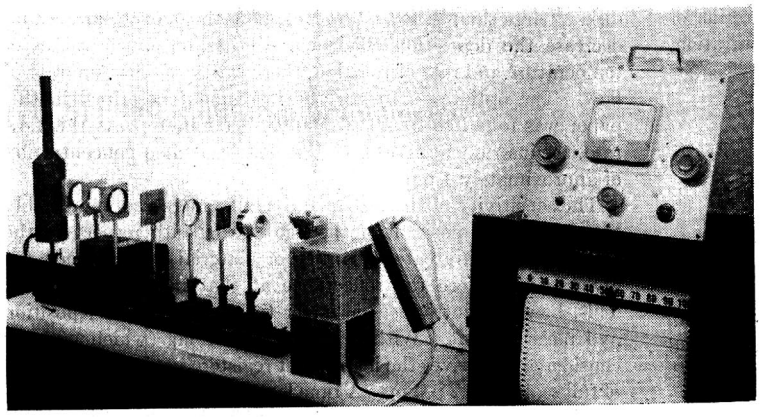

Figure 1. Recording nickel carbonyl detector

ponents is shown in Figure 3. The source of illumination is a General Electric lamp, Type 18A-T10P, operating on 6 volts at 18 amperes, supplied by a Sola constant voltage transformer. The light from the lamp is collimated by the first lens, passes through a limiting aperture, and is brought to a focus by the second lens. A second aperture, slightly smaller in diameter than the lamp filament, is located at the focal point of the second lens. This aperture is adjusted so that only the light from the center of the filament passes through it. The light from this aperture is recollimated by a third lens and passes through a wide band filter having its maximum transmittance at 5200 A. From the filter, the light passes through an adjustable slit and a Polaroid disk and emerges as a very narrow collimated beam of plane-polarized, green light.

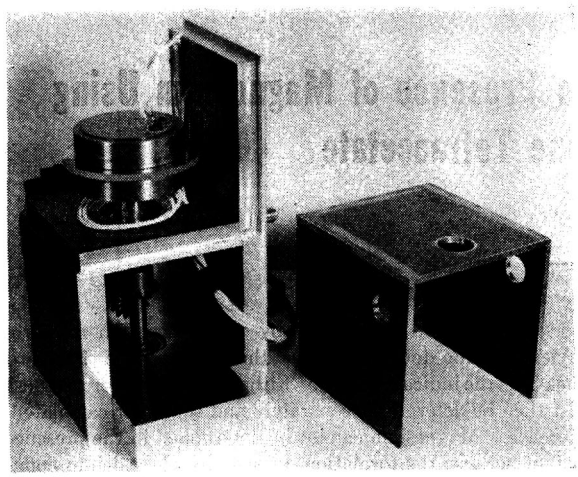

Figure 2. Mechanical unit

This beam of light is incident upon a first surface mirror, is reflected through a port in the mechanical unit, and falls upon the periphery of the disk. The mirror is adjustable for both rotation and translation, so that it is possible to direct the beam to a given point on the disk at a given angle of incidence. The mirror is adjusted so that the light strikes the disk directly below the nozzle at an angle of incidence equal to the Brewsterian angle for borosilicate glass. The Polaroid polarizer in the optical system is set with its polarizing plane perpendicular to that of the disk so that no light is reflected from the upper surface of the disk if it is clean. The lower surface of the disk is frosted so that the light which is transmitted by the glass is scattered and the amount reflected in any particular direction is negligible. Light will, however, be reflected by the deposit, since its Brewsterian angle is different from that of borosilicate glass. The light reflected by the deposit is measured by the detector unit.

Detector. The detector unit consists of a Photovolt line-operated photomultiplier photometer Model 520-M, operating into a Brown Electronik strip chart recorder.

The light which is reflected from the deposit falls upon the cathode of the photomultiplier, the output of which is measured by a vacuum tube voltmeter-recorder combination. The intensity of this reflected light is a function of the quantity of deposit on the disk and is, thus, also a function of the concentration of nickel carbonyl in the sample stream.

The recorder is calibrated in parts per million of nickel carbonyl and gives a continuous record of the concentration of nickel carbonyl in the atmosphere. A microswitch can be mounted on the recorder chassis in such a position that it is actuated at any predetermined concentration of nickel carbonyl. It is thus very easy to connect an alarm bell which can be made to ring at a predetermined set point.

CALIBRATION

Air containing a known concentration of nickel carbonyl for calibrating the instrument is obtained from a multiple dilution system.

Carbon monoxide from a cylinder is bubbled through a test tube of liquid nickel carbonyl which is maintained at 0° C. by an ice bath. In bubbling through the liquid, the carbon monoxide becomes saturated with nickel carbonyl vapor, so that the concentration, X, of nickel carbonyl in the resulting stream is given by

$$X = \frac{p}{P} \times 10^6 \text{ p.p.m.}$$

where p is the vapor pressure of nickel carbonyl at 0° C. and P is the total pressure of the system. Just after the saturator there is a relief valve, which consists of a glass tube inserted in a beaker of water. This relief valve keeps the system at atmospheric pressure, so that P may be determined by means of a barometer.

The stream of saturated carbon monoxide is passed through a size 08 Fischer-Porter Flowrator with a range of 0 to 58 cc. per minute, and is introduced into a stream of pure carbon monoxide from a size 01 Flowrator. The flow rate of this stream of pure carbon monoxide is held constant at 10,000 cc. per minute by means of a Moore flow controller, Model No. 63SU.

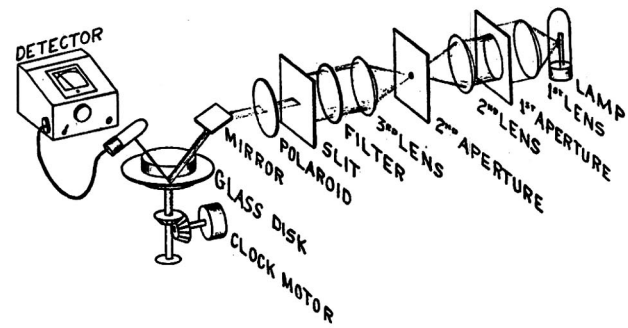

Figure 3. Optical system

Most of the resulting stream is exhausted into a fume hood, but a small part of it is passed through a second size 08 Flowrator and is mixed with a stream of air from a size 2L Flowrator. The air stream is held constant at 25,000 cc. per minute by a second Moore flow controller. Most of the resulting stream is again exhausted to the hood; only 500 cc. per minute is fed to the instrument nozzle as calibrating gas.

The flow rates through the two size 08 Flowrators are manually controlled by means of glass stopcocks. These flow rates de-

termine the two dilution ratios and, thus, the concentration of nickel carbonyl in the calibrating gas. The final concentration, X, is given by

$$X = \frac{p}{P} \times \frac{F_1F_3}{F_2F_4} \times 10^6 \text{ p.p.m.}$$

where p = vapor pressure of nickel carbonyl at 0° C.
P = atmospheric pressure
F_1 = flow rate through first 08 Flowrator
F_2 = flow rate through 01 Flowrator
F_3 = flow rate through second 08 Flowrator
F_4 = flow rate through 2L Flowrator

The vapor pressure, p, of nickel carbonyl at 0° C. is 134 mm., and F_2 and F_4 are held constant at 10,000 cc. per minute and 25,000 cc. per minute, respectively. If atmospheric pressure, P, is taken as 760 mm., the equation reduces to

$$X = 0.000705\ F_1F_3 \text{ p.p.m.}$$

Carbon monoxide is used in the bubbler and for the first dilution to prevent premature decomposition of the nickel carbonyl. Air is used for the second dilution to promote oxidation of the deposit on the hot disk.

No evidence of decomposition of nickel carbonyl in any part of the system except on the hot disk was found.

DISCUSSION

From the basic principles of operation, it is seen that the minimum concentration of nickel carbonyl that the instrument can detect depends upon the quantity of deposit that is allowed to build up on the surface of the disk. This, in turn, depends upon the rate of sample flow and upon the rate of rotation of the disk. Large sample flows, however, tend to cool the surface and decrease the deposition efficiency, so that for given values of temperature and rate of rotation, there exists an optimum rate of flow. The optimum rate for the instrument described in this paper was found to be about 500 cc. per minute. At this flow rate, the instrument gives full-scale deflection at a concentration of approximately 4 p.p.m.

The sensitivity of the instrument can be increased appreciably by altering the position of the light beam with respect to the nozzle so that a greater quantity of material is deposited at a given point before the reflectance at that point is measured. This, however, introduces a longer time lag between deposition and measurement. With a 5-minute time lag (which was not considered excessive) the instrument registered a deflection of 1% of full scale for 0.2 p.p.m. A time lag of 10 minutes results in 1% of full-scale deflection for 0.05 p.p.m. The instrument is capable of even greater sensitivity because, with the particular nozzle which was used, the time of deposition at any point on the periphery of the disk is approximately 40 minutes.

Although the instrument described in this paper was designed for the detection of nickel carbonyl, it is also sensitive to iron carbonyl and should be easily adapted to the detection of tetraethyllead and other metallo-organic gases and vapors.

LITERATURE CITED

(1) *Arch. Ind. Hyg. and Occupational Med.* **9**, 531 (1954).
(2) *Chem. Processing* (American Conference of Governmental Industrial Hygienists) **15**, 134 (1952).
(3) Garratt, A. P., Thompson, H. W., *J. Chem. Soc.* (*London*) **1934**, 1824.

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Indicator for Titration of Calcium in Presence of Magnesium Using Disodium Dihydrogen Ethylenediamine Tetraacetate

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A new indicator, designated calcein, has been prepared for the titration of calcium in the presence of magnesium with disodium dihydrogen ethylenediamine tetraacetate. No preliminary treatment is necessary beyond dissolving the sample and adjusting the pH to a value of 12. Excessively large amounts of sodium and magnesium cause the results for calcium to be slightly low. Interference by copper and iron is obviated by the addition of cyanide.

A NEW indicator for the titration of calcium with disodium dihydrogen ethylenediamine tetraacetate [the disodium salt of (ethylenedinitrilo)tetraacetic acid] in the presence of magnesium has been prepared by condensing iminodiacetic acid with fluorescein. This is a procedure analogous to that employed by Schwarzenbach and others for the preparation of the so-called metal phthaleins (*4*). In highly alkaline solution the indicator is brown and its calcium complex is a yellow-green. At lower pH values the free indicator is also yellow-green. Magnesium does not form a complex with the indicator. The indicator may be used for the determination of calcium in water, limestone, or other calcium compounds. It has been given the trivial name calcein.

In the analysis of limestone or water, the total calcium and magnesium can be determined by using disodium dihydrogen ethylenediamine tetraacetate as the titrant with Eriochrome Black T as the indicator (*1–3*). Either the calcium or magnesium must then be determined separately and the other calculated by difference. Magnesium cannot be determined in the presence of calcium because the formation constant of the calcium complex of ethylenediamine tetraacetate is two orders of magnitude greater than that of the magnesium complex. To determine calcium directly using disodium dihydrogen ethylenediamine tetraacetate as the titrant, the pH is made sufficiently high so that the magnesium is largely precipitated as the hydroxide and an indicator is used which combines with calcium only. Murexide is such an indicator (*5*, *6*), but the end point with it is rather indefinite and is made worse by increasing amounts of magnesium.

A sharper end point is obtained with calcein than with murexide, and larger quantities of magnesium may be present without impairing the end point. The magnesium may exceed the calcium by a factor of 20 to 30 without interference. Large amounts of sodium salts—2 to 3 grams of sodium chloride, for example—do not affect the titration. Strontium and barium interfere and are titrated along with calcium; the end point with either alone is the same as that with calcium. Copper and iron interfere with the end point, but such interference is easily obviated by the addition of cyanide. The titration of calcium may be performed in the presence of chloride, nitrate, acetate, and sulfate.

Below pH 12, both the indicator and its calcium complex have a yellow-green color. Above pH 12 the indicator is brown, but the calcium complex has the same yellow-green color. The titration is carried out at a pH above 12 so that the end point is marked by a change from yellow-green to brown. The indicator can be added either as the solid (one part of indicator mixed with 100 parts of potassium chloride) or as a 2% solution in dilute sodium hydroxide. A better end point is obtained if the solid indicator contains some charcoal (1 part of indicator, 10 parts of charcoal, and 100 parts of potassium chloride). The calcium complex appears much greener when this is done and, when large amounts of iron are present, the end point is much better. When the indicator is used alone or in conjunction with charcoal, it is completely reversible. The end point is better in diffuse light than in illumination of high intensity.

Table I. Analyses on Calcein

Preparation[a]	Neutralization Equivalent, NaOH in Water	% Nitrogen, Kjeldahl	Neutralization Equivalent, Bromination	Neutralization Equivalent, $HClO_4$ in Acetic Acid
1	172	3.85		
2	161	3.48	116	
3	253	4.34	102	
4	190	4.03		
5	176	3.69		
6	203	3.58		
7	187	4.16	88	194

[a] Various preparations obtained from numerous attempts at purification.

Owing to the high pH at which the titration is performed, some calcium is precipitated as the hydroxide at the beginning. Vigorous stirring is necessary to dissolve the hydroxide as the titration progresses. If the stirring is slow, false end points are obtained, the color returning after each change as the stirring is continued.

PREPARATION OF INDICATOR

Mix 100 grams (0.3 mole) of fluorescein, 300 ml. of ethyl alcohol, 150 ml. of distilled water, and 90 ml. of 30% sodium hydroxide. Add with stirring 87 grams (0.66 mole) of iminodiacetic acid, dissolved in 105 ml. of 30% sodium hydroxide plus 120 ml. of distilled water. Cool the mixture to 10° C. in an ice bath. Add dropwise 74 ml. (0.75 mole) of 37% formaldehyde, stirring vigorously. After all of the formaldehyde has been added, heat the mixture to 60° to 70° C. for 6 to 7 hours, stirring continuously. Allow the solution to cool, then dilute to 3 liters. Add 1 to 1 hydrochloric acid, precipitating the indicator as the free acid. Filter and wash with distilled water. Redissolve the material in 3 liters of water containing 120 grams of sodium acetate. Precipitate it again with hydrochloric acid, filter, and wash. Transfer the material into 2 liters of ethyl alcohol, stir for 1 hour, and filter. Repeat the ethyl alcohol washing, then dry the material in a vacuum. The product is bright yellow. When heated, it begins to decompose slowly at about 185° C. It is apparently a mixture, with a compound predominating which contains two iminodiacetic acid residues, because analyses performed on materials obtained from various purification processes gave variable results (Table I). The titration with sodium hydroxide gave a titration curve having a single sharp break with the end point around a pH of 7.5. Attempts to determine the molecular weight were unsuccessful.

Though admittedly not a pure product, the material so prepared functions well as an indicator. It is also available from the G. Frederick Smith Chemical Co., Columbus, Ohio.

DETERMINATION OF CALCIUM

Preparation of Indicator. SOLUTION. Dissolve 2 grams of the indicator in 25 ml. of 1*N* sodium hydroxide and dilute to 100 ml. with distilled water. Use 1 to 2 drops of this solution.

SOLID. Grind thoroughly 1 gram of the indicator with 100 grams of potassium chloride.

SOLID WITH CHARCOAL. Grind together 1 gram of the indicator, 10 grams of charcoal (Norite A is satisfactory), and 100 grams of potassium chloride.

It is convenient to measure out either of the solid indicators with a small metal scoop that holds about 0.07 gram of the mixture.

Calcium in Water. Pipet a 50-ml. sample into a conical flask. Add 1 or 2 drops of 2% indicator solution (or a scoop of a 1% solid mixture, about 0.07 gram) and 5 ml. of 1*M* sodium hydroxide containing 1 gram of sodium cyanide per 100 ml. Titrate with 0.02*N* disodium dihydrogen ethylenediamine tetraacetate until the color changes from yellow-green to brown. Vigorous stirring is necessary throughout the titration. Do not carry out the titration under a fluorescent lamp or in light of high intensity.

Standardize the disodium dihydrogen ethylenediamine tetraacetate solution against Iceland spar or a primary standard grade of calcium carbonate.

Table II. Analysis of Limestone and Gypsum

(Disodium dihydrogen ethylenediamine tetraacetate standardized against Iceland spar[a])

	NBS		Standard Sample Co.		
	1a	88	1099	1100	Selenite[b]
CaO reported, %	41.32	30.49	54.28	30.49	32.57[c]
MgO reported, %	2.19	21.48	0.85	21.83	[b]
CaO found, %	41.31	30.48	54.33	30.34	32.53
	41.37	30.37	54.17	30.48	32.46
	41.25	30.41	54.32	30.52	32.51
	41.26	30.45	54.30	30.56	32.54
	41.22	30.44	54.23	30.42	32.64
	41.36	30.56	54.18	30.38	32.50
	41.21	30.44	54.26	30.44	32.47
	41.20	30.50	54.20	30.51	32.54
	41.18				32.52
					32.60
					32.54
					32.46
Av.	41.26	30.46	54.25	30.45	32.52
Range	0.19	0.19	0.16	0.22	0.18
Av. dev.	0.056	0.044	0.054	0.061	0.039
Std. dev.	0.070	0.059	0.065	0.075	0.054

[a] Transparent crystals; magnesium content determined spectrographically to be 40 p.p.m.
[b] Transparent crystals of gypsum (selenite variety from Freedom, Okla.); magnesium content determined spectrographically to be 20 p.p.m.
[c] Theoretical CaO content for $CaSO_4 \cdot 2H_2O$.

Limestone. Weigh a sample of about 0.3 gram into a 400-ml. beaker. Add 20 ml. of 1 to 1 hydrochloric acid and evaporate to dryness. Redissolve the sample in 5 ml. of 1 to 10 hydrochloric acid and then dilute to 100 to 200 ml. with distilled water. To this add 1 to 2 drops of the indicator solution or a scoop of the solid indicator mixture and about 5 ml. of 10*M* sodium hydroxide containing 5 grams of sodium cyanide per 100 ml. Titrate with 0.1*N* disodium dihydrogen ethylenediamine tetraacetate under the same conditions as for calcium in water.

Table III. Titration of Calcium in Presence of Large Amounts of Magnesium and Sodium Salts

Calcium, Gram		Magnesium, Grams	Sodium Chloride, Grams	Calcium Recovered, %
Taken	Found			
0.1188	0.1188	..	1.0	100.00
0.1188	0.1191	..	3.6	100.29
0.1188	0.1178	..	3.0	99.18
0.1188	0.1190	8.4[a]	..	100.19
0.1188	0.1173	10.1[a]	..	98.74
0.0833	0.0827	2.2[b]	..	99.32
0.1175	0.1162	2.0[b]	2.0	98.91
0.0595	0.0585	10.9[a]	3.0	98.38
0.0595	0.0589	1.9[a]	..	98.99

[a] $MgCl_2 \cdot 6H_2O$.
[b] $Mg(C_2H_3O_2)_2 \cdot 4H_2O$.

RESULTS

Two limestone samples from the Bureau of Standards, two samples of limestone from the Standard Sample Co., Ames, Iowa, and a sample of selenite (transparent variety of gypsum),

obtained from the G. Frederick Smith Chemical Co., were analyzed for calcium. The limestones were analyzed by the procedure given above. The selenite was dissolved in an excess of standard disodium dihydrogen ethylenediamine tetraacetate at a pH of 12, the stirring being continued for 4 to 6 hours. Excess standard calcium chloride was then added and the solution titrated with disodium dihydrogen ethylenediamine tetraacetate.

The results reported were obtained using both the indicator solution and the solid indicator with and without charcoal. The results on the samples from the Bureau of Standards were corrected for the strontium present (0.05% in sample 1a and 0.01% in sample 88). The results are given in Table II.

Titrations of known amounts of calcium in the presence of magnesium and sodium chloride were carried out. The results of these titrations are given in Table III. No interference with the end point could be detected.

LITERATURE CITED

(1) Biedermann, W., Schwarzenbach, G., *Chimia.* (*Switz.*) **2**, 56 (1948).
(2) Diehl, H., Goetz, C. A., Hach, C. C., *J. Am. Water Works Assoc.* **42**, 40 (1950).
(3) Schwarzenbach, G., Ackermann, H., *Helv. Chim. Acta* **30**, 1798 (1947).
(4) Schwarzenbach, G., Anderegg, G., Flaschka, H., Salliman, R., *Ibid.*, **37**, 113 (1954).
(5) Schwarzenbach, G., Biedermann, W., Bangerter, F., *Ibid.*, **29**, 811 (1946).
(6) Schwarzenbach, G., Gysling, H., *Ibid.*, **32**, 1314 (1949).

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Fluorometric Micromethod for Determination of Tryptophan

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The measurement of fluorescence intensity of substances formed by the reaction of glucose and tryptophan is suggested as a method for the quantitative determination of microquantities of tryptophan. Tryptophan is separated from the other amino acids on a resin column of Dowex-50 (sodium form), and is made to react with glucose under optimum standard conditions. These conditions include the heating of 20 γ or less of tryptophan with 0.8 gram of glucose at pH 1.38 and a temperature of 118° C. for 4 hours. There is a linear relationship between tryptophan concentration and fluorescence intensity which is read at pH 1.80. The standard error is ±3% for 12 γ of tryptophan.

THE determination of tryptophan, particularly in materials containing carbohydrates, requires special analytical techniques as well as hydrolysis in an alkaline rather than in an acid medium. Both chemical and biological methods may be used for the analysis of tryptophan in pure protein.

The many available chemical methods for the determination of tryptophan involve reaction with oxidizing agents, condensation with aldehydes, or diazotization reactions. These methods have the common limitation that they are not sensitive enough to determine microquantities of tryptophan which occur in some biological materials. The ninhydrin reaction (*10*) which has been used for assay of many amino acids is not sufficiently sensitive to give satisfactory results for assay of tryptophan in the low concentrations present in flour.

Portner and Högl (*12*) reviewed the chemical methods used in the determination of tryptophan and concluded that the most advantageous method is that of Spies and Chambers (*14–17*). This method involves condensation of tryptophan and *p*-dimethylaminobenzaldehyde followed by oxidation with nitrous acid. While the Spies and Chambers method is satisfactory for tryptophan assay of proteins, it is not adaptable to the assay of flour and similar materials.

The limitations of available methods for the analysis of tryptophan in concentrations that occur in foods suggested that a new approach to the problem might be profitable. Tryptophan is known to react with reducing carbohydrates (*7*) to produce fluorescent material (*2, 4, 5*). Although most of the amino acids react with glucose to form fluorescent compounds, the fluorescence intensity of compounds resulting from the reaction of glucose and tryptophan is much greater than that for the other amino acids (*5*). This reaction, therefore, appeared to have several advantages over other methods. The object of the present study was to define the conditions affecting the development of fluorescent compounds resulting from the reaction of tryptophan with glucose and to apply the reaction to the quantitative estimation of tryptophan. The application of this method to the determination of tryptophan in biological materials will be published later.

APPARATUS AND MATERIALS

A Coleman electronic photofluorometer, Model 12C, was used for the fluorometric analyses. It was equipped with a No. 5874 Corning filter which transmits the 365-mμ mercury line, and another filter consisting of two Corning filters (Nos. 3398 and 4308) which absorbs below 425 mμ. A reference standard consisting of a solution of 0.1 γ per milliliter of sodium fluorescein in water was used to adjust the instrument to a given sensitivity. All values were calculated on the basis of a fixed value of 40 for sensitivity. Dilutions of the reaction mixtures used to establish the standard curve were made with the buffer in order to adjust them to the reading range of the photofluorometer and to adjust the pH to permit reading at maximum fluorescence. Commercially available chromatographic columns (10 × 300 mm.) were fitted into condenser jackets and used to remove tryptophan from other amino acids according to the procedure of Moore and Stein (*11*). One-ounce prescription bottles with Teflon inserts in the caps were used as containers for the solutions containing tryptophan and glucose, which were heated in the autoclave to develop fluorescent compounds.

A Beckman Model GS pH meter was used to measure the pH of all solutions. A saturated solution of potassium acid tartrate (*6*) was used as a standard buffer (pH 3.57). A Coleman standard buffer (pH 2.0) also was used.

Tryptophan. Analytically pure L-tryptophan was obtained from Nutritional Biochemicals Corp., Cleveland, Ohio. A sample of DL-tryptophan obtained from the Dow Chemical Co., Midland, Mich., was recrystallized twice from water and ethyl alcohol followed by a rinse with anhydrous ethyl ether. The purified DL-tryptophan was used as a standard.

Sources of Other Amino Acids. All of the amino acids other than tryptophan normally found in food products were obtained from Nutritional Biochemicals Corp. and were used without additional purification.

Dextrose. Anhydrous dextrose was obtained from Merck & Co., Inc., Rahway, N. J., and the Pfanstiehl Chemical Co., Waukegan, Ill.

Buffer Solutions. Sodium citrate buffer, pH 5.0, was prepared as a 0.2*M* stock solution by mixing 21.008 grams of reagent grade citric acid monohydrate with 200 ml. of 1.0*N* sodium hydroxide and diluting to 500 ml. This stock buffer was diluted 1 to 1 and to each liter 1.0 gram of disodium Versenate and 15 ml. of benzyl alcohol were added (*11*).

Phosphate buffer, pH 6.8, was prepared by mixing 500 ml. of 0.1*M* reagent grade disodium phosphate, 450 ml. of 0.1*M* reagent grade monosodium phosphate monohydrate, 1.0 gram of disodium Versenate, and 15 ml. of benzyl alcohol (*11*).

Sodium citrate–hydrochloric acid buffer, pH 1.8, was prepared by mixing 20 parts of 1.00*M* sodium citrate and 60 parts of 1.00*N* hydrochloric acid.

Sodium citrate–hydrochloric acid buffer, pH, 1.38, was prepared by mixing 20 parts of 1.00*M* sodium citrate and 62 parts of 1.00*N* hydrochloric acid.

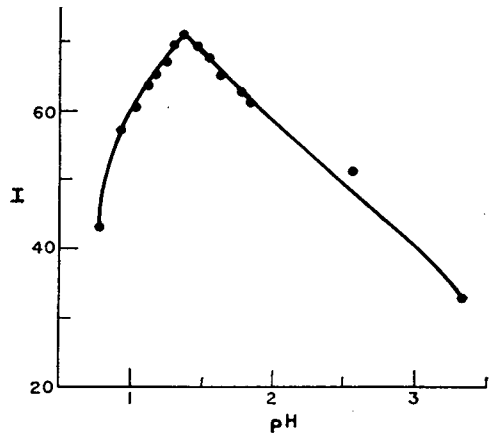

Figure 1. Fluorescence intensity of compounds formed by heating tryptophan and glucose at various pH values

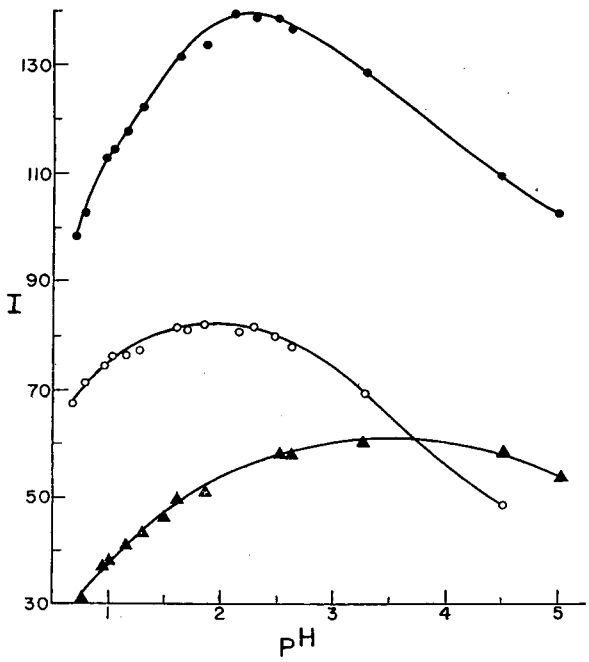

Figure 2. Influence of hydrogen ion concentration on fluorescence intensity of compounds formed by heating glucose and tryptophan

▲ Glucose, pH 1.38
● Glucose plus tryptophan, pH 1.38
○ Difference between glucose and glucose plus tryptophan curves

The various buffers were prepared in quantity, adjusted to the proper pH, if necessary, with hydrochloric acid or sodium hydroxide, and stored with a few crystals of thymol in the refrigerator.

RECOMMENDED PROCEDURES

The following recommended procedures were based on the results which were obtained from the data discussed below.

Separating Tryptophan from Other Amino Acids. The ion exchange procedure of Moore and Stein (*11*), employing 15-cm. chromatographic columns and a temperature of 25° C., was used to separate tryptophan from the other interfering amino acids. No polyoxyethylene lauryl alcohol (*11*) was used in the buffers because this material contributed to the fluorescence intensity. A 2-ml. aliquot of hydrolyzate (pH 4.0) containing a maximum of 6 mg. of amino acids and 2 ml. of stock sodium citrate buffer was added to the column. The amino acids were eluted with 45 to 50 ml. of citrate buffer and were discarded; the tryptophan was eluted with the first 40 to 50 ml. of phosphate buffer. This solution containing the tryptophan was adjusted to pH 1.38 and a volume of 50 ml.

Reaction of Tryptophan with Glucose. Triplicate aliquots of the phosphate buffer solution, containing a maximum of 20 γ of tryptophan, were mixed with 5 ml. of the sodium citrate–hydrochloric acid buffer (pH 1.38) in a 1-ounce prescription bottle. The citrate buffer contained 0.8 gram of glucose. If less than 5 ml. of the tryptophan solution was required, the difference, up to 5 ml. of phosphate buffer (adjusted to pH 1.38), was also added to give a maximum volume of 10 ml. Triplicate blanks were provided. These solutions were heated in a preheated autoclave for 4 hours at 118° C., cooled, and stored until read.

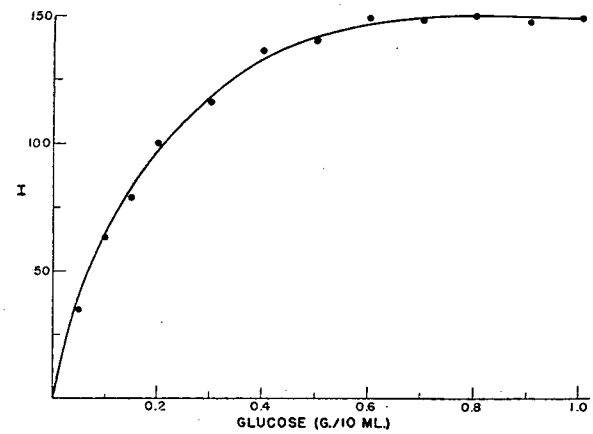

Figure 3. Influence of glucose concentration on fluorescence intensity of compounds formed by heating glucose and tryptophan at pH 1.38

Measurement of Fluorescence. Two-milliliter portions of the fluorescent solution were diluted to 25 ml. with sodium citrate–hydrochloric acid buffer (pH 1.8) and readings were taken in the photofluorometer. A solution of sodium fluorescein (0.1 p.p.m.) was used as a stable reference standard.

RESULTS AND DISCUSSION

The well-known Maillard or "browning" reaction was adapted to the quantitative estimation of tryptophan by making a critical evaluation of the effects of pH, temperature, time, sugar, and tryptophan concentrations. The variation in the reaction of the biologically active L-form and the DL-form of tryptophan, the purity of the tryptophan sample used for standard, the effect of grades of dextrose, and the interference of the other amino acids normally found in foods were investigated.

Influence of Hydrogen Ion Concentration on Development of Fluorescence. Mohammad and others (*9*) studied the reaction between glucose and proteins and found that the rate of browning was a function of pH. In the present study, the effect of pH was investigated by heating a solution composed of 20 γ of tryptophan in 5 ml. of phosphate buffer and 200 mg. of glucose in 5 ml. of sodium citrate–hydrochloric acid buffer. Various com-

binations of 1*M* sodium citrate and 1*N* hydrochloric acid were mixed to obtain pH values ranging from 0.78 to 3.33. Aliquots of the phosphate buffer were adjusted to similar values by adding concentrated hydrochloric acid.

To develop fluorescence, the solutions were placed in a preheated autoclave and heated at 118° C. for 4 hours. Simultaneously, solutions of glucose were heated at similar pH values to provide blank determinations. The data which indicate the development of maximum fluorescence intensity at a pH of 1.30 to 1.45 are shown in Figure 1. The ordinate represented by I indicates fluorescence intensity. Based on these data, a reaction pH of 1.38 was chosen for study of other factors that influenced the development of fluorescence in the glucose-tryptophan reaction. Experiments established that the presence of citrate buffer salts did not affect the development of fluorescence. Phosphate salts, however, did affect the fluorescence intensity. Therefore, the concentration of phosphate salts was maintained at a constant level.

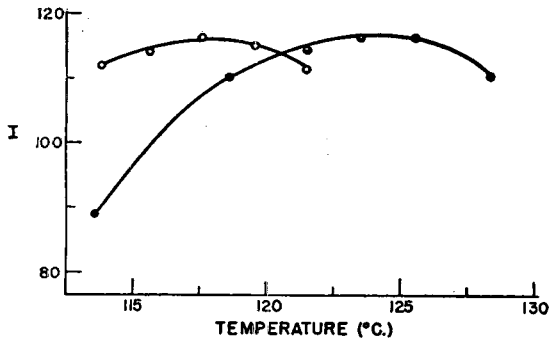

Figure 4. Influence of time and temperature on fluorescence intensity of compounds formed by heating glucose and tryptophan at pH 1.38

● 2.5 hours
○ 5 hours

Influence of Hydrogen Ion Concentration on Fluorescence Intensity. The hydrogen ion concentration exerts a marked influence on the fluorescence intensity of a solution. The effects of this variable on the fluorescence of heated glucose blank solutions and on heated tryptophan-glucose solutions are shown in Figure 2. The maxima of these two curves are at widely different pH values. This might be expected, because the compounds resulting from the degradation of glucose are probably different from those resulting from the reaction of tryptophan and glucose. The result was a maximum value at pH 1.30 to 1.45 for the difference in fluorescence intensity of heated blank and glucose-tryptophan solutions. This is also shown in Figure 2. The fluorescent material was irreversibly quenched above pH 7.0. Concomitant with the disappearance of the fluorescence, a brown color developed.

Influence of Glucose Concentration on Fluorescence Intensity. The relation between glucose concentration and the fluorescence intensity developed when 20 γ of tryptophan in 5 ml. of phosphate buffer (pH 1.38) is heated with 0 to 1.6 grams of glucose in 5 ml. of sodium citrate–hydrochloric acid buffer (pH 1.38) is shown in Figure 3. These results show that a minimum of 0.8 gram of glucose is required for 20 γ of tryptophan. This quantity of glucose was used as a standard amount for reaction with as much as 20 γ of tryptophan. Larger quantities of tryptophan would require larger quantities of glucose.

Several different lots and grades of glucose were investigated, including one of commercial grade. Only the analytical grades of glucose were satisfactory for use as a reagent in the glucose-tryptophan reaction.

Effect of Temperature and Time on Development of Fluorescence. Temperatures above 100° C. were chosen for this study in order to shorten the reaction time. Aliquots of a tryptophan solution were heated for 2.5 and 5 hours in a preheated autoclave at various temperatures ranging from 114° to 126° C. These data, shown in Figure 4, indicate that the development of fluorescence in the glucose-tryptophan reaction is a function of both time and temperature. A temperature of 118° C. was selected for further study.

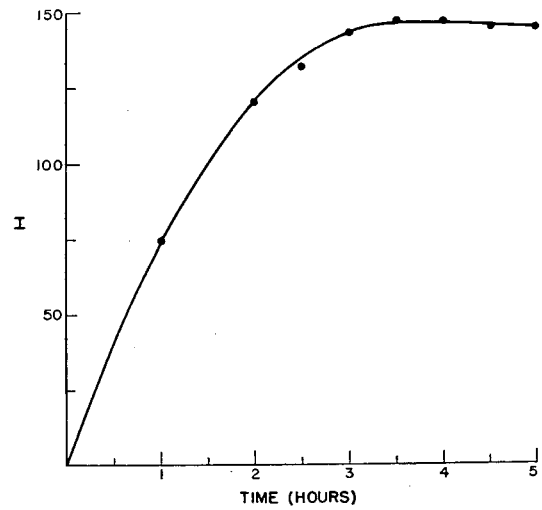

Figure 5. Influence of reaction time on fluorescence intensity of compounds produced by heating glucose plus tryptophan at 118° C.

The relationship of time of reaction and fluorescence intensity at 118° C. is shown in Figure 5. These data suggest that the development of fluorescence proceeds rapidly for the first 2 hours, after which there is a decrease in rate of reaction. The maximum fluorescence intensity was reached after 4 hours of heating. Heating as long as 10 hours caused a diminution in fluorescence intensity. This decrease was associated with the development of a black precipitate which became visible when the reaction mixture was cooled. This undoubtedly was a polymerized product typical of the latter stages of the Maillard reaction. In further experiments, solutions were heated for 4 hours at 118° C. There was no evidence of a polymerized product under these conditions.

Development of Fluorescence Due to Amino Acids Other Than Tryptophan. At a given molar concentration, tryptophan contributes more to fluorescence than any of the other amino acids occurring in protein (*5*). Some proteins, however, contain much more of certain amino acids than of tryptophan. Glutamic acid (*8*) makes up 35% of flour protein, while tryptophan comprises approximately 1.0% (*3*). When the reaction of glucose with all amino acids in the ratio in which they occur in wheat protein was studied, it was found that the individual amino acids other than tryptophan contributed a rather small amount of fluorescence. Collectively, however, the error contributed by the amino acids other than tryptophan amounted to as much as 25%.

Although Schram and others (*13*) indicated that chromatographic methods were not suitable for the analysis of tryptophan, this difficulty appeared to be due to techniques other than the separation of the amino acids. Accordingly, a chromatographic method (*11*) for separation of tryptophan from other amino acids was employed. Tryptophan recoveries of 100 ±2% from the chromatographic column were established. This agrees with the value of 100 ±3% reported by Moore and Stein (*11*).

Preparation of Standard Curve. Data for a standard curve were obtained by heating 0.8 gram of glucose in 5 ml. of sodium citrate–hydrochloric acid buffer (pH 1.38) with from 4 to 20 γ

of tryptophan in 5 ml. of phosphate buffer (pH 1.38) for 4 hours at 118° C. All samples were diluted 12.5 times with sodium citrate–hydrochloric acid buffer (pH 1.80) before reading in the photofluorometer. Data representing the average of 5 separate analyses were plotted and a linear relationship was shown to exist between the tryptophan concentration and the fluorescence intensity developed under standard conditions. A standard error of ±3.0% for 12 γ of tryptophan was calculated. The error of estimate was essentially constant over the entire range, up to 20 γ of tryptophan. There was no difference in the reactions of LD- and DL-tryptophan.

Normally the fluorescence intensity of a 2 to 25 dilution of the sample was determined. The fluorescent materials could be extracted with isobutyl alcohol with no loss of fluorescence, thus providing a means for concentrating these products and making the method more sensitive. This extraction technique is similar to that applied in the analysis of thiamin by the thiochrome method (*1*).

LITERATURE CITED

(1) Association of Vitamin Chemists, Inc., "Methods of Vitamin Assay," 2nd ed., p. 111, Interscience, New York, 1951.
(2) Friedman, L., Kline, O. L., *J. Biol. Chem.* **184**, 599 (1950).
(3) Gordon, M., Mitchell, H. K., *Ibid.*, **180**, 1065 (1949).
(4) Graham, W. D., Hsu, P. Y., McGinnis, J., *Science* **110**, 218 (1949).
(5) Haney, H. N., M. S. thesis, Kansas State College, Manhattan, Kan., 1952.
(6) Lingane, J. J., ANAL. CHEM. **19**, 810 (1947).
(7) Maillard, L. C., *Compt. rend.* **154**, 66 (1912).
(8) Miller, B. S., Seiffe, J. Y., Shellenberger, J. A., Miller, G. D., *Cereal Chem.* **27**, 96 (1950).
(9) Mohammad, A., Fraenkel-Conrat, H., Olcott, H. S., *Arch. Biochem.* **24**, 157 (1949).
(10) Moore, S., Stein, W. H., *J. Biol. Chem.* **176**, 367 (1948).
(11) *Ibid.*, **192**, 663 (1951).
(12) Portner, C., Högl, O., *Anal. Chim. Acta* **8**, 29 (1953).
(13) Schram, E., Dustin, J. P., Moore, S., Bigwood, E. J., *Ibid.*, **9**, 149 (1953).
(14) Spies, J. R., Chambers, D. C., ANAL. CHEM. **20**, 30 (1948).
(15) *Ibid.*, **21**, 1249 (1949).
(16) *Ibid.*, **22**, 1209 (1950).
(17) *Ibid.*, p. 1447.

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Determination of Nickel in Oxidized Films on Nickel Metal

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A rapid spectrophotometric method for the determination of nickel in oxidized films on nickel metal has been developed. The solvent used, 0.5% potassium cyanide, dissolves the oxidized film but not the nickel metal. The method is sensitive to microgram quantities of nickel.

THE formation of films of oxidized nickel on nickel metal has been studied by several investigators. In most cases the film was measured by either weight changes or x-ray or electron diffraction. The latter method requires removal of the film from the base metal. Techniques for stripping oxidized films from base metal have been developed by Evans and Stockdale (*1*), Phelps, Gulbransen, and Hickman (*3*), and Vernon, Wormwell, and Nurse (*5*). Both chemical and electrochemical methods have been employed.

Thin films of nickel hydroxide can be electroplated on nickel metal from a borate-buffered solution of nickel sulfate (*2*). A study of the electrochemical characteristics of films of this type made it necessary to determine the amount of nickel in the film.

When working with thin films on nickel metal, very small amounts of the base metal which may dissolve cause large errors in the film analysis. Therefore, the solvent used must be selective for the oxidized film.

A number of acids of varying concentrations were tried, but in all cases some of the base metal was dissolved. A 2% solution of formic acid was found most suitable of those tested. The oxidized film dissolved readily in this solution while the base metal dissolved very slowly. However, there was some difficulty in determining when the film was completely dissolved.

Some of the common nickel complexing agents were also tried. An aqueous solution of potassium cyanide was found to be specific for the oxidized film, provided all other oxygen was excluded from the solution. In addition to its specificity, it was useful in the analysis of solutions containing microquantities of nickel.

Prior to the use of the cyanide method, the nickel dissolved by the formic acid was determined spectrophotometrically with dimethylglyoxime (*4*). This method was not applicable to solutions of nickel cyanide, but ultraviolet absorption by the nickel cyanide complex solved the problem simply and satisfactorily.

NICKEL DETERMINATION

The ultraviolet absorption spectrum of a 0.5% solution of potassium cyanide containing nickel ion is shown in Figure 1. There are three absorption peaks, which occur at 208, 268, and 286 mμ. A much weaker absorption peak appears at 310 mμ. A calibration curve was prepared for the absorption peak at 268 mμ; a Beer's law curve was obtained in the range from

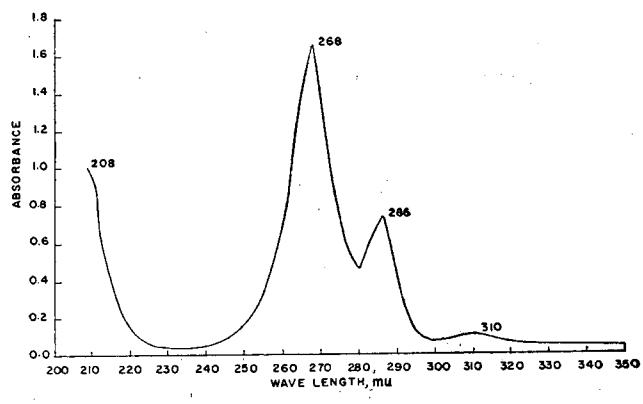

Figure 1. Absorption spectrum of nickel in 0.5% potassium cyanide solution

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2×10^{-7} to 25×10^{-7} gram of nickel per milliliter. This offers a sensitive method of analysis in the part per million range.

SELECTIVITY OF POTASSIUM CYANIDE

A nickel screen approximately $2^1/_2$ by $2^3/_4$ inches was used to determine the selectivity of aqueous potassium cyanide for the oxidized film. The screen was first cleaned in concentrated hydrochloric acid and washed with distilled water. It was then dipped in potassium cyanide solutions for varying lengths of time and the amount of nickel dissolved was determined. The hydrochloric acid dip, followed by a water wash, was repeated after each potassium cyanide dip. The amount of nickel dissolved by successive dips increased up to a contact point of 1 minute. Longer contact periods failed to dissolve more nickel. The surface of the metal appeared to have a soluble film which could be removed by the cyanide solution, after which nothing more was dissolved.

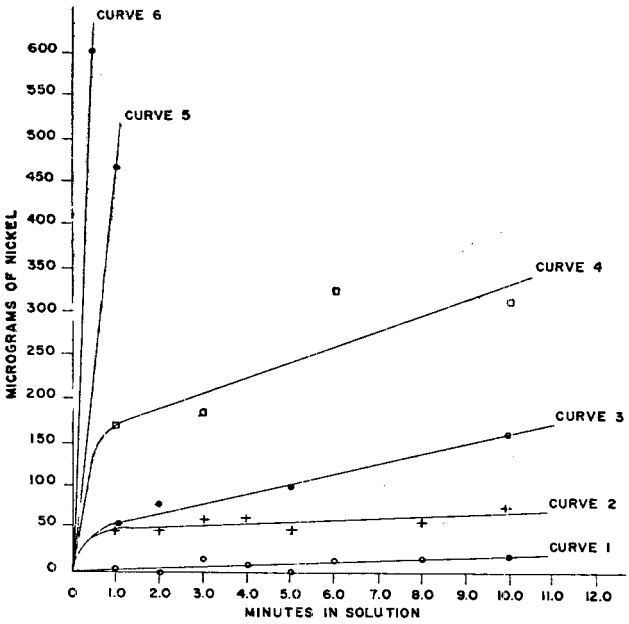

Figure 2. Amount of nickel dissolved in 0.5% potassium cyanide solutions under various conditions

1. Screen removed from aqueous potassium cyanide into nitrogen atmosphere, then dipped into fresh, nitrogen-flushed potassium cyanide solution
2. Screen cleaned in concentrated hydrochloric acid and air-dried, then placed in nitrogen atmosphere, and dipped into nitrogen-flushed potassium cyanide
3. Screen cleaned in concentrated hydrochloric acid and air-dried, then dipped in potassium cyanide which had been nitrogen-flushed in contact with air
4. Screen cleaned in concentrated hydrochloric acid, then dipped into potassium cyanide with nitrogen flush
5. Screen cleaned in concentrated hydrochloric acid and dipped into potassium cyanide saturated with oxygen
6. Screen cleaned in concentrated hydrochloric acid, then dipped into potassium cyanide with oxygen bubbling over screen

The conclusion was reached that a fairly reproducible film of oxide or hydroxide was formed on the nickel when it was exposed to air. Additional tests showed that, when the sample was moved as rapidly as possible from the first potassium cyanide solution to the second, there was still an appreciable amount of nickel dissolved. The rate of film formation was rapid.

The experiment was then repeated with a nickel screen and a solid nickel plate in a nitrogen atmosphere to avoid exposure to oxygen. With both samples nickel was found in the first solution after 2 minutes' contact, but not in the second, third, or fourth solutions. It was concluded that the nickel found in the first solution amounted to the air-oxidized film on the nickel. Because the samples were not exposed to air between the second, third, and fourth solutions, no oxidation could occur and, therefore, no nickel was dissolved.

Continued work on the determination indicated that the amount of nickel which dissolved was sensitive to the amount of oxygen present in the potassium cyanide solution. To clarify this point, several experiments were performed, starting with solutions free of dissolved oxygen and continuing to solutions saturated with oxygen. The data obtained are shown in Figure 2.

These experiments show that elemental nickel is insoluble in a 0.5% potassium cyanide solution if oxygen is not present in the solution. In order to prove conclusively the usefulness of the method, it should also be shown that an oxidized film is completely dissolved in the potassium cyanide solution. This is difficult because the stoichiometry of oxidized films is indefinite, a fact which makes it difficult to prepare a film containing a known amount of nickel. Although a known sample cannot be prepared, complete solution of the oxidized film in potassium cyanide solution was demonstrated by showing in the following way that the base metal was free of appreciable quantities of oxidized film after the potassium cyanide dip.

A sample was prepared from 16 strips of nickel metal, 3 mils thick and 1 by 3 inches; this offered a large surface area. Approximately 10 to 12 mg. of oxidized film was plated from a borate-buffered nickel sulfate solution. The film was then dissolved in potassium cyanide and the electrode thoroughly washed with water, dried, and weighed. It was then reduced in a hydrogen atmosphere for 1 hour at 950° C. and again weighed. The change in weight resulting from the hydrogen reduction was ±0.2 mg., which is approximately the experimental error for samples of this type. If the oxidized film had not been completely removed by the potassium cyanide dip, there would have been an appreciable loss in weight resulting from the hydrogen reduction.

PROCEDURE

Aqueous potassium cyanide, .0.5%, was flushed 10 minutes with nitrogen gas. The sample was immersed in the solution for 2 to 5 minutes, depending on the film thickness, and the nitrogen flush was continued during the contact period. The resulting solution was diluted with 0.5% potassium cyanide to a concentration suitable for analysis and the absorbance was measured at 268 mμ, using 0.5% potassium cyanide for a blank.

DISCUSSION

When elemental nickel is exposed to air, an oxidized film very rapidly forms on the surface. This film is soluble in aqueous solutions of potassium cyanide. The oxidized film deposited electrolytically on nickel metal from a borate-buffered nickel sulfate solution is also completely soluble in aqueous potassium cyanide. Both of these films can be dissolved without dissolving the base metal if oxygen is excluded from the solutions. For very accurate work a nitrogen box can be used to provide a nitrogen atmosphere. However, good results can be obtained by flushing the solution with nitrogen before addition of the sample and then continuing the flush during the contact period.

This method may have general applicability to the study of corrosion rates of nickel metal.

LITERATURE CITED

(1) Evans, U. R., Stockdale, J., *J. Chem. Soc.* **1929**, 2651.
(2) Haïssinsky, M., Quesney, M., *Compt. rend.* **223**, 792–4 (1946).
(3) Phelps, R. T., Gulbransen, E. A., Hickman, J. W., IND. ENG. CHEM., ANAL. ED. **18**, 391 (1946).
(4) Porcelain Enamel Institute, Quality Development Committee, Washington, D. C., Bull. T-16.
(5) Vernon, W. H. J., Wormwell, F., Nurse, T. J., *J. Chem. Soc.* **1939**, 621.

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Stable High Frequency Titration Apparatus in the 100-Mc. Frequency Range

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Replacement of the multiturn coil by a coaxial half-wave line in the oscillator circuit of a high frequency titration apparatus raises the operating frequency to 130 mc. By following the oscillator tube grid current change, using a Model XXI Sargent Polarograph, practical instrument response is observed with aqueous solutions in the titration cell varying in concentration from 0.001- to 1*M* in sodium chloride. With perchloric acid in glacial acetic acid solutions, response is observed for concentrations up to 0.04*M* in perchloric acid.

DURING consideration of the construction of a high frequency titration apparatus for a systematic study of possible applications of high frequency titrations, the instrument described by Anderson, Bettis, and Revinson (*1*) was selected on the basis of its stability, sensitivity, simplicity of construction, and flexibility for possible modifications. Its performance as a "capacitative" type instrument operating in the frequency range from 12 to 130 mc. is described. Oscillator frequency change is the response measured. Response curves for an instrument operating at 130 mc., obtained by measuring the oscillator tube grid current change, are also included. Extension of the operating frequency to the 130-mc. range was accomplished by replacing the conventional multiturn coil in the oscillator circuit with a coaxial half-wave line. The term "capacitative" is used to denote the type of cell employed and not the response measured. The term is suggested to differentiate between the type of cell in which the cell bands or plates are in contact with the walls of the titration vessel, constituting capacitor construction (the type used in this study), and the type in which the titration vessel is placed in the coil of the oscillator circuit, a "coil loading" type instrument originally designed by Jensen and Parrack (*5*).

FREQUENCY MEASURING INSTRUMENT

Instrument and Cell. A capacitative-type instrument similar to the prototype mentioned above (*1*) was constructed with some changes in mechanical arrangement and cell design. Essentially the instrument circuit was that shown in Figure 1, without R_2 and C_3. For operating frequencies up to 60 mc., L_1 was a multiturn coil, and in the 120-mc. region L_1 was a half-wave line constructed of RG 8/U coaxial cable. Three individual coils were wound so that the instrument operated at 12.2, 38.8, or 60 mc. (45 turns of No. 30, 15 turns of No. 24, 8 turns of No. 24 insulated copper wire, respectively). Each coil was wound on a $^1/_2$-inch coil form mounted on a substantial insulating base. Two banana plugs were attached to this base, and the ends of the coil were terminated in the plugs. The coil shields were made of heavy gage copper and were firmly attached to the bases of the coil forms. The coil and shield, as well as band-type cell construction, are shown in Figure 2. When plugged into position, a spring collar attached to the chassis gripped the lower edge of the shield firmly, completing the shielding to the chassis, as well as preventing any physical movement of the coil assembly during a titration.

The half-wave line was a 90-cm. length of RG 8/U flexible coaxial cable, the ends of which were terminated in Amphenol type PL-259 male coaxial cable connectors. The cable was bent back upon itself to bring the two cable connectors side by side, and then the parallel sections of cable were taped together. Added rigidity was given to the line by taping it to a short length of $^1/_2$-inch wooden dowling. The male coaxial cable connectors at the ends of the line plugged into female coaxial receptacles mounted on a brass plate $^3/_8$-inch thick. The coaxial receptacles were so spaced that the spacing of the banana plugs soldered to the center conductors of the female receptacles was the same as the spacing of the terminal plugs of the coils previously used. Figure 3 shows the half-wave line mounted in position. During operation the line was clamped to a vertical rod fastened to the work bench.

The 955 tube, V_1, and its associated components were mounted on a small subchassis of $^3/_{16}$-inch aluminum plate attached to the main chassis of the instrument by means of suitable brass standoffs. Access to the tube was provided through a hole in the top of the main chassis around which a suitable shield assembly was secured.

The titration vessel, 6 cm. in diameter and 8.5 cm. high, was constructed from a 250-ml. polyethylene bottle. A collar located about 2 cm. from the top of the vessel regulated the volume of the vessel which could be lowered into the bands. Two copper bands, 1 cm. high and 6 cm. in inside diameter, were mounted 2 cm. apart by polystyrene retainers; the retainers were rigidly mounted inside a metal box which served as a shield for the bands and the titration vessel. Leads from the two bands were soldered to banana plugs insulated from the box. A third plug connected to the box grounded the box to the chassis of the oscillator. The cell assembly was designed so that the polyethylene vessel could be removed by simply sliding it vertically out of the bands and the remainder of the cell assembly. The fit was snug enough to prevent any movement of the vessel relative to its surroundings during a titration.

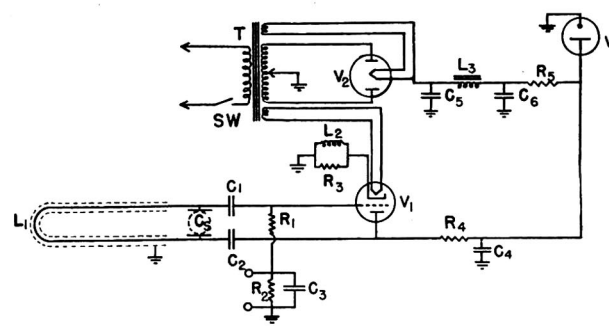

Figure 1. Schematic diagram of 120-mc. titration apparatus

C_8. Cell assembly
C_1, C_2, C_3, C_4. 100 micromicrofarads, mica
C_5, C_6. 20 microfarads, 450 volts
L_1. RG 8/U coaxial half-wave line, approximately 90-cm. total length (see text)
L_2. 10 turns No. 22 wire wound around R_3.
L_3. Filter choke, Gracoil 200925
R_1, R_4. 15,000-ohm, 1-watt
R_2. 1000-ohm, 1-watt
R_3. 100-ohm, 2 watts
R_5. 5000 ohms, 10 watts
SW. SPST toggle switch
T. 350-0-350, 70 ma.; 5 volts, 3a; 6.3 volts, 3a.
V_1. 955
V_2. 5Y3
V_3. VR 150/30

For operation at 120 mc., the bands around the titration vessel provided more capacitance than could be tolerated. They were replaced by two plates 3 cm. high, 2.5 cm. long, and 2.5 cm. apart. The plates were mounted in the same plane and curved to fit the contours of the polyethylene vessel. The polystyrene spacers that had previously supported the bands were left in position to center the vessel and to prevent its physical displacement during a titration. Details of component arrangements are shown in Figure 4.

With an empty titration vessel in the cell assembly and the

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cell assembly plugged into the oscillator, the operating frequency of the titration apparatus was measured with a U. S. Army BC-1255-S heterodyne frequency monitor. The frequency was found to be 120 mc., a good check on the frequency expected on the basis of the length of the half-wave line used.

The frequency change of the oscillator resulting from solution composition change in the titration vessel was followed. A U. S. Army BC-221-D heterodyne frequency meter was employed. Because the highest fundamental frequency of the calibrated oscillator in the frequency meter was 20 mc., higher frequencies necessitated that higher harmonics of the frequency meter oscillator be used to obtain an audible beat note. A short antenna

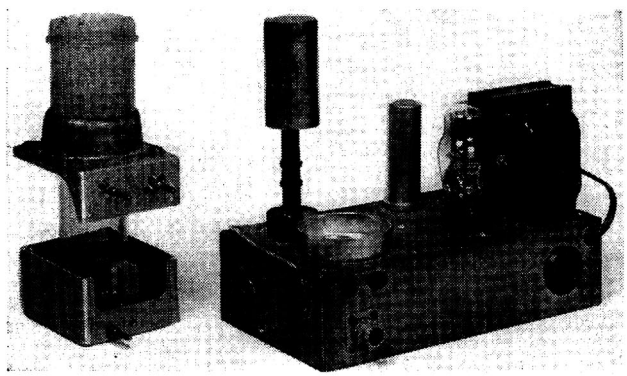

Figure 2. Coil, shield, and cell construction

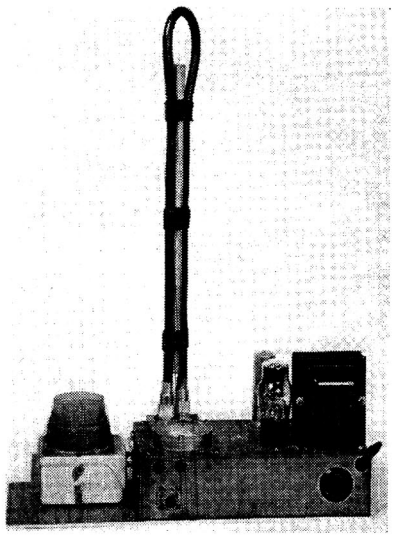

Figure 3. Half-wave line and connection to titration apparatus

connected to the frequency meter provided sufficient signal for measuring purposes at the three lower frequencies listed. For frequency change measurements at 120 mc., a loose coupling between the plate of the oscillator tube and the frequency meter mixer was made through a small capacitor. Connection was made by means of coaxial cable from the frequency meter to the titration apparatus at the female coaxial receptacle located on the side of the chassis shown in Figure 3. No pulling of the oscillators was detected. The output of the frequency meter was amplified in the audio channel of an R.C.A. Rider Channelyst. Sufficient volume was provided in the earphones after amplification. A visual indication of zero beat conditions could be obtained on the appropriate magic eye in the Channelyst if desired.

Sensitivity and Stability. A sensitivity curve for the 120-mc. titration apparatus was obtained for sodium chloride in water and is shown as curve *A* in Figure 5. Practical sensitivity is attained for a specific conductance range corresponding to that of sodium chloride concentrations from 0.005 to 0.08*M* in aqueous solutions, as should be attained according to Blaedel and Malmstadt (*2, 4*).

Frequency drift of the frequency meter oscillator and the titration apparatus oscillator combination was followed for a 30-minute period. An increase in frequency was noted during the first and last 5-minute intervals. The frequency appeared to remain constant during the time interval from 9 to 22 minutes. Slight scattering of experimental points in the titration curves indicates desired stability of the instrument.

Frequency drift was observed when the filled titration vessel was inserted into position in the cell assembly. It was assumed that temperature change was the cause. The effect was detected by following the temperature change of a dilute sodium chloride solution in the vessel inserted in the cell assembly and the corresponding frequency change of the oscillator of the titration apparatus. After the first 15 minutes, the temperature rise and the frequency increase had passed through a maximum and then leveled off. The maximum was reached at 5 minutes, during which the temperature rise was 0.47° C. and the meter reading increase was approximately 1 division. For the next 15 minutes, the solution temperature remained constant at 0.11° C. above the initial temperature, and the frequency meter reading remained at about 0.3 division above the initial reading. The results of this experiment are presented in Figure 6. On subsequent titrations the vessel containing the solution was introduced into the cell assembly about 10 minutes prior to reagent additions.

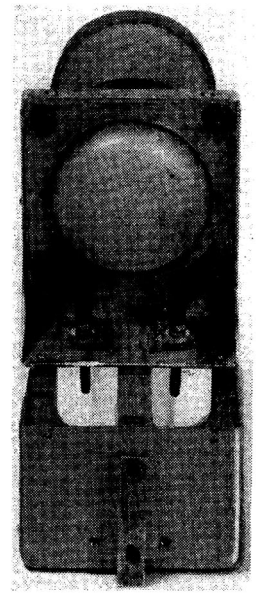

Figure 4. Cell construction details for operation at 120 mc.

GRID CURRENT MEASURING INSTRUMENT

The frequency measuring instrument was modified so that grid current changes could be measured. The parallel combination of R_2 and C_3 was placed in

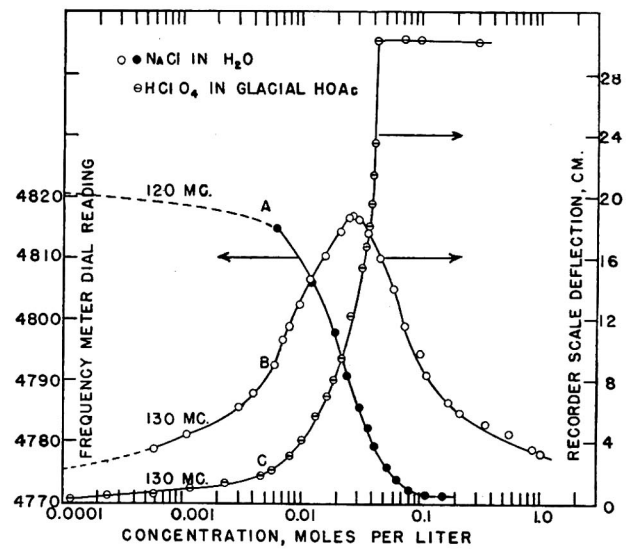

Figure 5. Response curves

Frequency change response at 120 mc. and grid current change at 130 mc.

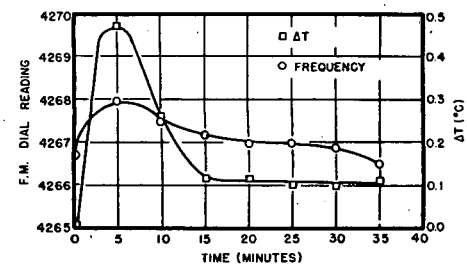

Figure 6. Frequency change associated with temperature change of solution in titration cell assembly

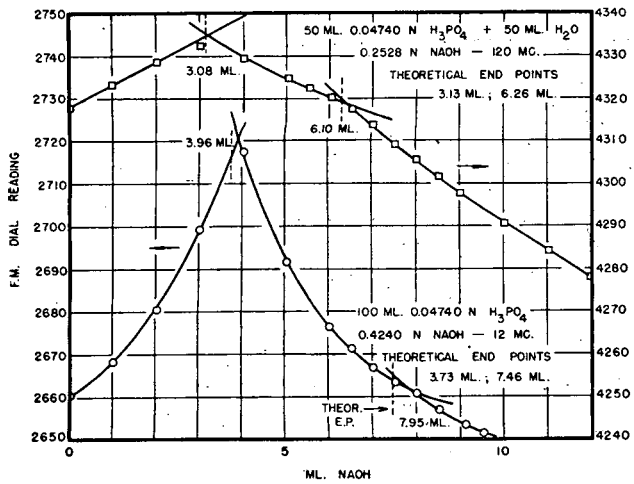

Figure 7. High frequency titration of phosphoric acid with sodium hydroxide

series with the grid leak resistor, R_1 (Figure 1). A part of the grid bias developed by the oscillator appears at the terminal of R_2 and is normally less than 0.2 volt. The wall-type galvanometer and its associated circuitry as used by Anderson and others (*1*) for measuring grid current changes was replaced by the Sargent Model XXI Polarograph. Leads from the polarograph normally connected to the polarographic electrode assembly were connected to be terminals of R_2. With a span voltage of 1 volt on the polarograph, any voltage of the same polarity as the bias voltage between 0 and 1 volt could be placed across R_2 through the leads. At the beginning of a titration, the applied voltage and current measuring sensitivity were selected so that the recorder indicator could be moved to such a position that the response encountered in the titration covered the 280-mm. range of recorder scale without any readjustments. By preliminary titrations or experience the proper initial adjustments were made.

Sensitivity. Sensitivity curves for operation of the instrument at 130 mc. (half-wave line approximately 85 cm.) were obtained with aqueous sodium chloride and with perchloric acid in glacial acetic acid. The results are shown as curves *B* and *C* in Figure 5.

TITRATIONS

Solutions prepared and standardized by common methods were used for various types of titrations with the frequency measuring instrument operating at 12.2, 38.8, or 60 mc. The appearance of the titration curves was similar to those reported by Anderson and others (*1*). Figure 7 compares the titration of phosphoric acid with sodium hydroxide at 12.2 mc. to a titration at 120 mc. The discrepancy between the theoretical and the experimental end points is due to the fact that phosphoric acid was standardized to the phenolphthalein end point. Subsequent check titrations show much better agreement of the high frequency end point with other titration methods. Results are listed in Table I.

Table I. Titration of Phosphoric Acid[a] with Sodium Hydroxide

Series[b]	Type of Titration	NaOH, Ml.
A	HF, 137 mc.	2.75
	HF, 137 mc.	2.75
	pH	2.72
	pH	2.75
	Methyl orange indicator	2.75
B	HF, 137 mc.	2.65
	pH	2.65

[a] 100 ml. of phosphoric acid stock solution + 35 ml. of water titrated with 0.1958*N* sodium hydroxide.
[b] A and B, different solutions of phosphoric acid.

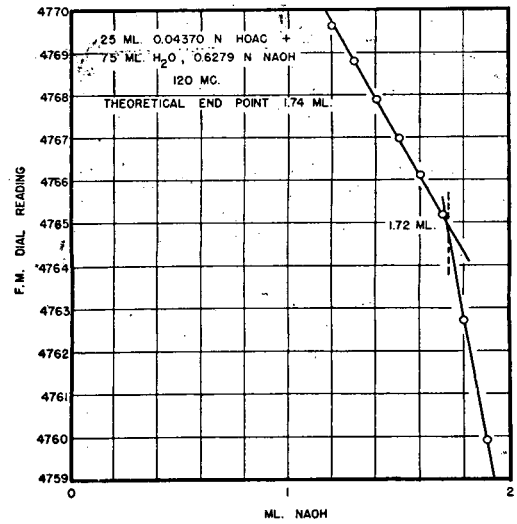

Figure 8. High frequency titration of acetic acid with sodium hydroxide

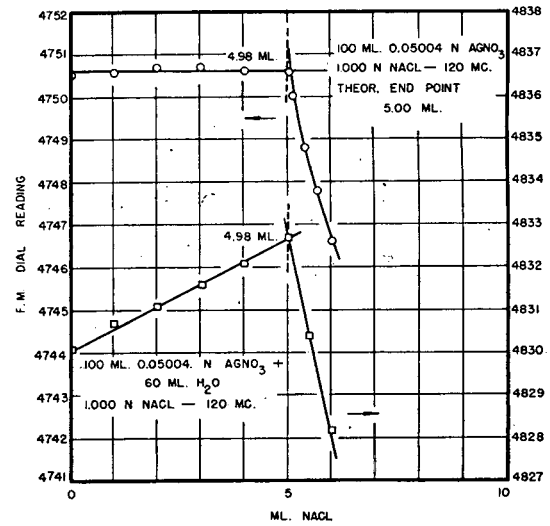

Figure 9. High frequency titration of silver nitrate with sodium chloride

The titration curves show that straighter lines are obtained at 120 mc., facilitating the location of the end point. Figure 8 is the reproduction of a curve obtained for the titration of acetic acid with sodium hydroxide at 120 mc. Perfectly straight lines

were obtained. Figure 9 shows titration curves obtained for titrations of silver nitrate with sodium chloride.

DISCUSSION

Blaedel and Malmstadt (*2, 3*) had shown that, in order to extend the useful range of frequency measuring instruments to a more practical region of conductivities, frequencies of 100 mc. and above were necessary. The highest frequency obtainable using conventional multiturn coils in the instrument reported here is approximately 80 mc. Substitution of a half-wave coaxial line for the conventional coil resulted in an operating frequency of 130 mc. Higher frequencies could probably be attained with shorter lengths of coaxial cable and other slight modifications. This will be investigated.

There is no radical departure here from the ordinary simple oscillator constructional practices. All electronic components are readily available and any mechanical fabrication is easily performed. Construction of a duplicate oscillator in which some changes in layout were made required only 6 hours.

The adaptability of a recording polarograph to grid current change measurements reduces construction time. Also, it becomes apparent that by synchronizing the movement of the plunger of an automatic buret with the chart drive of the recorder, a recording automatic titration apparatus could result.

ACKNOWLEDGMENT

The authors wish to thank R. E. Hooser and D. A. Costanzo for carrying out the check phosphoric acid titrations.

LITERATURE CITED

(1) Anderson, K., Bettis, E. S., Revinson, D., ANAL. CHEM. **22**, 743–6 (1950).
(2) Blaedel, W. J., Malmstadt, H. V., *Ibid.*, **22**, 734–42 (1950).
(3) *Ibid.*, pp. 1413–17.
(4) Blaedel, W. J., Malmstadt, H. V., Petitjean, D. L., Anderson, W. K., *Ibid.*, **24**, 1240–4 (1952).
(5) Jensen, F. W., Parrack, A. L., IND. ENG. CHEM., ANAL. ED. **18**, 595–9 (1946).

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Determination of Hydroxyalkyl Groups in Low-Substituted Starch Ethers

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By modifying Morgan's alkoxyl apparatus, the degree of substitution of low-substituted hydroxyalkyl starch ethers may be determined quantitatively. In this procedure hot, constant-boiling hydriodic acid cleaves ether linkages and the hydroxyalkyl groups decompose quantitatively into ethyl iodide and ethylene. Ethyl iodide and ethylene may be determined volumetrically in standard solutions of silver nitrate and bromine, respectively. Along with modifications of apparatus the sample size was adjusted as well as the quantity of hydriodic acid to accommodate samples with small amounts of ether substitution in the range of 0.005 to 0.20 hydroxyalkyl group per anhydroglucose unit.

DURING the past several years the commercial importance of hydroxyalkyl starch ethers has become increasingly significant. In particular, the low-substituted hydroxyethyl starch ethers in the granule state (*6*) have found wide acceptance as sizes in the paper and textile industry. As previously published analytical methods were not suitable for low-substituted starch ethers, there was a need for a quantitative analytical method for such materials. Cellulose ethers as well as glycol ethers and esters have also found widespread application in the past 15 years and a number of analytical methods have been published for these materials.

About 10 years ago Morgan (*7*) gave an extensive review of the problem as well as modifications and improvements of previous methods. He described a modified alkoxyl analysis on a semimicro scale for the determination of ethers and esters of ethylene glycol. Without exception he found that olefins were a product of the reaction as well as alkyl iodides. In recovering both products quantitatively he accounted for the starting material. He suggested an adjustment in sample size and a reaction flask of doubled capacity to accommodate samples of less than 10% glycol residues. He reported substitution values as low as 3.6% as ethylene oxide. This method was applied successfully to

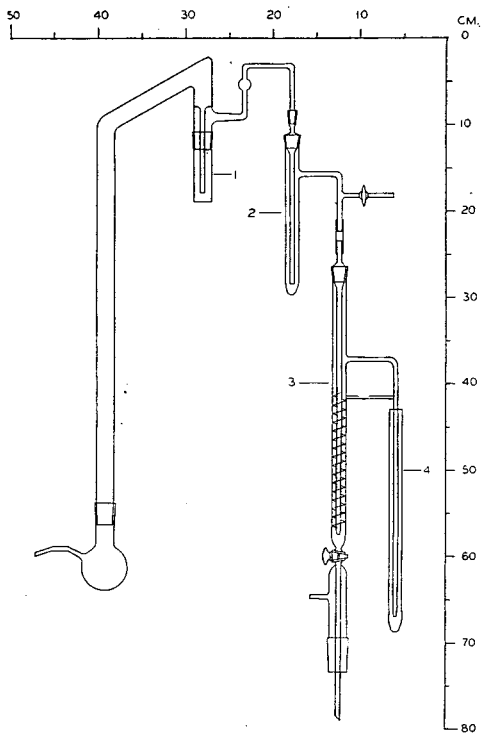

Figure 1. Diagram of apparatus for low-substituted ethers

poly(ethylene glycol) ethers, solvents, plasticizers, and hydroxyethyl celluloses.

In attempting to analyze samples of low-substituted hydroxyethyl starch, with 1 to 3% substitution as ethylene oxide, by Morgan's (*7*) method considerable difficulty was encountered. Larger samples, about 1 gram, of low-substituted ethers require about 40 ml. of hydriodic acid. This necessitated a reaction flask of 100-ml. capacity as well as an enlarged reflux column and trap arrangement. Also, to ensure greater reproducibility, all but one of the rubber joints were replaced with standard-taper glass joints; the reaction temperature was thermostatically controlled; and the carbon dioxide was passed through a tube of Drierite prior to introduction into the apparatus to avoid the addition of moisture. It was found convenient to add the sample to the reaction flask by means of a gelatin capsule. This allowed a more uniform reaction with the hydriodic acid as the gelatin capsule was gradually dissolved and the presence of gelatin did not interfere with the reaction. Of the various bromide-solvent mixtures suggested (*7*) the bromine–acetic acid solution was found most satisfactory.

APPARATUS

Shown in Figure 1 is an arrangement of the apparatus for low-substituted ethers and Figure 2 is a photograph of the assembled apparatus. The apparatus is constructed entirely of borosilicate glass with the dimensions shown by scale in Figure 1. Less obvious dimensions which were found convenient are as follows: round-bottomed reaction flask with capillary inlet, 100-ml. capacity equipped with a 24/25 standard-taper joint; inlets to traps 1 and 2, capillary, 1 mm. in inside diameter; joint for trap 1, 19/22 standard-taper, joints for trap 2, 10/18 and 14/20 standard-taper respectively; joint for trap 3, 14/20 standard-taper; trap 3, a spiral adsorption tube similar to that in a Widmer distillation column; joint for the receiver below trap 3, 24/40 standard-taper; all joints equipped with hooks for holding springs.

REAGENTS

Hydriodic acid. One of the most important considerations in this determination is an especially purified, constant-boiling hydriodic acid having a specific gravity of 1.70 and boiling point of 126–27° C. Purification steps should provide an acid sufficiently free of hypophosphorous acid and phosphine to provide consistent results on blank determinations, as pointed out by Steyermark (*9*). Samsel and McHard (*8*) outline a method for the preparation of a suitable reagent. For the purpose of this analysis a reagent grade hydriodic acid for methoxyl determination supplied by Merck & Co., Inc., was found satisfactory.

Alcoholic silver nitrate solution. Fifteen grams of silver nitrate are dissolved in 28 ml. of water and added to 422 ml. of 95% ethyl alcohol. Several drops of concentrated nitric acid are added. A brown bottle is desirable for storage. The solution is stable and is standardized against 0.05*N* ammonium thiocyanate by the Volhard (*11*) method.

Ammonium thiocyanate standard solution, 0.05*N*.

Ferric sulfate indicator. A saturated aqueous solution is prepared and sufficient concentrated nitric acid is added to remove the brown color, thus providing a sharper end point.

Bromine–acetic acid solution. To 600 ml. of glacial acetic acid saturated with dry potassium bromide (about 10 grams) are added 2 ml. of bromine. This solution should be stored in a very clean, brown bottle in a cool dark place. Care should be given to the bottle closure; a screw-type cap with a paraffin or glass lining is satisfactory. The solution becomes reasonably stable after standing for several days. It is advisable to standardize the solution daily during use. About 40 ml. of 0.05*N* sodium thiosulfate are required by 15 ml. of the bromine–acetic acid solution.

Sodium thiosulfate standard solution, 0.05*N*.

Potassium iodide, 10% aqueous solution.

Starch indicator, 1% aqueous solution.

Cadmium sulfate, 5% aqueous solution.

Red phosphorus, amorphous powder.

PROCEDURE

Safety precautions are best observed when the apparatus is set up in a safety hood. The apparatus should be thoroughly cleaned and dried. Acetone as an aid in drying the apparatus is undesirable, as such residues would interfere with the results.

To trap 1 are added about 0.5 gram of red phosphorus and sufficient 5% cadmium sulfate solution to cover the inlet tube at least 3 cm. Into trap 2 are pipetted 10 ml. of alcoholic silver nitrate, and into the spiral absorption tube (trap 3) are pipetted 15 ml. of bromine solution. Into the final trap, No. 4, are placed about 15 ml. of the 10% potassium iodide solution. The spiral absorption tube is fitted into a 500-ml. Erlenmeyer flask containing 10 ml. of 10% potassium iodide solution and about 150 ml. of water.

The sample is weighed into a 000 gelatin capsule; about 1 gram of dry sample is satisfactory for ethers of 1 to 2% substitution. However, samples of 3 to 4% substitution should be reduced to about 0.5 gram. The weighed sample is placed in the reaction flask along with a Hengar boiling granule and about 40 ml. of hydriodic acid. As the flask is connected to the reflux column several drops of hydriodic acid are placed on the glass joint as a seal. Carbon dioxide, which has been passed through a U-tube of Drierite, is admitted through the capillary inlet tube of the reaction flask at the rate of about one bubble per second. The flask is heated in an oil bath to between 140° and 145° C. and thermostatically controlled in this optimum range for this apparatus.

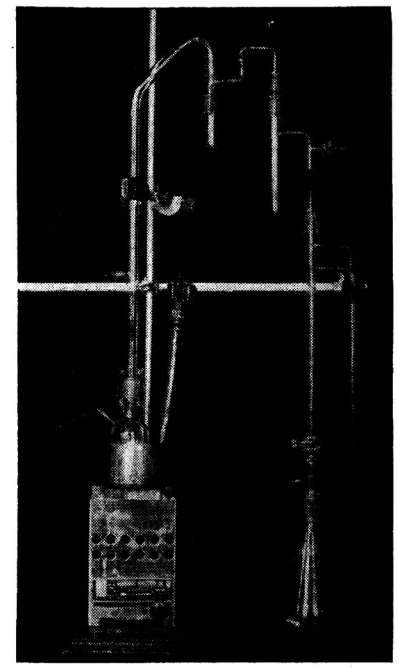

Figure 2. Assembled apparatus

After about 1 hour at the optimum temperature most reactions are complete; rarely is more time required. During the early part of the reaction the supernatant alcoholic silver nitrate is cloudy; near the completion of the reaction the supernatant liquid becomes clear and the precipitate is well settled. If at the end of the reaction the silver nitrate trap is below 40° C., it should be heated slightly prior to removal to drive out any dissolved olefin into the bromine solution. This may be done by immersing the trap in a beaker of warm water.

When the decomposition is complete, the stopcock between the silver nitrate trap and the bromine absorption tube is opened very slowly. An abrupt opening may cause alcoholic silver nitrate to be carried over into the bromine tube. After the bromine absorption tube is disconnected the alcoholic silver nitrate tube is also disconnected. The carbon dioxide is then disconnected and the heating is terminated. The contents of spiral absorption tube, side arm, and trap 4 are quantitatively transferred with washing into the attached Erlenmeyer receiving flask. After the stopper is placed in the flask the solution is allowed to stand no more than 5 minutes before titrating with 0.05*N* sodium thiosulfate. About 2 ml. of starch indicator are added when only a faint tinge of yellow remains. Sufficient sodium thiosulfate is added to remove the blue color, which should not reappear for at least 30 seconds.

The contents of alcoholic silver nitrate trap 2 are rinsed into a 500-ml. Erlenmeyer flask containing about 150 ml. of water. After being heated to boiling and cooled to room temperature, the solution is titrated with 0.05*N* ammonium thiocyanate using 3 to 5 ml. of ferric sulfate solution as an indicator. The first

Table I. Analysis of Known Hydroxyethyl Starch Ethers

Sample	Ethylene Oxide, % Added[a]	Found As C_2H_5I	Found As C_2H_4	Found Total	Hydroxyethyl Groups per Anhydroglucose Unit Added	Found
A	0.159	0.134	0.026	0.160	0.00586	0.00590
	0.159	0.106	0.052	0.158	0.00586	0.00582
B	1.09	0.869	0.211	1.08	0.0409	0.0402
	1.09	0.704	0.311	1.02	0.0409	0.0379
C	4.31	3.62	0.80	4.42	0.166	0.170
	4.31	3.34	0.95	4.29	0.166	0.165
D	10.1	8.20	1.94	10.1	0.413	0.413
	10.1	7.95	1.49	9.44	0.413	0.384

[a] $\frac{\text{weight } C_2H_4O \times 100}{\text{weight } C_2H_4O + \text{weight of starch}}$

tinge of pink is considered a suitable end point. For both titrations the use of a magnetic stirrer in the receiving flask has been most helpful.

If it is desirable to analyze samples with substitution values in excess of 5%, the semimicromethod described by Morgan (*7*) is most suitable.

CALCULATIONS

In making calculations for this type of quantitative organic determination it is necessary to choose a convenient unit by which the results are to be expressed. Low-substituted hydroxyethyl starch ethers, for example, could be calculated on the basis of the hydroxyethyl group, $HOCH_2CH_2-$ or the ethylene oxide unit, C_2H_4O. The latter has been chosen, because it is commonly used in the commercial preparation of starch ethers in the granule state (*6*).

Using the molecular weight of 44.05 for ethylene oxide, the following calculations suggested by Morgan (*7*) are applicable.

$$\frac{\text{Difference in ml. of } Na_2S_2O_3 \times N \times 2.203}{\text{grams of sample}} = \%\ C_2H_4O \text{ as } C_2H_4$$

$$\frac{\text{Difference in ml. of } NH_4SCN \times N \times 4.405}{\text{grams of sample}} = \%\ C_2H_4O \text{ as } C_2H_5I$$

The difference in milliliters of the standard solutions for the above equations is found by subtracting the titration values for the unused alcoholic silver nitrate and the unreacted bromine–acetic acid solution from their corresponding blank titrations. Repeated blank determinations on the reagents with 1 gram of dry, unmodified, granule starch in a gelatin capsule required no silver nitrate solution; however, an average of 0.4 ml. of 0.05*N* sodium thiosulfate was required to account for the loss in the bromine–acetic acid solution. This blank was used as part of all calculations for titrations of the bromine–acetic acid solution.

In some instances it may be desirable to express the degree of substitution in terms of hydroxyethyl groups per anhydroglucose unit. This conversion of per cent substitution may then be put on a molar basis as follows:

$$\frac{\%\ \text{ethylene oxide found}}{(100 - \%\ \text{ethylene oxide found})} \times \frac{162}{44.05} = \text{hydroxyethyl groups per anhydroglucose unit}$$

where 162 equals the molecular weight of anhydroglucose and 44.05 equals the molecular weight of ethylene oxide.

RESULTS

As known, mixtures of poly(ethylene glycol) and unmodified starch showed good reproducible recovery of the ether in mixtures containing 1 to 2% poly(ethylene glycol). Further, known starch ethers were analyzed. These samples were carefully prepared by making starch react with a known ratio of ethylene oxide in a closed system (*6*), to varying levels of substitution as measured by the weight gain of the starch. In Table I the results are shown for dry, known samples.

In Table II are shown the results of analysis of hydroxyethyl starch ether derivatives in the granule state after being thoroughly dried. Many of these samples are representative of starch ethers prepared commercially (*6*) under the trade name of Penford Gum (Penick and Ford, Ltd., Inc.). Various types of modification are shown with good reproducibility of results as applied to typical commercial products.

The results of numerous determinations and those shown in Table II indicate that the average variation between duplicate determinations is ±3% of the mean amount of ethylene oxide found.

DISCUSSION

Interference may be encountered with any sample containing ethers, esters, or alcohols which react with hydriodic acid to form volatile alkyl iodides or olefins. Most sulfur-containing compounds are sufficiently eliminated with cadmium sulfate in the phosphorus trap.

Best results were obtained when the samples were thoroughly dried prior to analysis. The efficiency of the starch-ether cleavage by constant-boiling hydriodic acid was decreased as the moisture of the sample increased.

The data in Table III compare the analytical reaction efficiencies obtained on samples which were identical except for moisture content.

From the data in Table III it may be concluded that a dry sample is preferable if the maximum degree of accuracy and reproducibility of results are desired. There is some evidence in the literature as to the lowered efficiency of hydriodic acid ether cleavage when the specific gravity of the acid is less than 1.7 (constant boiling 126–27° C.). Apparently, varying amounts of water in the sample tend to change this equilibrium.

No difficulty was encountered in regard to the solubility of the starch ethers in hot hydriodic acid. In analyzing samples of poor

Table II. Analysis of Hydroxyethyl Starch Ethers

Sample	C_2H_4O Found, % As C_2H_5I	As C_2H_4	Total	Hydroxyethyl Groups per Anhydroglucose Unit, Found
E, high viscosity starch ether	1.34	0.30	1.64	0.0615
	1.34	0.31	1.65	0.0620
F, high viscosity starch ether	2.68	0.51	3.19	0.121
	2.66	0.56	3.22	0.123
G, medium viscosity starch ether	1.42	0.71	2.13	0.0801
	1.46	0.58	2.04	0.0766
H, medium viscosity starch ether	2.38	0.64	3.02	0.115
	2.47	0.62	3.09	0.117
I, medium viscosity starch ether	11.8	2.4	14.2	0.610
	12.1	1.6	13.7	0.594
J, low viscosity starch ether	0.912	0.943	1.86	0.0700
	1.00	0.890	1.87	0.0704
K, low viscosity starch ether	1.54	0.860	2.40	0.0905
	1.23	1.48	2.71	0.102

Table III. Analytical Reaction Efficiency of Samples of Different Moisture Content

% Moisture in Sample	C_2H_4O Found, % As C_2H_5I	As C_2H_4	Total	Calcd. on dry substance basis	% Efficiency[a]
0	0.99	0.98	1.97	1.97	101
0	0.93	1.00	1.93	1.93	99
10.0	0.84	0.90	1.74	1.93	99
10.0	0.76	0.90	1.66	1.84	94
21.6	0.73	0.41	1.14	1.46	75

[a] $\frac{\text{Total \% } C_2H_4O \text{ found on dry substance basis} \times 100}{\text{average of 1.97 and 1.93\% found in dry sample}}$

solubility the recommendations of Elek (*3*) concerning the addition of phenol and propionic anhydride to the samples should be considered.

APPLICATION OF METHOD

In 1946 Morgan (*7*) recognized that ethylene as well as ethyl iodide was a reaction product of hydroxyethylcellulose and hydriodic acid. This made possible the first reliable determination of materials containing hydroxyethyl groups as well as ethers and esters of ethylene glycol. His work paved the way for many valuable advances in the analysis of glycol ethers. In studying tosylated hydroxyethylcellulose Tasker and Purves (*10*) analyzed their reaction products for ethylene oxide content. They reported values as low as 1.49%, but no degree of accuracy was indicated. Cohen and Haas (*1*) analyzed hydroxyethylcellulose with substitution values as low as 0.44 mole or about 10%. Gloor, Mahlman, and Ulrich (*4*) also used this method to measure the amount of substitution of hydroxyethylcellulose. Haas, Cohen, Oglesby, and Karlin (*5*) prepared hydroxyethyl derivatives of nylon and found this method useful in determining the ethylene oxide content of the poly(ethylene) glycol branches. By analyzing a suitable low molecular weight analog of *N*-hydroxyethyl nylon, they found that the carbon-nitrogen bond is not cleaved by this method; thus the combined ethylene oxide group attached directly to the amide nitrogen is not attacked. Cohen and Haas (*2*) applied this method satisfactorily to hydroxyethyl nylon and tosylated hydroxyethyl nylon.

The modifications of apparatus and methods outlined in this paper may be used for low-substituted ethers of materials other than starch, such as cellulose. Ethers and esters of ethylene glycol or the types of material mentioned by Morgan (*7*) which have less than 5% substitution may be analyzed readily by this method. In general alkyl groups of greater chain length than propyl, such as hydroxybutyl ether, do not give sufficiently volatile alkyl iodides to be distilled over from the constant-boiling hydriodic acid. Satisfactory determinations were made on hydroxypropyl starch ether and the degree of substitution was calculated by using the appropriate molecular weight values.

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LITERATURE CITED

(1) Cohen, S. G., Haas, H. C., *J. Am. Chem. Soc.* **72**, 3954 (1950).
(2) Cohen, S. G., Haas, H. C., *J. Polymer Sci.* **11**, 193 (1953).
(3) Elek, A., IND. ENG. CHEM., ANAL. ED. **11**, 174 (1939).
(4) Gloor, W. E., Mahlman, B. H., Ulrich, R. D., *Ind. Eng. Chem.* **42**, 2150 (1950).
(5) Haas, H. C., Cohen, S. G., Oglesby, A. C., Karlin, E. R., *J. Polymer Sci.* **15**, 427 (1955).
(6) Kesler, C. C., Hjermstad, E. T. (to Penick and Ford, Ltd., Inc.), U. S. Patents **2,516,632, 2,516,633, 2,516,634** (July 25, 1950).
(7) Morgan, P. W., IND. ENG. CHEM., ANAL. ED. **18**, 500 (1946).
(8) Samsel, E. P., McHard, J. A., *Ibid.*, **14**, 750 (1942).
(9) Steyermark, Al, ANAL. CHEM. **20**, 368 (1948).
(10) Tasker, C. W., Purves, C. B., *J. Am. Chem. Soc.* **71**, 1023 (1949).
(11) Volhard, J., *Ann.* **190**, 23 (1878); *J. prakt. Chem.* **117**, 217 (1874).

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Modified Ultraviolet Spectrophotometric Micromethod for Determination of Amino Acids and Peptides as Copper Complexes

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The usefulness of the Spies spectrophotometric micromethod for determining amino acids and peptides has been confirmed. The method has been simplified, the blank value has been reduced approximately two thirds, and the accuracy with small samples (below 1 micromole) has been increased.

THE need for a micromethod to determine tyrosine and other amino acids in mixtures of amino acids and peptides led to a study of the Spies (*1*) ultraviolet spectrophotometric procedure. This method appeared to offer distinct advantages in that it is simple and rapid, requires small samples (0.5 to 5 micromoles), distinguishes between free amino acids and peptides, and yields reasonably accurate values for copper-reacting nitrogen. As in preliminary experiments the results with samples below 1 micromole were less dependable than had been anticipated, the sources of error were ascertained and an improved method was devised.

EXPERIMENTAL

The following method of analysis was employed by Spies (*1*).

To 5.0 ml. of buffer solution in a 25-ml., glass-stoppered Erlenmeyer flask were added 5.0 ml. of a water solution of an appropriate concentration of the test substance; 0.1 ml. of copper chloride solution was then added, and the flask was shaken and let stand for 10 minutes at a convenient room temperature, the same temperature (±1°) being used for all determinations. The suspension was centrifuged in a capped 12-ml. tube for 5 minutes, and the clear solution was carefully decanted into a clean flask. Transmittancy of the solution was determined at 230 mμ. Control solutions were made by adding 5.0 ml. of the test solution to 5.0 ml. of buffer solution. A control analysis of freshly prepared alanine solution was made with each series of tests to check for possible slight variations in the absorbancy due to the distilled water or other causes.

Spies' procedure was modified by adding 0.1 ml. of distilled water to the control solution to compensate for the 0.1 ml. of cupric chloride solution added to the test solution, employing aliquots of only one solution (all reagents except cupric chloride) in preparing both the control and test solutions, centrifuging (International centrifuge Model 1C) at 3000 r.p.m., approximately 1800 × gravity, and increasing the centrifuging time from 5 to 20 minutes. Absorbances were determined using 1-cm. cells. The effect of centrifuging times from 5 to 60 minutes on absorbances was investigated.

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Table I. Ultraviolet Absorbances of Copper Complexes of Certain Amino Acids and Peptides

Substance[a]	Alanine Equivalence[b] at 235 Mμ, %	Absorbance Maximum, Mμ	Wave-Length Range[c], Mμ
Alanine	100	235	234–238
Arginine[d]	112	235	234–238
Aspartic acid	93	236	233–238
Cystine	84	239	234–243
Glutamic acid	103	236	233–238
Glycine	93	232	229–233
Histidine[d]	92	255	225–255
Hydroxyproline	91	245	235–250
Isoleucine	100	238	234–240
Leucine	107	237	234–239
Lysine[d]	108	235	233–238
Methionine	108	237	233–239
Phenylalanine	88	240	235–253
Proline	87	240	235–253
Serine[e]	97	236	233–238
Threonine	103	235	233–239
Tryptophan	ca. 110[f]	[f]	232[g]
Tyrosine	ca. 87[f]	235	233–236
Valine	102	235	234–238
Alanylglycine	72	208	207–236[g]
Glycylleucine[e]	73	210	207–236[g]

[a] Two samples of each compound (A.P. grades of Amino Acid Manufactures and H. M. Chemical Co.) were weighed quantitatively and four replicate solutions of each compound were prepared at each of two concentrations (150 and 300μ*M* for cystine and peptides; 300 and 600μ*M* for all others).
[b] Concentration of solutions of cystine and peptides was 150μ*M* and of all other compounds 300μ*M*. It was assumed that the latter substances react with copper to form principally complexes of the general formula CuA_2, and cystine and peptides CuA.
[c] Absorbances of all compounds except tryptophan and peptides were within 1% of that at 235 mμ.
[d] Hydrochloride was used, but absorbance was calculated for free amino acid.
[e] Not tested by Spies.
[f] Accurate determination was difficult, owing to high absorbance and impossibility of adjusting the blank to zero absorbance of the control.
[g] Absorbances could not be measured accurately, especially at lower wave lengths.

RESULTS AND DISCUSSION

Photometric readings (Beckman DU spectrophotometer) deviated 0.1 to 5%, with 10% deviation in three cases. The blank absorbance values at 230 mμ were reduced from an average of 0.086 obtained with Spies' procedure to as low as 0.024 and an average of 0.034 with the authors' modified method. All compounds except tyrosine and tryptophan obeyed Beer's law from zero to the highest concentration tested. The authors' data are shown in Table I.

The sharp rise in the blank absorbance value observed by Spies (*1*) is eliminated by application of the authors' modified procedure and the wave length of maximum absorbance of the copper-alanine complex is shifted from 230 to 235 mμ, in agreement with the corrected curve (Figure 2) of Spies. Shifts of the absorbance maxima ranging from 1 to 30 mμ were observed for the copper complexes of 17 other amino acids and two peptides. On the other hand, the copper alanine equivalences found for these compounds at 235 mμ diverged by less than 10% from those at 230 mμ reported by Spies.

The reduction in the blank absorbance values is explained, apparently, by the more complete removal of suspended cupric hydroxide. It was found, by inspecting the clarified solutions in a Tyndall beam, that filtration on a funnel fitted with paper or medium porosity fritted glass was as effective as centrifugation in removing suspended cupric hydroxide. The filtrates, however, gave erratic absorbance values, apparently due to absorbing materials extracted from the filter paper or the fritted glass.

The present authors' results confirmed those of Spies in unsuccessful attempts to determine combined copper of amino acids and peptides as the copper salt of alanine by an ultraviolet spectrophotometric adaptation of the visible photometric procedure of Spies and Chambers (*2*).

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The authors are indebted to Joseph R. Spies for valuable suggestions (*3*).

LITERATURE CITED

(1) Spies, J. R., *J. Biol. Chem.* 195, 65 (1952).
(2) Spies, J. R., Chambers, D. C., *J. Biol. Chem.* 191, 787 (1951).
(3) Spies, J. R., private communication, Sept. 28, 1953.

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Investigation of the Franke Method of Determining Free Calcium Hydroxide and Free Calcium Oxide

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The Franke method (or acetoacetic ester method) for the determination of uncombined calcium hydroxide and calcium oxide in the presence of hydrated and anhydrous substances was investigated. Repeated extractions gave the correct values for calcium hydroxide in the presence of a calcium silicate hydrate in which the calcium was firmly bound; however, some calcium was removed from another calcium silicate hydrate in which the binding was weaker. A modification of the method gave accurate results for free lime in anhydrous substances, such as portland cements. The precision of the Franke method was about the same as that of the Lerch and Bogue method (or glycerol-alcohol method), but the former presented certain advantages.

IN THE investigations of the kinetics and thermodynamics of the hydration of the calcium silicates, conducted at this laboratory, one of the problems was to obtain a reliable method for the determination of calcium hydroxide in the presence of hydrated calcium silicates. This problem has received considerable attention in the past two decades because of its importance in the investigations of portland cements, pozzolanic cements, and other industrial materials. The main difficulty is that the analytical methods proposed for the determination of uncombined calcium hydroxide involve its solution in some organic solvent, and this is usually accompanied by the solution of some of the combined calcium.

The difficulties may be illustrated with two examples taken from the literature. In 1938 Bessey (*4*) reported uncombined calcium hydroxide values for four hydrated portland cements

determined by four different methods. Two of the methods involved dissolving the uncombined calcium hydroxide in organic solvents: the glycerol-alcohol method of Lerch and Bogue (*10, 14*) and the ethylene glycol method of Schläpfer and Bukowski (*16*). The other two methods, proposed by Bessey himself, did not involve the use of organic solvents. The values obtained by the four methods ranged from 13.7 to 16.5% calcium oxide for the cement which gave the narrowest range, and from 10.7 to 18.4% calcium oxide for the cement which gave the widest range. (It is customary to report the uncombined calcium hydroxide as per cent calcium oxide, rather than per cent calcium hydroxide.) The glycerol-alcohol method gave the highest uncombined calcium hydroxide value for two of the cements and the lowest for one; the ethylene glycol method gave the highest value for one of the cements and the lowest for one. As the true values of calcium hydroxide in the hydrated portland cements were unknown, it was impossible to tell whether any of the methods gave values close to the true values.

In 1954 Assarsson (*3*) reported his investigations on the determination of uncombined calcium hydroxide in the presence of calcium silicate hydrates. He investigated four methods: the glycerol-alcohol method, using barium chloride as an accelerator (*6*), the same with strontium nitrate as an accelerator (*9*), the ethylene glycol method, and the acetoacetic ester–isobutyl alcohol method of Franke (*11*). He prepared calcium silicate hydrates by hydrothermal reaction between calcium oxide and silica gel at 120°, 180°, and 240° C. He found that the organic solvents always attacked the calcium silicate hydrates; the hydrates prepared at lower temperatures and with higher molar CaO/SiO_2 ratios were attacked more than the others. The worst case was that of the hydrate prepared with a molar CaO/SiO_2 ratio of 1.5 at 120° C.; the four methods, in above order, gave values of 11.5, 13.0, 16.5, and 18.5% calcium oxide in one extraction, and 13.6, 15.4, 20.5, and 22.6% calcium oxide in four extractions.

In the present work, primarily the method of Franke was investigated, because it was found in early tests that it gave better results for uncombined calcium hydroxide in the presence of a calcium silicate hydrate prepared at room temperature than the ASTM standard test (*2*) (essentially the method of Lerch and Bogue), or the modification of that test proposed by Bogue and Lerch (*5*). A modified Franke method gave accurate results for calcium hydroxide in the presence of a calcium silicate hydrate in which the calcium was firmly bound, but the solvents attacked the calcium when it was weakly bound. The glycerol-alcohol method, without modifications, usually gave too low results for the first case and as unreliable results as the Franke method for the second. Probably, with proper modifications, this method would also have given accurate results for the first case; however, that problem was not investigated. It is doubtful whether the problem can be solved for the second case by means of organic solvents. The only safe method is to determine the calcium hydroxide *in situ*, which can be done, for example, by means of x-ray line intensity measurements (*7*).

The applicability of a modification of the Franke method to the determination of free lime in anhydrous systems was also investigated. The method gave results in good agreement with the results of the ASTM standard test, and it presented certain advantages over the latter.

CALCIUM SILICATE HYDRATE-CALCIUM HYDROXIDE MIXTURES

The mixtures used in these experiments were prepared by hydrating tricalcium silicate in two ways: in a small steel ball mill and in the form of "paste." The term "paste", as used by cement chemists, and as used here, means a plastic or semifluid mixture of a hydraulic material, such as portland cement or tricalcium silicate, with water. After a few hours the paste sets and then hardens; the present investigations were carried out on hardened pastes.

The experiments were conducted in a constant temperature room, kept at 23° ± 0.5° C. In the ball mill hydration an H_2O/Ca_3SiO_5 weight ratio of 9 was used. Complete hydration was obtained in 6 days, and the reaction progressed according to the equation

$$2Ca_3SiO_5 + 6H_2O = Ca_3Si_2O_7.3H_2O(A) + 3Ca(OH)_2 \quad (1)$$

The calcium silicate hydrate, $Ca_3Si_2O_7.3H_2O(A)$, was afwillite. The paste was hydrated for 2 years, at the end of which it contained only about 0.5% unhydrated tricalcium silicate. The reaction took place according to the equation

$$2Ca_3SiO_5 + 6H_2O = Ca_3Si_2O_7.3H_2O(T) + 3Ca(OH)_2 \quad (2)$$

The calcium silicate hydrate produced, $Ca_3Si_2O_7.3H_2O(T)$, was similar in many respects to the natural mineral tobermorite, and it will be called tobermorite. The products of both types of hydration were identified by x-ray diffraction patterns, differential thermal analysis curves, and density and index of refraction measurements. These experiments were reported by Brunauer, Copeland, and Bragg (*7*).

The tricalcium silicate preparations contained approximately 96% tricalcium silicate, 3% dicalcium silicate, and 1% of other impurities, including some free calcium oxide. In the calculations of the theoretical calcium hydroxide contents of the hydrated materials, it was assumed that β-dicalcium silicate hydrated according to the equation

$$2Ca_2SiO_4 + 4H_2O = Ca_3Si_2O_7.3H_2O + Ca(OH)_2 \quad (3)$$

There is evidence in the literature (*8*) that (1) the number of molecules of water reacting with one molecule of dicalcium silicate is one less than the number reacting with one molecule of tricalcium silicate and (2) the number of molecules of calcium hydroxide produced in the hydration of dicalcium silicate is one less than the number produced by one molecule of tricalcium silicate. This indicates the stoichiometry represented by Equation 3.

Pure tricalcium silicate, completely hydrated, should produce 39.36% calcium hydroxide in the hydration products according to either Equation 1 or 2. Expressed in terms of calcium oxide and the ignited weight of the hydration products, the theoretical uncombined lime content is 36.85%. Because the tricalcium silicate preparations contained a few per cent of dicalcium silicate, the theoretical values were usually between 35 and 36%. It will be shown that repeated extractions by the modified Franke method gave values close to the theoretical for the ball mill–hydrated materials, but greater than the theoretical values for the paste-hydrated materials. Apparently, the calcium in afwillite is firmly bound, whereas tobermorite gives up at least a part of its calcium to the organic solvents.

EXPLORATORY TESTS BY FRANKE METHOD

Franke (*11*) proposed the following method for the determination of uncombined calcium oxide and calcium hydroxide.

Place a finely ground sample, weighing 0.05 to 1.0 gram, in a 200-ml. Erlenmeyer flask; add 3 ml. of acetoacetic ester and 20 ml. of isobutyl alcohol; and reflux for 1 hour on a hot plate, protecting the system with soda lime and calcium chloride to prevent entry of moisture or carbon dioxide. Cool the mixture and filter with suction, using a dense filter. Wash the flask and residue with 20 ml. of isobutyl alcohol. The calcium oxide or calcium hydroxide that was originally uncombined is now in the filtrate, and it can be determined by various methods.

For extracting uncombined calcium hydroxide from hydrated materials, which might decompose at the refluxing temperature of the solvent mixture, Franke recommended the addition of ether to the solvent solution. He did not specify the quantity of ether to be used.

For the determination of the calcium in the filtrate, Franke

proposed three alternative methods. The method he favored consisted in mixing 20 ml. of methanol with the filtrate, and titrating with 0.1*N* hydrochloric acid, using bromophenol blue as the indicator. The other methods involved precipitation of the calcium as the oxalate or the sulfate.

The results of preliminary tests on samples of ball mill–hydrated tricalcium silicate indicated incomplete recovery of the uncombined calcium hydroxide in one extraction even when higher ratios of solvent volume to sample size were used than Franke recommended. Because of this, the residues were returned to the extraction flasks, and repeated extractions were made with like volumes of new solvent solutions. Frequently, three or more extractions were needed to recover all of the uncombined calcium hydroxide. After various volumes and proportions had been tested, a mixture of 9 ml. of acetoacetic ester (ethyl acetoacetate), 60 ml. of isobutyl alcohol, and 15 ml. of ether was adopted as standard solvent for samples of 1 gram or less. For larger samples, the volumes of the solvents were increased proportionately.

Table I. Uncombined Calcium Hydroxide in Ball Mill–Hydrated Tricalcium Silicate (D-11)

(Reported as % calcium oxide on ignited weight basis)

	Volumetric			Gravimetric		
First extraction	35.66	35.46	35.71	35.26	35.02	35.36
Second extraction	0.24	0.28	0.31	0.41	0.36	0.48
Total	35.90	35.74	36.02	35.67	35.38	35.84
Average		35.89			35.63	
Theoretical			35.74			

The refluxing temperature of the above mixture was about 70° C. In some of the present work ether was omitted; this, according to Franke, gave a refluxing temperature of 106° C. Refluxing periods longer than 1 hour gave higher recoveries of uncombined calcium hydroxide; the practice of refluxing for 3 hours was, therefore, adopted.

For the determinations of the calcium in the filtrate the method of titration with 0.1*N* hydrochloric acid was first tried; however, the end point was too uncertain. The results were checked, therefore, by precipitating the calcium in the filtrate as the oxalate after the titration, and weighing as the oxide. The gravimetric results were in fair agreement with the volumetric results, but the method was cumbersome; it seemed desirable, therefore, to improve the volumetric method by the use of other indicators and acids. It became apparent that, for best results, both the indicator and the acid should be contained in nonaqueous media compatible with the extract solutions (*1, 13*). A solution of thymol blue in isobutyl alcohol showed a satisfactory color change from yellow to red; and perchloric acid, dissolved in isobutyl alcohol, held a stable titrating value for 2 months and longer. A 0.2*N* solution of perchloric acid in isobutyl alcohol was adopted as standard for the titration, with thymol blue as the indicator.

RESULTS ON BALL MILL–HYDRATED TRICALCIUM SILICATE

Afwillite, the calcium silicate hydrate produced in the ball mill–hydration of tricalcium silicate, is not attacked appreciably by the Franke solvents. Eight batches were hydrated in the ball mill and the uncombined calcium hydroxide was determined by repeated Franke extractions.

The results obtained for the first batch, D-11, are shown in Table I. Triplicate samples of approximately 0.2 gram each were used, and the uncombined calcium hydroxide in each sample was determined volumetrically and gravimetrically. Theoretical value was based on Equations 1 and 3.

Another batch of ball mill–hydrated tricalcium silicate, D-23, was divided into 16 samples of approximately 1 gram each, and a Franke extraction was performed on each sample. The 16 residues were then combined into four samples, and two more extractions were performed on each of the combined residues. After the third extraction, the x-ray powder diffraction patterns showed no calcium hydroxide lines. The results are shown in Table II.

Franke used only a single extraction for the determination of uncombined calcium hydroxide. As Table II shows, the first extraction did not remove all the uncombined calcium hydroxide from any of the 16 samples; actually, on the average, only 94% was removed. This was true in spite of the fact that the volume of solvents used was three times as large as that proposed by Franke, and the refluxing period was three times as long. The total uncombined calcium hydroxide obtained in three extractions was 99.4% of the theoretical value. On the basis of the results of the three extractions, a fourth extraction would, probably, have yielded only a slight additional amount of calcium hydroxide.

The results on a third batch of ball mill–hydrated tricalcium silicate, D-21, are shown in Table III. Eight samples were used, approximately 2 grams each. After the first extraction the residues were combined for samples 1 to 4 and for samples 5 to 8, and the second and third extractions were performed on the combined residues. The calcium hydroxide obtained in the third extraction was not much less than that obtained in the second; it is possible, therefore, that subsequent extractions would have indicated some decomposition of the afwillite by the Franke solvents.

On another portion of the same batch of hydrated tricalcium silicate, D-21, Franke extractions were performed at room temperature. Instead of being refluxed, the mixture was shaken on a rotating table for 24 hours. Determinations were made on 24 samples of about 1 gram each. After the first extraction the residues were combined into five samples, and a second extraction was performed on them, under the same conditions. The total obtained in the two extractions was 35.33%. The carbon dioxide content of the residue after the two cold extractions was less than one third of that of the residue after three hot extractions. Thus the cold method appeared promising at first; however, it was found that its applicability was limited. In the determination of the uncombined calcium hydroxide content of paste hydrated tricalcium silicate, seven cold extractions gave a lower value than two hot extractions; and in the investigation of anhydrous materials (portland cements, tricalcium silicate, and dicalcium silicate) a single hot extraction gave the correct free lime value, whereas a single cold extraction always gave too low a value.

Table II. Uncombined Calcium Hydroxide in Ball Mill–Hydrated Tricalcium Silicate (D-23)

(Reported as % calcium oxide on ignited weight basis)

	Gravimetric Method							
Sample	1	2	3	4	5	6	7	8
First extraction	33.65	33.91	34.23	35.71	34.49	35.10	34.97	34.69
			34.23				34.80	
Second extraction			1.31				0.70	
Third extraction			0.26				0.29	
Total			35.80				35.79	
Sample	9	10	11	12	13	14	15	16
First extraction	33.40	33.92	33.39	33.16	32.40	33.14	33.34	34.50
			33.51				33.31	
Second extraction			1.85				2.15	
Third extraction			0.30				0.32	
Total			35.66				35.78	
Total of three extractions				35.76				
Theoretical value				35.98				

Table III. Uncombined Calcium Hydroxide in Ball Mill-Hydrated Tricalcium Silicate (D-21)

(Reported as % calcium oxide on ignited weight basis)

Sample	Gravimetric Method 1	2	3	4	5	6	7	8
First extraction	34.51	34.20	34.41	35.06	34.44	34.61	34.24	34.87
Second extraction	0.49	0.49	0.49	0.49	0.57	0.57	0.57	0.57
Third extraction	0.31	0.31	0.31	0.31	0.38	0.38	0.38	0.38
Total	35.31	35.00	35.21	35.86	35.39	35.56	35.19	35.82
Total of three extractions				35.42				
Theoretical value				35.30				

Table IV. Uncombined Calcium Hydroxide in Paste-Hydrated Tricalcium Silicate (C-13)

(Reported as % calcium oxide on ignited weight basis)

Sample	Gravimetric Method 1	2	3	4	5	6	7	8
First extraction	27.46	26.62	27.17	27.09	27.72	27.50	25.78	27.12
Second extraction	8.98	8.98	8.98	8.98	8.13	8.13	8.13	8.13
Third extraction	1.98	1.98	1.98	1.98	2.30	2.30	2.30	2.30
Fourth extraction	1.59	1.59	1.59	1.59	1.79	1.79	1.79	1.79
Total	40.01	39.17	39.72	39.64	39.94	39.72	38.00	39.34
Total of four extractions				39.45				
Theoretical value				35.24				

For one batch of ball mill-hydrated tricalcium silicate, D-14, an independent check was obtained on the total uncombined calcium hydroxide extracted. First, the total calcium oxide content of the batch was determined on duplicate samples by the standard ASTM procedure (*2*). The batch was then divided into nine samples, and four Franke extractions were performed on each sample. The residues from the nine samples were combined and homogenized; and the total calcium oxide was determined on duplicate samples by the standard ASTM method. The total calcium oxide in the residue thus obtained was 58.79%. The calculated total calcium oxide in the residue, obtained by subtracting the total uncombined lime removed in the 36 extractions from the initial total lime, was 58.77%.

There is no need to report the experiments performed on other batches of ball mill-hydrated tricalcium silicate, since the results and conclusions were similar to those already discussed. Repeated Franke extractions, either hot or cold, gave results close to the theoretical values; but a single extraction always gave too low a value.

RESULTS ON PASTE-HYDRATED TRICALCIUM SILICATE

Tobermorite, the calcium silicate hydrate produced in the paste hydration of tricalcium silicate, is attacked by the Franke solvents (Table IV). Approximately 2-gram samples of batch C-13 were used. After the first extraction, the residues were combined for samples 1 to 4 and for samples 5 to 8; and three other extractions were performed on the combined residues. The uncombined calcium hydroxide obtained in the four extractions was 39.45%; the theoretical value, according to Equations 2 and 3, was 35.24%. The first extraction gave values far below the theoretical; the second extraction gave a total of 35.55%, which was slightly above the theoretical value. The high values obtained in the fourth extraction clearly indicated that additional extractions would have resulted in further decomposition of tobermorite.

On another batch of paste-hydrated tricalcium silicate, C-14, two Franke extractions were performed. The total uncombined calcium hydroxide removed corresponded approximately to the stoichiometric amount, but the x-ray diffraction pattern of the residue still showed distinctly the lines of calcium hydroxide. This indicated that the Franke solvents had begun to decompose tobermorite before all the uncombined calcium hydroxide was removed. In spite of this fact, however, the uncombined calcium hydroxide in the original hydrated mixture can be evaluated by a roundabout method, utilizing the experimental fact that the Franke solvents attack tobermorite much more slowly than they attack the calcium hydroxide. The method, discussed below, gave an uncombined calcium hydroxide content of 1.43 moles per mole of silica for a batch of paste-hydrated tricalcium silicate, C-19. The theoretical value, based on Equations 2 and 3, was 1.45 moles.

Four samples of paste-hydrated tricalcium silicate, C-19, were taken, and 42 successive Franke extractions were performed on each sample. The results are given in Table V, column 2; each value in this column is the average obtained for the four samples. Seventy milliliters of the solvent mixture were used in each extraction, consisting of acetoacetic ester and isobutyl alcohol in the proportion of 3 to 20. No ether was used, except in the 13th extraction, in which 15 ml. of ether were added to

Table V. Extraction of Calcium Hydroxide from Paste-Hydrated Tricalcium Silicate (C-19)

(Reported as % calcium oxide on ignited weight basis, volumetric method)

1 Extraction No.	2 % Removed: Extraction	3 % Removed: Cumulative	4 Moles Removed: Extraction, ΔC	5 Moles Removed: Cumulative	6 Moles Remaining	7 Corr. for Impurities	8 Moles Remaining (Corr.), C_{II}	9 K
0	0.00	0.00	0.0000	0.0000	2.9788	0.0354	2.9434	...
1	23.09	23.09	0.9388	0.9388	2.0399	0.0732	1.9667	0.4773
2	9.89	32.98	0.4022	1.3410	1.6378	0.0895	1.5483	0.2598
3	1.93	34.91	0.0784	1.4194	1.5593	0.0927	1.4666	0.0535
4	1.22	36.13	0.0496	1.4690	1.5097	0.0947	1.4150	0.0351
5	0.95	37.08	0.0387	1.5077	1.4711	0.0960	1.3751	0.0281
6	0.79	37.87	0.0321	1.5398	1.4390	0.0976	1.3414	0.0239
7	0.68	38.55	0.0276	1.5674	1.4113	0.0984	1.3129	0.0208
8	0.70	39.25	0.0285	1.5959	1.3828	0.0996	1.2832	0.0220
9	0.63	39.88	0.0256	1.6215	1.3572	0.1008	1.2564	0.0202
10	0.65	40.53	0.0264	1.6479	1.3308	0.1016	1.2292	0.0212
11	0.61	41.14	0.0249	1.6728	1.3060	0.1029	1.2031	0.0205
12	0.55	41.69	0.0223	1.6951	1.2836	0.1037	1.1799	0.0187
13	0.40	42.09	0.0163	1.7114	1.2674	0.1045	1.1629	0.0139[a]
14	0.55	42.64	0.0223	1.7337	1.2450	0.1053	1.1397	0.0194
15	0.54	43.18	0.0220	1.7557	1.2231	0.1061	1.1170	0.0195
16	0.58	43.76	0.0236	1.7793	1.1995	0.1069	1.0926	0.0214
17	0.67	44.43	0.0272	1.8065	1.1722	0.1082	1.0640	0.0126[b]
18	0.48	44.91	0.0195	1.8260	1.1527	0.1090	1.0437	0.0185
19	0.34	45.25	0.0139	1.8399	1.1389	0.1094	1.0295	0.0134
20	0.34	45.59	0.0138	1.8537	1.1251	0.1102	1.0149	0.0135
21	0.21	45.80	0.0085	1.8622	1.1165	0.1106	1.0059	0.0084
22	0.37	46.17	0.0151	1.8773	1.1015	0.1110	0.9905	0.0151
23	0.27	46.44	0.0110	1.8883	1.0905	0.1114	0.9791	0.0112
24	0.21	46.65	0.0085	1.8969	1.0820	0.1118	0.9702	0.0087
25	0.22	46.87	0.0089	1.9057	1.0730	0.1122	0.9608	0.0092
26	0.34	47.21	0.0139	1.9196	1.0592	0.1126	0.9466	0.0146
27	0.34	47.55	0.0138	1.9334	1.0454	0.1134	0.9320	0.0147
28	0.33	47.88	0.0134	1.9464	1.0320	0.1138	0.9182	0.0145
29	0.25	48.13	0.0102	1.9570	1.0218	0.1143	0.9075	0.0112
30	0.32	48.45	0.0130	1.9700	1.0088	0.1147	0.8941	0.0144
31	0.27	48.72	0.0110	1.9810	0.9978	0.1151	0.8827	0.0124
32	0.32	49.04	0.0130	1.9940	0.9848	0.1159	0.8689	0.0148
33	0.28	49.32	0.0114	2.0054	0.9734	0.1163	0.8571	0.0132
34	0.32	49.64	0.0130	2.0184	0.9604	0.1167	0.8437	0.0153
35	0.34	49.98	0.0138	2.0322	0.9466	0.1171	0.8295	0.0165
36	0.29	50.27	0.0118	2.0440	0.9348	0.1179	0.8169	0.0143
37	0.26	50.53	0.0105	2.0545	0.9242	0.1183	0.8059	0.0129
38	0.21	50.74	0.0086	2.0631	0.9157	0.1183	0.7974	0.0107
39	0.15	50.89	0.0061	2.0692	0.9096	0.1187	0.7909	0.0076
40	0.17	51.06	0.0069	2.0761	0.9027	0.1191	0.7836	0.0088
41	0.24	51.30	0.0098	2.0859	0.8929	0.1195	0.7734	0.0126
42	0.27	51.57	0.0109	2.0968	0.8819	0.1199	0.7620	0.0142

[a] 15 ml. of ether included in solvent.
[b] 140 ml. of solvent used.

the solvent. In the 17th extraction 140 ml. of solvent were used, instead of 70 ml.

The Franke solvents react with calcium hydroxide much more rapidly than with tobermorite; consequently, after a certain number of extractions only a negligible amount of calcium hydroxide remains in the system, and thereafter all of the extracted calcium comes from the tobermorite. It will be assumed that the rate of extraction of calcium from tobermorite follows a first-order law—i.e., the rate of removal is proportional to the amount present in the solid, or

$$-\frac{dc}{dt} = kc \tag{4}$$

where c is the quantity in the tobermorite.

In one extraction only about 2% of the lime is removed from the tobermorite; the approximation may, therefore, be used

$$-\overline{\frac{dc}{dt}} = k\frac{C_I + C_{II}}{2} \tag{5}$$

where $\overline{\frac{dc}{dt}}$ is the average rate during the extraction, and C_I and C_{II} are the initial and final quantities of lime in the solid. The amount of lime extracted during the 3 hours of extraction, ΔC, is proportional to the average rate; consequently,

$$\Delta C = K\frac{C_I + C_{II}}{2} \tag{6}$$

In Table V, columns 4, 8, and 9 show the values of ΔC, C_{II}, and K, respectively, for each extraction. For any given extraction, C_I is equal to the C_{II} of the previous extraction. The quantities in columns 4 to 8 are expressed in units of moles per mole of silica.

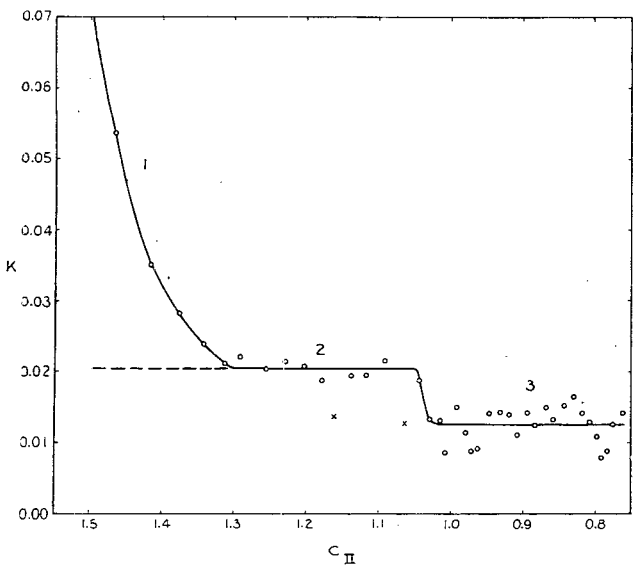

Figure 1. Variation of K with C_{II}

Figure 1 shows the variation of K with C_{II}. The K values for the first two extractions are not shown. The points obtained for the 13th and 17th extractions, in which the solvent was altered, are represented by crosses. Three regions can be distinguished on the curve. In the first region K drops sharply in each extraction. This is the region in which the uncombined calcium hydroxide was removed from the hydrated paste. From the seventh to the 18th extraction the value of K is approximately constant. This constant, K_2, is a characteristic of tobermorite only; its value is 0.0202 ± 0.0013.

If it is assumed (1) that in the first six extractions the reaction between the solvent and tobermorite progressed simultaneously with the reaction between the solvent and uncombined calcium hydroxide, and (2) that both reactions were sufficiently far from equilibrium to proceed without influencing each other, one can calculate the molar CaO/SiO_2 ratio in the tobermorite prior to the first extraction. Combining Equation 6 with the equation

$$\Delta C = C_I - C_{II} \tag{7}$$

and using the value $K_2 = 0.0202$, one obtains

$$C_I = 1.020\ C_{II} \tag{8}$$

At the end of the sixth extraction, the molar CaO/SiO_2 ratio in the tobermorite was 1.34. According to Equation 8, the molar CaO/SiO_2 ratio prior to the first extraction was $(1.020)^6(1.34) = 1.51$. Since the total CaO/SiO_2 ratio in the hydrated paste was 2.94, the moles of calcium hydroxide per mole of silica was 1.43. The theoretical value, as stated earlier, was 1.45 moles.

After the 18th extraction, K dropped to a lower value, as shown in Figure 1. The transition from the second to the third region may not have been so sharp as drawn in Figure 1; it is not difficult to visualize a more gradual transition on the basis of the experimental points. The value of K in the third region, as drawn, is 0.0126; however, the experimental points are too scattered to establish definitely that K is constant in this region. Nevertheless, it seems clear that some changes had occurred in the tobermorite (possibly a partial conversion to afwillite). Further experiments would be needed to clarify this point, but such experiments would have little or no bearing on the subject of the present investigations, the determination of uncombined calcium hydroxide.

LERCH AND BOGUE DETERMINATIONS ON HYDRATED TRICALCIUM SILICATE

For comparison, the uncombined calcium hydroxide contents of several batches of ball mill– and paste–hydrated tricalcium silicate were determined by means of the Bogue and Lerch (*5*) modifications of the method of Lerch and Bogue (*10, 14*). This consists in heating the material at 540° C. for 1 hour, then applying the standard ASTM procedure (the method of Lerch and Bogue) for free lime determination. Seven samples of a batch of ball mill–hydrated tricalcium silicate, D-15, gave the results 31.29, 30.60, 32.66, 29.65, 31.11, 30.49, and 32.38% calcium oxide; the average was 31.17% calcium oxide. These values were lower than those obtained in the first Franke extraction, which is in agreement with the findings of Assarsson (*3*). Repeated extractions were not attempted, but it seems likely that repeated extractions with glycerol-alcohol would also have led to the correct results for ball mill–hydrated tricalcium silicate.

On another batch of ball mill–hydrated tricalcium silicate, D-21, similar determinations were made; however, instead of the recommended heating at 540° C. for 1 hour, two samples were heated at 500° and two at 600° C. for 1 hour. The results were 33.69 and 33.31% calcium oxide for the former, and 33.36 and 33.06% for the latter. The Franke extraction results for this batch were given in Table III. (The samples were not heated prior to the Franke extractions.) In this instance again the Lerch and Bogue results were lower than those obtained in the first Franke extraction. However, this was not true in every case. Occasionally, the Lerch and Bogue determination gave a higher value than the first Franke extraction, and in one instance it gave as high as the theoretical uncombined calcium hydroxide value.

On some samples of ball mill–hydrated tricalcium silicate, the uncombined calcium hydroxide was determined by the Lerch and Bogue method, without initial heating. The values obtained were approximately the same as those obtained with initial

Table VI. Free Calcium Oxide in Anhydrous Substances

1	% Free Calcium Oxide						
	2	3	4	5	6	7	8
		Franke Method					
		Solvent 9-60-15		Solvent 9-60-0		70 Ml. Solvent	
Substance	Lerch and Bogue method (ASTM standard)	Vol.	Grav.	Vol.	Grav.	Vol.	Grav.
Portland cement I, Lot 18716	3.71	3.72	3.65	3.88	3.74	3.78	3.78
	3.85	3.87	3.66	3.77	3.80	3.81	3.75
	3.79						
Av.	3.78	3.80	3.66	3.83	3.77	3.80	3.77
Portland cement II, Lot 18961	2.25	2.35	2.28	2.40	2.40	2.38	2.31
	2.13	2.38	2.34	2.39	2.36	2.37	2.32
Av.	2.19	2.37	2.31	2.40	2.38	2.38	2.32
Portland cement III, Lot 15366	1.30	1.33	1.29	1.24	1.28	1.18	1.11
	1.14	1.18	1.15	1.23	1.21	1.22	1.13
Av.	1.22	1.26	1.22	1.24	1.25	1.20	1.12
Tricalcium silicate, Lot 18943	1.20	1.27	1.26	1.30	1.24	1.20	1.15
	1.16	1.14	1.08	1.26	1.24	1.21	1.19
Av.	1.18	1.21	1.17	1.28	1.24	1.21	1.17
Portland cement IV, Lot 17596	1.20	1.34	1.24	1.12	1.06	1.29	1.24
	1.02	1.26	1.23	1.25	1.25	1.26	1.17
	1.18						
Av.	1.13	1.30	1.24	1.19	1.16	1.28	1.20
Portland cement V, Lot 18822	0.60	0.60	0.50	0.67	0.58	0.61	0.57
	0.57	0.63	0.54	0.73	0.56	0.60	0.40
	0.63						
Av.	0.60	0.62	0.52	0.70	0.57	0.61	0.49
β-Dicalcium silicate, Lot 18944	0.11	0.19	0.10	0.22	0.21	0.22	0.16
	0.09	0.19	0.10	0.23	0.16	0.22	0.17
Av.	0.10	0.19	0.10	0.23	0.19	0.22	0.17

heating at 540° C.; some values were slightly lower, others somewhat higher. In contrast to this, heating made a great difference in the values obtained for paste-hydrated tricalcium silicate.

On a batch of tricalcium silicate, hydrated in the paste form for about 2.5 years (batch C-13) and then ball milled for several days, the uncombined calcium hydroxide content was determined by the Lerch and Bogue method, without initial heating. The results on duplicate samples were 31.35 and 30.79% calcium oxide. These values are greater than those obtained in the first Franke extraction, but smaller than those obtained in two extractions. Heating other samples first at 540° C. for 1 hour led to much lower values, 18.96 and 17.67% calcium oxide. It is clear that the heating resulted in the removal of a part of the uncombined calcium hydroxide. Calculations based on some results of Bogue and Lerch (*5*) and of Meyers (*15*) together with some results obtained in the present investigations indicated that tobermorite and calcium hydroxide reacted with each other according to the equation

$$Ca_3Si_2O_7.3H_2O(T) + 3Ca(OH)_2 = 2Ca_2SiO_4 + 2CaO + 6H_2O \quad (9)$$

Actually, the uncombined calcium hydroxide obtained for batch C-13 after heating was only 59% of that obtained prior to heating, instead of the 67% indicated by Equation 9. However, correction for carbonation during heating accounted almost quantitatively for the discrepancy.

In another set of experiments on paste-hydrated tricalcium silicate, batch C-19, not subjected to subsequent ball milling, the uncombined calcium hydroxide obtained after heating was 74% of that obtained before heating. Apparently, in this case, the reaction represented by Equation 9 did not progress to completion during the 1-hour heating.

As indicated by the results cited for ball mill–hydrated tricalcium silicate, afwillite did not react appreciably with calcium hydroxide in the temperature range 500° to 600° C. At higher temperatures, however, reaction did take place. Batch D-15 gave an average of 31.17% calcium oxide after heating at 540° C. for 1 hour; two other samples of batch D-15, heated at 950° C. for 1 hour, gave values of 19.78 and 18.70% calcium oxide. This corresponds to about 62% of the value obtained after heating at 540° C.

The Franke extraction results for ball mill–hydrated tricalcium silicate, batch D-23, are given in Table II. Two samples of the same batch were heated at 1000° C. for 1 hour, and then the uncombined calcium hydroxide was determined by the Franke method. The first extraction gave 22.04 and 22.02% calcium oxide, the second 0.62 and 0.70%, and the third 0.04 and 0.02%; the totals were 22.70 and 22.74%. The experimental value, prior to heating, was 35.76%. Thus, after heating, the uncombined calcium hydroxide content was 64% of that before heating. These experiments indicate that at higher temperatures a reaction took place between afwillite and calcium hydroxide, probably according to the equation

$$Ca_3Si_2O_7.3H_2O(A) + 3Ca(OH)_2 = 2Ca_2SiO_4 + 2CaO + 6H_2O \quad (10)$$

Whereas tobermorite and calcium hydroxide reacted with each other in the temperature range 500° to 600° C., afwillite and calcium hydroxide reacted at 900° to 1000° C.

From the above experiments it may be concluded that (1) a single Lerch and Bogue determination, without previous heating, usually gives a low value for uncombined calcium hydroxide in the hydrated systems investigated; and (2) heating of the hydration products is not safe; it may alter the uncombined calcium hydroxide content of the system.

RESULTS ON ANHYDROUS MATERIALS

Cooperative studies of methods of determining free lime in portland cements show wide variations in the results obtained by different laboratories for the same cements. Hanna, Hicks, and Saeger (*12*) reported on a cooperative study in which 37 laboratories participated; six modifications of the glycerol-alcohol method and two modifications of the ethylene glycol method were tried on five different portland cements. The free lime values for the same cement and by the same method differed by factors of 2 to 10, and even greater, from laboratory to laboratory. Because of such difficulties, it seemed worth while to try the Franke method for the determination of free lime in portland cements.

The results for seven anhydrous substances are shown in Table VI. Three modifications of the Franke method were investigated. In one of these the solvent consisted of 9 ml. of acetoacetic ester (ethyl acetoacetate), 60 ml. of isobutyl alcohol, and 15 ml. of ether (columns 3 and 4); in the second the ether was omitted, and a fresh solvent mixture was made up for each determination (columns 5 and 6); in the third a larger quantity of the solvent mixture, consisting of acetoacetic ester and isobutyl alcohol in the proportion of 3 to 20, was made up, and after standing for 1 month or longer, 70-ml. portions were used for the free lime determinations (columns 7 and 8). Accurately measured 1-gram samples of the materials were used; and the free lime, after the extraction, was determined first volumetrically and then gravimetrically for each sample. Duplicate or triplicate Lerch and Bogue (ASTM standard) determinations were performed on each substance.

First the Franke volumetric results will be considered. Comparison of columns 3 and 5 shows that omission of ether increased the free lime values of the seven substances by 0.03% on the average. This is not significant; consequently, the ether can be safely omitted, and the hazards accompanying its use in industrial laboratories can thus be avoided. Comparison of columns 5 and 7 shows that the use of the old solvent in place of the freshly prepared solvent decreased the free lime values of the seven substances by 0.03%, on the average. This, again, is not significant; consequently, for convenience, the solvent can be made up in quantity. Apparently, no segregation of the components or deterioration of the solvent mixture occurs even during 2 months of standing.

Comparison between the Franke volumetric and gravimetric results shows a systematic difference; the latter, on the average, are 0.06% smaller than the former. The gravimetric determination was always made on the solution after the volumetric determination; and the lower values are due to incomplete recovery of the calcium under the experimental conditions. This point was further investigated on two portland cements. Three samples of each cement were extracted; first, volumetric, then gravimetric determinations were made on each; the six ignited precipitates of calcium oxide were then extracted; and again first volumetric then gravimetric determinations were made. The results are shown in Table VII. Free lime quantities, averaging 0.09%, were lost in each operation. Losses 1 and 2 indicate that the volume of Franke solvents used retains roughly 1 mg. of calcium oxide in solution. Loss 2 may have been due to a slight carbonation of the samples during extraction or filtration. The treatment of the samples prior to the initial titration parallels that of the samples of calcium oxide used for standardizing; hence the first titration should give a correct evaluation of the quantities of calcium oxide in the filtrates.

Comparison of columns 2 and 7 of Table VI shows that the Franke method gave 0.10% higher free lime values, on the average, than the standard ASTM method (*2*). This difference is significant only for substances having free lime contents of the order of a few tenths of 1%. The results indicate that a single Franke extraction recovers all the free lime for anhydrous substances having free lime contents of 4% or less. The Lerch and Bogue method and the Franke method appear to be about equally precise; and the good agreement between the values obtained by the two methods indicates that they measure the free lime contents accurately.

Although the above data show that the Franke method presents no significant improvement in precision over the ASTM method for the determination of free lime in portland cement, it seems to present certain other advantages worth consideration. The method is more rapid, and it requires only supervisory attention during the extraction period. The filtration provides a clear solution for titration; consequently, there is less uncertainty in the end point. In some cases it may also be an advantage that the Franke method avoids the use of alcohol requiring a government permit. The Franke reagents are less common and more expensive than the Lerch and Bogue reagents, but they are not hazardous for use. The use of 60% perchloric acid in the titration solution is not considered hazardous, as it is not heated. The adoption of the method would require additional equipment for filtration with vacuum.

A cooperative study of the Franke method would ascertain its reproducibility from laboratory to laboratory, and it seems desirable that such a study be undertaken.

For the convenience of those investigators who may wish to test the method, the essential requirements are outlined below.

MODIFIED FRANKE METHOD FOR DETERMINATION OF FREE LIME IN PORTLAND CEMENTS

Reagents. Acetoacetic ester (ethyl acetoacetate), anhydrous grade, such as Eastman 111.

Isobutyl alcohol, anhydrous grade, such as Eastman 303.

Thymol blue indicator; chemical name, thymol sulfonphthalein.

Perchloric acid, C.P., 60%.

Solution Preparations. Solvent, 450 ml. of ethyl acetoacetate and 3000 ml. of isobutyl alcohol.

Indicator, 0.1 gram of thymol blue indicator powder dissolved in 100 ml. of isobutyl alcohol.

Titration, for approximately 0.2*N* perchloric acid 21.8 ml. of 60% perchloric acid is made up to 1000 ml. with isobutyl alcohol.

The method of standardizing the acid solution follows, in principle, the ASTM method of standardizing ammonium acetate solutions, C114-53; Sec. 31(c). In the present work ignited iceland spar was used as the source of calcium oxide, and its purity was determined by dissolving 0.1-gram samples in a hydrochloric acid solution and precipitating and weighing according to ASTM method C114-53; Sec. 13(b) and (c). For titration, companion samples of approximately 0.1 gram were used and they were extracted with 70 ml. of the prepared Franke solvent solution. The extraction, filtration, and titration operations were performed as described below for samples of cements.

Equipment. Water-cooled condensers, preferably with standard-taper inner joints to fit 200-ml. Erlenmeyer flasks; and upper adapter tubes to fit absorption tubes containing soda-lime and Ascarite.

Büchner-type fritted borosilicate glass disk filtration funnel of 350-ml. capacity and F grade porosity.

Equipment for vacuum filtration.

Table VII. Losses in Franke Determinations

Substance	First Titration, %	First Weight, %	Loss 1, %	Second Titration, %	Loss 2, %	Second Weight, %	Loss 3, %
Portland cement I, Lot 18716	3.87	3.66	0.21	3.65	0.01	3.50	0.15
	3.77	3.80	−0.03	3.68	0.12	3.53	0.15
	3.81	3.75	0.06	3.59	0.16	3.62	−0.03
Av.	3.82	3.74	0.08	3.64	0.10	3.55	0.09
Portland cement V, Lot 18822	0.63	0.54	0.09	0.50	0.04	0.47	0.03
	0.73	0.56	0.17	0.48	0.08	0.43	0.05
	0.60	0.40	0.20	0.33	0.07	0.27	0.06
Av.	0.65	0.50	0.15	0.44	0.06	0.39	0.05
Combined averages			0.12		0.08		0.07

Procedure. Sieve and grind the sample of cement according to the standard ASTM procedure for free lime determination. Measure 70 ml. of the prepared solvent solution containing acetoacetic ester and isobutyl alcohol in the proportion of 3 to 20, and transfer into a 200-ml. Erlenmeyer flask. Weigh accurately 1 gram of the prepared sample of cement and transfer into the flask.

Adjust the flask in position to a water-cooled condenser, fitted with an upper adapter tube containing soda-lime and Ascarite, and reflux at boiling temperature on a hot plate for 3 hours. Remove the flask, stopper, and cool, then filter with vacuum on a 350-ml. fritted disk filter funnel, receiving the filtrate in a second flask. Wash the flask and residue with 50 ml. of isobutyl alcohol, using a policeman to guide the flow.

Add 10 to 12 drops of the indicator solution to the filtrate and titrate with the standard 0.2*N* perchloric acid solution to a distinct reddish tinge.

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LITERATURE CITED

(1) Acree, S. F., Fawcett, E. H., IND. ENG. CHEM., ANAL. ED. 2, 78 (1930).
(2) Am. Soc. Testing Materials Standards on Cement, C114-53, Sec. 31 and 32, May 1954.
(3) Assarsson, G., *Zement-Kalk-Gips* 7, 167 (1954).
(4) Bessey, G. E., Proc. Symposium on Chemistry of Cements, Stockholm, 1938, p. 285, Ingeniörsvetenskapsakademien, Stockholm, 1939.
(5) Bogue, R. H., Lerch, W., *Ind. Eng. Chem.* 26, 837 (1934).
(6) Brandenburg, H. R., *Rock Products* 34, 68 (March 1931).
(7) Brunauer, S., Copeland, L. E., Bragg, R. H., *J. Phys. Chem.* 60, 112, 116 (1956).
(8) Brunauer, S., Hayes, J. C., Hass, W. E., *Ibid.*, 58, 279 (1954).
(9) Dennis, J. W., *Rock Products* 41, 43 (December 1938).
(10) Emley, W. E., *Trans. Am. Ceram. Soc.* 17, 720 (1915).
(11) Franke, B., *Z. anorg. allgem. Chem.* 247, 180 (1941).
(12) Hanna, W. C., Hicks, T. A., Saeger, G. A., *ASTM Bull.* 94, 47 (October 1938).
(13) Kolthoff, I. M., Rosenblum, C., "Acid-Base Indicators," p. 325, Macmillan, New York, 1937.
(14) Lerch, W., Bogue, R. H., IND. ENG. CHEM., ANAL. ED. 2, 296 (1930).
(15) Meyers, S. L., *Pit and Quarry* 35, 97 (July 1942).
(16) Schläpfer, P., Bukowski, R., "Eidgenössische Matèrialprüfungsanstalt an der E. T. H. Zürich," Report No. 63, Zurich, 1933.

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Qualitative and Semiquantitative Tests for Amine Hydrochlorides on Paper Chromatograms

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Two simple tests based on reaction of chloride ion with silver ion were devised in order to locate and obtain a semiquantitative estimate of amine hydrochloride spots on paper chromatograms. Spots which contain as little as 3 γ of chloride ion can be located by the qualitative test. The semiquantitative test, based on titration of the chloride ion, showed 88% recovery with good precision. The tests require only that the amines be in the hydrochloride form; they apparently are not dependent upon the amine structure.

ALTHOUGH mixtures of mono- and diamine hydrochlorides can be readily separated by paper chromatography, there is no generally applicable test for the qualitative and semiquantitative determination of these materials. The colorimetric tests summarized by Block and others (*1*) and Lederer and Lederer (*3*) were found not entirely suitable, because they were either time-consuming or did not give a satisfactory test for both the mono- and diamines. The tests described here are based on the detection of chloride ion by reaction with silver ion. Qualitatively, the amine hydrochloride spots are detected by the reduction of silver chloride to metallic silver. The chloride content of the spots from a separate chromatogram may be titrated by the sensitive potentiometric procedure of Kolthoff and Kuroda (*2*).

QUALITATIVE TEST

After the paper chromatogram has been developed to give the desired separation of amine hydrochlorides, the solvent is allowed to evaporate either spontaneously or with gentle heating. When the paper appears dry, it is uniformly and lightly sprayed with a 1% ammoniacal silver nitrate solution. Care should be taken to keep the reagent from running; otherwise the amine spots will blur. The wet paper is first dipped in 10% aqueous acetic acid and then in fresh distilled water. Black metallic silver is deposited in the amine hydrochloride spots upon exposing the wet paper to ultraviolet light.

Table I. Analysis of Pyrrolidine-Ethylenediamine Mixtures

Pyrrolidine in Sample			
Known, %	Added, γ	Found, γ	Recovery, %
7.6	64.6	48.8	78.5
14.1	118.5	84.8	74.4
19.7	164.1	145.6	92.2
24.7	203.1	176.8	90.5
33.0	266.6	242.0	94.4
45.1	355.5	321.7	94.1
73.2	546.9	487.7	92.7

Table II. Precision of Semiquantitative Test

Silver Nitrate[a], Ml.		Pyrrolidine.HCl	
Paper blank	Pyrrolidine.HCl spot (uncorr.)	Found, γ	Found in sample, %
0.28	6.73	356	22.1
0.28	6.78	358	22.2
0.31	6.75	355	22.0
0.31	6.61	347	21.6
0.31	6.65	350	21.7
0.25	6.78	360	22.4
0.25	7.17	382	23.7
0.25	6.61	351	21.8

[a] Silver nitrate, $5.13 \times 10^{-4} N$.

SEMIQUANTITATIVE TEST

The amine hydrochloride spots are cut out of the paper chromatogram, using the silver spot test on a duplicate chromatogram as a guide. R_f values may be used as guides, although they do not give information concerning the area or shape of the spot to be excised. The paper cutout is placed in a 20-ml. beaker containing 5 ml. of 0.1M potassium nitrate which is stirred by a slow stream of nitrogen. Approximately $5 \times 10^{-4} N$ standard aqueous silver nitrate is used to titrate the chloride ion from the paper. The potentiometric technique of Kolthoff and Kuroda (*2*), using the silver–silver chloride indicator electrode and the saturated calomel electrode as reference, is used to follow the titration.

A paper blank of nearly identical area is analyzed in the same manner. The blank cutout is taken from an area of the chromatogram which contains no solutes but which was exposed to the developing solvent.

RESULTS

Some data from the determination of pyrrolidine in synthetic mixtures of pyrrolidine (Matheson) and ethylenediamine (Eastman Kodak) hydrochlorides are given below to illustrate the application of these procedures. The qualitative spot test was used to establish that separation of these salts could be made at 25° C. on strips of Whatman No. 1 paper with water-saturated butanol as the developing solvent. With the ascending technique, complete separation resulted after the solvent traveled 13 cm. In this system the hydrochlorides of pyrrolidine and ethylenediamine exhibited R_f values of 0.30 and 0.01, respectively.

A series of known mixtures was analyzed by the semiquantitative technique, with the results shown in Table I. Between 750 and 850 γ of the various mixtures in 10 μl. of solution was spotted on the paper for each analysis.

An average recovery of 88.1% with a standard deviation of 8.2% was obtained for the mixtures in Table I. No effort was made to correct the cause of incomplete recovery other than to apply a 3.8% calibration correction for the 10-μl. micropipet. A series of samples containing smaller amounts of pyrrolidine was also analyzed. These results showed that as little as 3 γ of chloride ion could be detected by the qualitative and semiquantitative tests. In the above case, this would represent 0.4 mole % pyrrolidine in 15 μl. of a 1M amine solution.

The reproducibility of the semiquantitative test was found to be surprisingly good, as shown in Table II. These data represent eight analyses for one component in a single unknown amine mixture. A total of 1610 γ of the amine hydrochloride mixture in 10 μl. of solution was applied to each paper strip. The first column in Table II lists the blank values which were used to correct the titration results. A standard deviation of 0.7% was calculated from these data.

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LITERATURE CITED

(1) Block, R. J., LeStrange, R., Zweig, F., "Paper Chromatography," 1st ed., Academic Press, New York, 1952.
(2) Kolthoff, I. M., Kuroda, P. K., ANAL. CHEM. **23**, 1304 (1951).
(3) Lederer, E., Lederer, M., "Chromatography," 1st ed., Elsevier, New York, 1953.

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Chamber Size and R_f Values of Amino Acids

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Because of the extreme importance of R_f values as physical constants, it is unfortunate that they are not more reproducible. This study was undertaken in an attempt to determine the effect of chamber size on the R_f values of amino acids. The term, "critical volume," has been introduced to define the volume of solvent necessary to saturate a specific chamber under a given set of experimental conditions. It has been found that when the amount of developing solvent used exceeds the critical volume, the R_f values will be constant and at a minimum; below the critical volume the values will be at a constant maximum. Notation of these volumes in future communications would greatly aid in the standardization of paper chromatography.

THE importance of paper chromatography as a research tool is unquestioned. In many instances, the R_f value has become as important a physical constant as the melting point. It is unfortunate, therefore, that it is frequently extremely difficult, if not impossible, to reproduce published R_f values with a given solvent system. This difficulty is generally circumvented by a comparison of the R_f value of a standard sample with the R_f of the compound in question. Unfortunately, such an approach is not always possible.

No attempt will be made in this communication to survey the literature completely with respect to variables in paper chromatography. Strain's review article on chromatography (*8*), as well as the articles by Cassidy (*2*), Kowkabany and Cassidy (*4*), and Block, Le Strange, and Zweig (*1*) serve well in this capacity. These authors discuss the influence of such factors as type of paper, pH, purity of solvents, and temperature. Landua, Fuerst, and Awapara (*5*) reported on the effect of the pH of the sample on the observed R_f values of amino acids. McFarren (*6*), working with buffered developing solvents, showed the variation of R_f values of amino acids with the pH of the developing solvent. Underwood and Rockland (*7, 9*) reported on the effect of the aforementioned factors on small scale paper chromatography. However, even though all these variables be rigidly controlled, R_f values, with a given solvent system, have been found to vary as much as 50% from one laboratory to another. A marked deviation in R_f values of amino acids with varying chamber sizes was first reported by Clayton and Strong (*3*). The present work was undertaken in an attempt to elucidate further the relationships between chamber and solvent volumes and R_f values. The common *n*-butyl alcohol–acetic acid–water system was chosen for this study.

EXPERIMENTAL

Preparation of Samples. The following amino acids were used in this study: L-tyrosine, DL-methionine, DL-leucine, DL-tryptophan, L-cysteine, L-cystine, L-arginine, DL-alanine, L-histidine, DL-valine, L-glutamic acid, DL-aspartic acid, and glycine. Approximately 20 mg. of each amino acid was dissolved in 3.0 meq. of hydrochloric acid and diluted to 10 ml. with water.

Operating Procedure. Whatman No. 1 paper was used as supplied by the manufacturer. Two to 4 γ of each amino acid was applied to the paper at the base line at points 1 cm. apart. Ascending chromatograms were developed for 12 to 16 hours with a solvent system which was prepared by equilibrating 300 ml. of *n*-butyl alcohol, 300 ml. of water, and 72 ml. of glacial acetic acid in a separatory funnel. After thorough shaking, the two-phase system was allowed to stand for 15 minutes; the upper layer was then used. Because on standing this solvent system propagates a fourth component, butyl acetate, the solvent was always prepared immediately before use. In order to study the influence of chamber size on R_f values, three papers were developed simultaneously in different-sized chambers of volumes of 37, 14, and 8 liters, hereafter referred to as chambers *A*, *B*, and *C*, respectively. The distance from the surface of the developing solvent to the base line was kept constant. All experimental work was carried out in a constant temperature room at 25° ± 1° C.

After development, the air-dried chromatograms were sprayed with a butyl alcohol solution of ninhydrin (0.125%) in the usual manner. Over 100 chromatograms were run (13 amino acids per paper) in an attempt to clarify the influence of chamber size on R_f values.

RESULTS AND DISCUSSION

To report over 1300 R_f values would, in this author's opinion, be inadvisable. In an attempt to present these data in a clear and concise manner, the results obtained with five representative amino acids under varying conditions of chamber size and solvent volume have been reported. The effect of chamber size on R_f values is typified by the data in Table I. From these data, it appears that when a given volume of solvent (150 ml.) is used in each chamber, the R_f values of the amino acids increase as the volume of the chamber increases. These increases were fairly constant with the 13 amino acids studied, varying from 20 to 27%.

The effect of varying the solvent volume with a given chamber was studied next. The results of such a study as carried out with chamber *A* are shown in Table II. These data show that the R_f values are constant and at a maximum when 300 ml. of solvent (or less) is used, and are at constant minima when more than 375 ml. of solvent is used. This volume of solvent, above which the R_f value is at a minimum, and below which it is at a maximum, has been termed the critical volume.

When such a study was made with chamber *B*, no discrete critical volume was found. When less than 75 ml. of developing solvent was used, R_f values were at constant maxima, in good agreement with the maxima obtained in chamber *A*. Above 175 ml. of developing solvent, the values were at the same constant minima, as observed in the larger chamber. However, between 75 and 175 ml., the R_f values were approximately midway between the maxima and minima. At first it was thought that

Table I. Effect of Chamber Volume on R_f Values

(150 ml. of developing solvent)

	$R_f \times 100$		
	A (37 liters)	*B* (14 liters)	*C* (8 liters)
Leucine	81	71	67
Tryptophan	61	56	51
Methionine	62	55	49
Valine	64	55	52
Tyrosine	50	48	41

Table II. Effect of Solvent Volume on R_f Values in Different Chambers

	Chamber *A*					Chamber *B*					
	Ml. of Solvent					Ml. of Solvent					
	150	300	375	400	700	50	75	100	150	175	200
	$R_f \times 100$					$R_f \times 100$					
Leucine	81	80	68	66	67	79	78	73	71	65	66
Tryptophan	61	61	54	53	54	60	62	57	56	53	53
Methionine	62	63	51	49	50	62	65	54	55	49	49
Valine	64	65	51	51	51	63	62	56	55	52	50
Tyrosine	50	50	44	44	44	50	51	48	48	43	43

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Table III. Constancy of R_f Values with Respect to Critical Volumes

	Above Critical Volume			Below Critical Volume	
	A (375 ml.)	*B* (175 ml.)	*C*	*A* (350 ml.)	*B* (50 ml.)
	$R_f \times 100$				
Leucine	68	65	67	81	79
Tryptophan	54	53	51	61	60
Methionine	51	49	49	62	62
Valine	51	52	52	64	63
Tyrosine	44	43	41	50	50

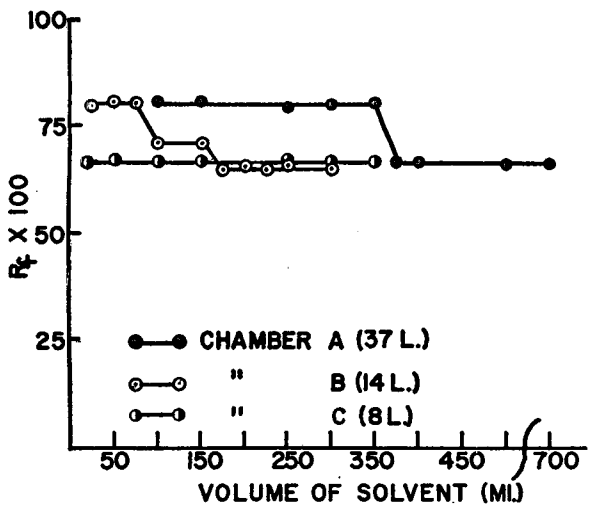

Figure 1. Comparison of R_f values of leucine in three different-sized chambers with varying amounts of developing solvent

Maximum R_f, 81; minimum, 65

these differences were not significant, but repeated investigation showed otherwise. These data are also presented in Table II.

When the effect of varying the solvent volume was studied with the smallest chamber, it was found that the critical solvent volume for this 8-liter chamber was so small that it was, for all practical purposes, always exceeded. Thus, when as little as 20 ml. of solvent was used, the R_f values for the 13 amino acids studied were at the same constant minima as obtained in the two larger chambers.

Figure 1 shows the variation in R_f value of the amino acid leucine in the three chambers with different amounts of developing solvent. The maximum and minimum values obtained in the three chambers are in good agreement, but the critical solvent volume differs markedly according to the volume of the chamber.

If these variations in R_f values are due to critical volumes alone, then one should be able to observe constant values in any chamber, regardless of its volume, if the critical volume of that chamber is known. The constancy of R_f values with respect to critical volumes is shown in Table III. In this table, the R_f values obtained in the three different chambers are compared when the volume of solvent in each chamber is either above or below the critical volume of that chamber.

Pre-equilibration and Chamber Volumes. The effect of pre-equilibration on chamber size phenomena was investigated. With descending paper chromatography, probably the most common equilibration technique is to suspend the papers in a sealed chamber above a given volume of solvent for 2 to 4 hours, and then to introduce additional solvent into the trough. Because the results of such a study would depend on the amounts of developing solvent used—i.e., whether it was above or below the critical volume of the chamber—this technique could not be used in connection with this work. Instead, the paper was suspended above the developing solvent in a sealed chamber for 3 hours, after which it was lowered into the developing fluid. Development time was varied from 7 to 13 hours with no significant R_f differences with a given volume of solvent. The effect of pre-equilibration was studied in the three chambers when the amount of developing solvent was above and below the critical volumes of the chambers. When the amount of solvent was above the critical volumes of the three chambers, there were no significant differences between the values obtained with or without pre-equilibration. Of the 13 amino acids tested, only tyrosine and tryptophan showed a variation with pre-equilibration. The R_f values of these two amino acids increased about 8% when pre-equilibrated. No significant change was observed with the other amino acids tested.

Likewise, when the amount of solvent used was less than the critical volume of chamber *A*, pre-equilibration had no effect on the R_f values. However, with chamber *B* there was a marked decrease in R_f values for leucine, methionine, and valine under these conditions. Slight decreases were also observed for tryptophan and tyrosine, but it is questionable whether these changes are significant. These data are presented in Table IV.

It is difficult to rationalize these results. It is known that butyl acetate forms under the experimental conditions within a short time, and it might be argued that the introduction of relatively large amounts of this fourth component at the beginning of the developing period would be expected to alter observed values. It also seems tenable that such an alteration could result in variations with some amino acids and not with others. However, one must also explain the lack of variation in R_f values with and without pre-equilibration in chambers *A* and *C*, and indeed, the absence of a pre-equilibration effect in chamber *B* when the volume of solvent was above the critical volume of that chamber. It is felt that these pre-equilibration studies only emphasize the importance of chamber size because the variations in R_f values caused by these differences in chamber volumes cannot be circumvented by pre-equilibration.

THEORY

With most water-soluble compounds, the R_f values increase as the water content of the developing solvent is increased. In this system, butyl alcohol is much more volatile than water, and unequal vaporization of the components of the solvent system results. Because the composition of the vapor and liquid phases at equilibrium is dependent on the partial pressures and mole fraction of each component, factors which would favor removal of the most volatile component would result in a developing liquid richer in water. When a given volume of solvent is used, the larger the chamber, the greater the vaporization of the butyl alcohol, and the richer the liquid phase becomes with respect to water. Below the critical volume, unequal vaporization of the solvent components results in a developing liquid richer in water, and maximum R_f values are observed. The constancy of R_f values below the critical solvent volume must be ascribed to the partition coefficients of the amino acid between the mobile and stationary phases, and to the maximum amount of water which can be held by the paper under the prevailing experimental conditions.

Table IV. Effect of Pre-equilibration on R_f Values in Chamber B

(50 ml. of solvent)

	No Equilibration	3-Hour Equilibration
	$R_f \times 100$	
Leucine	79	68
Tryptophan	60	56
Methionine	62	52
Valine	63	52
Tyrosine	50	47

SUMMARY

The relationship between chamber volume and solvent volume has been investigated. A new term, critical volume, has been introduced which defines the volume of solvent necessary to saturate a specific chamber under a given set of experimental conditions. The R_f values of amino acids and presumably of other water-soluble compounds (preliminary work with carbohydrates gave results similar to those reported here) vary with a given solvent system according to the volume of the chamber, the volume of developing solvent, and the volatility of the organic solvent. (In preliminary work only slight variations in the R_f values of amino acids were observed when a less volatile organic solvent—i.e., phenol-water—was used.) These variations are not eliminated by pre-equilibration. It is felt that the most reproducible chromatograms can be obtained when small chambers are used because the amount of solvent generally exceeds the critical volume of the chamber. In view of this evidence, the difficulty in reproducing R_f values from one laboratory to another might be greatly mitigated if authors report chamber and solvent volumes as part of the description of experimental conditions.

LITERATURE CITED

(1) Block, R. J., Le Strange, R., Zweig, G., "Paper Chromatography, A Laboratory Manual," Academic Press, New York, 1952.
(2) Cassidy, H. G., ANAL. CHEM. 24, 1415 (1952).
(3) Clayton, R. A., Strong, F. M., *Ibid.*, 26, 1362 (1954).
(4) Kowkabany, G. N., Cassidy, H. G., *Ibid.*, 22, 817 (1950).
(5) Landua, A. J., Fuerst, R., Awapara, J., *Ibid.*, 23, 162 (1951).
(6) McFarren, E. F., *Ibid.*, 23, 168 (1951).
(7) Rockland, L. B., Underwood, J. C., *Ibid.*, 26, 1557 (1954).
(8) Strain, H. H., *Ibid.*, 23, 25 (1951).
(9) Underwood, J. C., Rockland, L. B., *Ibid.*, 26, 1553 (1954).

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Radiotracer Studies of Analytical Methods for Styrenated Oil Acids and Esters

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Most analytical methods for styrenated paint vehicles fail to separate neutral polystyrene and the oil-acid-styrene copolymer. Armitage and Kut's method for such fractionation, which depends on differential solubility of the copolymer's calcium soaps and neutral polystyrene in wet ethyl acetate, was investigated using radiotracer techniques in polymers prepared from styrene tagged on the alpha-carbon with carbon-14. This procedure does not always work. Apparently no method yet exists for determining polymeric species without supplementary analysis of the separated fractions. Fractionation data suggest that extensive copolymerization of styrene and fatty acids can occur, but that its extent varies with conditions of resin synthesis.

STYRENE drying oil reaction products have been used commercially in paint vehicles for more than 10 years, but the precise chemical nature of these "copolymer oils" is yet unknown. Process conditions of manufacture greatly alter their paint formulation properties, and exact reproducibility of polymerization recipes is a serious problem. Studies of these factors have been hindered by the lack of precise analytical methods for fractionating and estimating the amount of homopolymer and copolymer in the product oils.

Various fractionation schemes have been proposed. Kappelmeier's (*4*) extensive studies led him to conclude that very little of the drying oil acid escapes reaction with styrene, and probably very little neutral polystyrene is formed—i.e., polystyrene unreacted with fatty acid. On the other hand, Armitage and Kut (*1*) and others (*6*) claim to have found a fractionation technique, which, at least for the reaction product of β-eleostearic acid and styrene, shows the presence of considerable quantities of nearly neutral polystyrene. If the latter claim is correct, the Armitage-Kut fractionation procedure might be a useful supplement to the Kappelmeier method of analysis of styrenated oils and alkyds. This would be true if neutral polystyrene and the acid copolymer can always be separated in commercial polymers, and if the Armitage-Kut procedure effects clean-cut separation. Because of the importance of this issue to analytical methods for polymerization research and production control, the combined Kappelmeier-Armitage-Kut procedures were investigated by special techniques using radiotracer carbon-14.

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SYNTHESIS OF RADIOSTYRENE

The styrene was synthesized by carbonation of benzyl magnesium chloride, followed by reduction of the resulting phenylacetic acid to the alcohol with lithium aluminum hydride, and subsequent dehydration of the phenyl ethyl alcohol with molten potassium hydroxide.

The procedure followed in the preparation of the phenylacetic acid was similar to that of Dauben, Reid, and Yankwich (*2*). Carbon-14 was introduced in the carbon dioxide used to carbonate the Grignard reagent. The carbon dioxide was generated by addition of concentrated sulfuric acid to 14.8 grams of barium carbonate which included 0.052 gram of barium radiocarbonate having a total activity of 2.51 millicuries of carbon-14. The barium radiocarbonate was placed on the inactive carbonate without mixing, so that the radiocarbon dioxide would react with the Grignard during the early stages of chemical combination when the rate of reaction was high and near theoretical yields were possible in recovering the carbon-14. Grignard reagent from 12.7 grams (0.1 mole) of benzyl chloride was carbonated. To ensure complete carbon dioxide generation, the acid slurry was heated until gas evolution stopped. The carbonated Grignard was alternately frozen in dry ice–acetone and thawed three times until the initial pressure of 400 mm. in the reaction flash dropped to 3 to 4 mm. of mercury. Phenylacetic acid was obtained by acidification of the reaction product with hydrochloric acid. The acid was purified by crystallization from the water solutions obtained on repeated extraction and separation from the ether layers.

Phenyl ethyl alcohol was prepared by reduction of phenylacetic acid with lithium hydride according to the procedure of Nystrom and Brown (*5*). The crude radioactive phenyl ethyl alcohol was directly converted to styrene by addition of 10 drops per minute to molten potassium hydroxide at 225° ± 5° C. at a pressure of about 100 mm. of mercury. A small amount of picric acid was present to inhibit polymerization. After the reaction, the system was purged with three 2- to 3-ml. portions of pure, inactive phenyl ethyl alcohol. After distillation of the azeotrope stopped, the crude distillate of wet styrene was diluted with ether, dried over magnesium sulfate, and filtered. The dry product was distilled at 60° to 61° C. under 40 mm. of vacuum, with hydroquinone present in the still pot. The still was flushed by distilla-

Table I. Fractionation of Fatty Acid Styrene Reaction Products

Sample No.	1	2	3	4	5	6	7[a]	8[b]
Distillation Products								
A. Reaction product, grams	86.80	317	403	403	72.16	75.74	539	510
B. Distillate from A, grams	24.0	23.0	116	54.4	18.82	35.5	...	...
C. Radioassay-styrene in B, grams	22.8	23.0	86	45.0	18.90	35.0	....	...
% styrene in B	95	100	74	83	100	100	...	...
D. Resin distillation residue of A, grams	62.7	273	270	346	52.8	40.5	539	510
E. Radioassay-styrene in D, grams	11.2	143	114	155	21.2	5.0	236	228
% styrene in D	18	53	43	45	40	11	46	45
Moles styrene per mole acid in E	0.59	3.0	2.0	2.2	2.0	0.38	2.0	2.2
Ethyl acetate solubles in residue								
F. D retained in EtOAc, Fraction IV, grams	48.6	242	195	64.3[b]	18.1	22.1	146[b]	165[b]
D retained in Fraction IV, %	77.5	88.6	72.0	18.6	34.2	54.5	27.1	32.3
G. Radioassay-styrene in F	11.0	140	109	74.4	12.3	2.8	184	196
H. Acids in F (F-G), grams	37.6	102	86	...	5.8	19.3	...	...
% styrene in F	23	58	56	>90	67	12	>90	>90
Moles styrene per mole acid in F	0.80	3.7	3.4	<0.3	5.9	0.40	<0.3	<0.3
Ether solubles in residue								
I. D recovered in Et_2O, Fraction V, grams	10.3	13.8	80	271	33.8	18.4	342	296
D recovered in Fraction V, %	16.4	5.1	29.6	78.3	64.0	45.5	63.5	38.0
J. Radioassay-styrene in Fraction V	0.0	0.0	3.1	58.9	8.4	2.2	46.7	57.4
K. Acids in I (I–J), grams	10.3	13.8	77	212	25.4	16.2	295	239
% styrene in I	0	0	3.9	22	25	12	14	20
Material balances								
% sample recovered, $\frac{F+I}{D} \times 100$	93.9	93.7	101.6	96.9	98.2	100.0	90.6	90.3
% styrene recovered, $\frac{G+J}{E} \times 100$	98	98	98	86	98	100	98	110
% acid recovered, $\frac{H+K}{D-E} \times 100$	93	90	104	102	99	100	97	85

[a] Fatty acid–polystyrene mixture recovered after removal of phthalic anhydride and glycerol by Kappelmeier method (4). Radioassay showed no styrene loss into any analytical reagents used in the analysis of phthalic, nor in the phthalic precipitate, or in water from which acids are recovered by neutralizing K soaps.

[b] Determination of nonvolatile content of samples containing less than 10% fatty acid is prone to serious error. Such samples seem to contain considerable low molecular weight polymer or are unstable in vacuum and mild heat (80° C.), and cannot be brought to constant weight. These low figures which do not fit the material balance resulted by procedures that seemed satisfactory for the other resins. Here data in rows H are estimated minimal and maximal values.

tion of inactive Dow styrene which was added to the radioactive distillate. An over-all yield of about 40% was obtained, while a slightly higher yield was indicated by radioactivity measurements.

SYNTHESIS OF RADIOSTYRENE RESINS

Prior to laboratory synthesis of the radioactive resins, a number of runs with dehydrated castor fatty acid, utilizing inactive styrene, were made to determine reaction conditions. After the proper reaction conditions were established, several runs were made with radioactive styrene. Synthesis methods are described below. Resin numbers correspond to the data of Table I.

The syntheses were performed in three-necked, glass flasks fitted with agitator, thermometer, condenser, and nitrogen sparger, and heated with electric mantles.

The starting composition of reaction mixtures for resins 1 to 4 contained equal parts by weight of styrene and dehydrated castor oil fatty acids (Woburn Chemical Co , isoline acids), with cumene hydroperoxide as catalyst (1.6% of styrene content). The synthesis schedules varied. For resins 1 and 3 the reaction mixture was warmed to about 145° C., where the exothermic reaction started, the flask was cooled just sufficiently to permit vigorous reflux without flooding of the spiral condenser with styrene monomer, and the reaction mixture was cooled after the exothermic reaction was spent. In the instance of resins 2 and 4, following the exothermic reaction the mixture was maintained at reflux by raising the temperature gradually to about 160° C. for 2 hours. At the end point of each reaction schedule, the mixture was vacuum distilled at 10 to 15 mm. of mercury and the distillate was collected in an ice-salt-cooled flask and dry ice trap. Distillation was stopped when the temperature of the residue resin reached 250° C.

Samples 5 and 6 used no peroxide catalyst. Sample 5 contained dehydrated castor acids, and sample 6 contained tung oil fatty acids. Equal parts of styrene and these oils were charged into silica flasks, purged with nitrogen, stoppered under a positive nitrogen pressure, and exposed to sunlight and heat on the roof top for approximately 100 days of the summer months (temperature variances 18° to 40° C.). These samples are of particular interest because no oxidizing catalysts are present and the polymerization proceeded at lower temperatures, probably through a photo and/or thermal initiation mechanism.

Sample 7 was prepared by reaction of 300 grams of dehydrated castor acids, 300 grams of styrene, and 4.8 grams of cumene hydroperoxide according to the schedule for resins 2 and 4. After vacuum distillation, an alkyd resin was formed by addition of 122.4 grams of glycerol and 180 grams of phthalic anhydride. The temperature was raised to 240° to 250° C. while a very slight positive flow of carbon dioxide was maintained through the mixture. The reaction was then continued at 260° C., accompanied by a vigorous sparging with carbon dioxide, until the acid number of the resin had dropped to 11 and a 50% solution of the resin in xylene had a viscosity of G (Gardner-Holdt scale at 77° F.).

Sample 8 was prepared by first forming an alkyd resin from 300 grams of dehydrated castor acids, 118.8 grams of glycerol, and 180 grams of phthalic anhydride, and heating at 250° to 260° C. until the resin possessed an acid value of about 20. This resin was diluted with 300 grams of styrene and 4.8 grams of cumene hydroperoxide, and warmed until the exothermic reaction started. The temperature was maintained at reflux for 2 hours after the exothermic period, during which time the temperature rose from 160° to 200° C. A 50% xylene solution of this resin had a viscosity of M. The acid value of the nonvolatile resin was 11.

In summary, the obvious sample differences are:

A. Peroxide *vs.* photothermal initiation: 1–4, 7, 8 *vs.* 5, 6.
B. Longer *vs.* shorter reaction time at higher temperatures: 2, 4, 7, 8 *vs.* 1, 3.
C. Prestyrenation *vs.* poststyrenation of fatty acids in alkyd esters: 7 *vs.* 8.

DECOMPOSITION AND FRACTIONATION OF STYRENATED RESINS

The entire styrenated fatty acid resin was first vacuum distilled at 200° C. and 12 mm. of mercury pressure to recover the unchanged monomeric styrene and other volatiles. A 25.0-gram analytical sample of the distillation residue in benzene solution was then treated with 1*N* alcoholic potassium hydroxide at room temperature for about 1 hour to convert all the fatty acid groups to their potassium salts. The reaction product was recovered in solid form by evaporation of the solvent in a nitrogen atmosphere. It was dissolved in water-saturated ethyl acetate and the pH was adjusted to 9.0 by adding 1*N* potassium hydroxide. An excess of saturated calcium chloride solution was then added. This solution was allowed to stand overnight, during which period the calcium salts of the acids were precipitated. The precipitate was recovered by filtration or centrifugation and washed thoroughly with ethyl acetate to remove residual polystyrene. The free fatty acids were recovered by treating the calcium salts with an excess of aqueous 2*N* hydrochloric acid, extracting the aqueous solution several times with ether. The ether solution was washed free of hydrochloric acid with water and then dried by filtration through anhydrous sodium sulfate. The ether extract contained the fatty acids, and the water layer retained the inorganic salts introduced in the analysis. The combined fraction of the ethyl acetate filtrate and washings from the calcium salts precipitate was concentrated by evaporation and treated repeatedly with calcium chloride until no further precipitation of salts was observed, then dried by filtration through anhydrous sodium sulfate. This ethyl acetate layer contained the polystyrene, which should be free of fatty acids if their separation as calcium salts is truly quantitative . Usually four or five precipitation fractionations were made of the ethyl acetate solution. The acids recovered from the calcium precipitate were redissolved in water-

saturated ethyl acetate and again recovered by precipitation with calcium chloride. The ethyl acetate solution from which this recovery was made was combined with the original ethyl acetate fraction and washings.

The styrenated esters (triglyceride ester or alkyds) were saponified according to the Kappelmeier method (*4*). An insoluble precipitate of potassium phthalate, $K_2C_6H_4(COO)_2 . C_2H_5OH$, was recovered quantitatively. The filtrate was evaporated to eliminate the benzene and alcohol, acidified with 2*N* hydrochloric acid, and extracted with ether. The water layer contained the glycerol. The ether layer was evaporated to dryness under vacuum at 60° to 80° C. to remove ether and monomeric styrene. The solid residue was dissolved in water-saturated ethyl acetate. The remainder of the analysis follows that of the fatty acid–styrene resins.

RADIOCHEMICAL ASSAY

A review of the literature shows that several workers (*7–10*) have used radiotracer elements in the field of polymerization. The work of Ingley and others (*3*) on the polymerization of carbon-14–labeled monomers indicated no change in the copolymerization ratio of the labeled to the normal compound. In addition, specific work by this team on the polymerization of radioactive styrene showed a relative reactivity of 0.980 for the carbon-14–labeled styrene compared with normal styrene. It is thus evident that in solutions of low radiostyrene concentration the reactivity of the labeled styrene may be assumed to be that of ordinary styrene.

The extremely high rate of volatilization of styrene prevents counting of the monomer directly. The easiest way to circumvent this volatility problem would be to reduce the vapor pressure by polymerization of monomer in the product to substantially 100% conversion. Workers at Oak Ridge (*9*) and the Dow Chemical Co. (*8*) have proceeded along this line and developed an assay method utilizing a rather thick (0.01 inch or more) circular plate (0.875 inch in diameter) of polystyrene for counting. The very nature of the analysis scheme described in the preceding section prohibits using this counting method, largely because the quantities of styrene required to cast each sample plate were excessive and impractical for this study. In this work the sample size of the analytical fractions assayed would be very small, probably 1 to 2 grams of 1 to 100% styrene content. If larger samples were needed, the analytical procedure would be impractical because of the quantities and cost of raw materials handled. The use of the isotopic dilution technique was also considered for the determination of styrene in the various fractions; however, it was soon evident that this important radiotracer technique would be uncertain for these systems because the exact chemical form of the styrene and polystyrene is unknown.

For only samples which contained monomer, the first step of this radioactive assay method involved procedures for polymerization of the monomer styrene. Conversion was accomplished by using a phenolic resin (Bakelite BR-254) as a molecular weight modifier, and a catalyst made of mixed oxalic and boric acid. Conversion of pure monomer as well as samples containing as little as 10% of monomer was approximately 98% complete in sealed ampoules after 3 days at 125° C. This low molecular weight polymer here formed was easily soluble in toluene (in contrast to the condition that results from most other polymerization formulas). Microvolumetric quantities of toluene solutions of the resins were evaporated to dryness in an aluminum planchet (1.125 inches in diameter and 0.05 inch deep) and counted. These planchets are standard sample mounts and are used in quenching gas-type flow counters. The flow counter is a radiation detection device which allows the sample to be placed inside the counting chamber. Such an instrument is extremely sensitive to soft radiations such as from carbon-14. Inherent with any weak beta emitter is the problem of self-absorption, which was eliminated by assaying samples of varying size and thickness to correct for any film absorption and back-scatter phenomena. In all cases, samples of the styrene polymer were counted for approximately 10,000 counts in order to reduce the statistical counting error to a value less than the *other errors* of the analytical procedure.

DISCUSSION OF RESULTS

A summary of the data on fractionation of the styrene oil–fatty acid reaction product for eight different samples is given in Table I. Examination of the experimental results suggests the following conclusion.

Under some circumstances synthesis yields a resin that contains a large part of the polymer as nearly neutral polystyrene (samples 6, 7, and 8). In such instances, at least 90% of acids can be separated which contain at most 25% of polymeric styrene which resists further separation from the acids by repeated solution and reprecipitation with calcium in water-saturated ethyl acetate. With respect to these three samples, experience confirms the results obtained by Armitage and Kut (*1*) on the reaction product of β-eleostearic acid and styrene.

The reaction product (sample 6) of styrene and mixed tung oil acids does not behave like the eleostearic-styrene sample which was quoted as an illustration of the effectiveness of the A-K fractionation procedure. The soluble and insoluble fractions contained about the same styrene-acid molar ratio, and the sample was distributed about equally between the two fractions.

For the reaction products of styrene and dehydrated castor acids, the fractionation was even more indeterminate. The soluble fraction contained more styrene, while the precipitate was nearly always richer in acids. Unknown variables in composition of the copolymer appear to affect its solubility, so as to make the A-K procedure a very dubious method for ascertaining what proportion of the copolymer is especially rich in styrene.

In general, the procedure of Armitage and Kut appears to work only under special conditions, and no principles are obvious which predict those conditions. Unless the process controls precisely the extent of copolymerization and molecular weight, supplementary methods such as spectroscopy will always be needed to determine the styrene content of the fractions. Experience in these laboratories has shown that ultraviolet spectroscopy can determine styrene quantitatively in the presence of fatty acids or oils, but that it provides no certain knowledge of the extent of copolymerization. Mixtures of oil and low molecular weight homopolymer styrene in appropriate solvents are indistinguishable from copolymer mixtures having the same mole ratio of styrene to acids. Further experiments are in progress to determine whether a distinction can be made by more precise infrared or ultraviolet techniques. Such work is greatly hampered by the problem of developing spectroscopic standards. The fact that the calcium-precipitated acids contain styrene which resists further separation by differential solubi ity leads to the belief that this fraction at least provides a limited quantity of fatty acid copolymer which contains no neutral polystyrene. This may be of assistance to future research in trying to identify and characterize the spectroscopic character of the covalent bond between vegetable oil acids and styrene.

LITERATURE CITED

(1) Armitage, F., Kut, S., *Offic. Dig. Federation Paint & Varnish Clubs* **333**, 671 (1952); *Paint Varnish Production* **43**, 33 (1953).
(2) Dauben, W., Reid, J., Yankwich, P., ANAL. CHEM. **19**, 828 (1947).
(3) Ingley, F., others, papers presented at the Milwaukee Meeting, 121st Meeting ACS, Division of Paint, Varnish, and Plastic Chemistry, p. 150, 1952.
(4) Kappelmeier, C. P. A., *Paint, Oil Chem. Rev.* **114**, 16 (1951).
(5) Nystrom, R., Brown, W., *J. Am. Chem. Soc.* **69**, 2548 (1947).
(6) Petit, J., Fournier, P., *Peintures, pigments, vernis* **26**, 357 (1950).
(7) Smith, W., *J. Am. Chem. Soc.* **71**, 4077 (1949).
(8) Smith, W., Campbell, H., *J. Chem. Phys.* **15**, 338 (1949).
(9) U. S. Atomic Energy Commission, "Isotopics," vol. 1, No. 3, p. 12.
(10) Walling, C., *J. Am. Chem. Soc.* **70**, 2561 (1948).

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Colorimetric Determination of Hydroxyproline

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Some of the principal factors influencing the colorimetric determination of hydroxyproline were studied. An improved procedure and its application to the determination of hydroxyproline in gelatin and collagen are given.

BECAUSE of the uniquely high content of hydroxyproline in collagen, colorimetric determinations of hydroxyproline have been employed almost exclusively in the analysis of collagen in the connective tissue of muscle. Since the colorimetric method was first introduced by Neuman and Logan in 1950 (*3*), valuable contributions have been made by Baker, Lampitt, and Brown (*2*) and Wierbicki and Deatherage (*5*) on factors affecting the accuracy of the method. Nevertheless, the factors specifically associated with the processes of color development have never been reported.

During the course of analysis of beef muscle for collagen and elastin by the Neuman and Logan method, instability of the color was found to limit the precision of the measurements. To obtain better precision, a study was made of the factors associated with color development. On the basis of the results obtained, a modification of the Neuman and Logan method was made. This modified method was applied to the analysis of available samples of collagen and gelatin and the results are compared with other values found in the literature.

MATERIALS AND EQUIPMENT

Copper sulfate, 0.01*M*.
Sodium hydroxide, 2.00*N*.
Hydrogen peroxide, 6%.
Sulfuric acid, 1.5, 3.0, and 9.0*N*.

Amino acids (tryosine, tryptophan, and hydroxyproline) were obtained from the Nutritional Biochemicals Corp. and made into solutions of required concentrations.

p-Dimethylaminobenzaldehyde (Eastman grade organic chemical) was recrystallized twice from ethyl alcohol according to the directions of Neuman and Logan (*3*).

1-Propanol was also an Eastman grade organic chemical, and the fraction distilling between 96° and 97° C. was used.

The 5% *p*-dimethylaminobenzaldehyde reagent was made by dissolving 5 grams of *p*-dimethylaminobenzaldehyde in 1-propanol and diluting to 100 ml.

Collagen samples were obtained from cattle hide (*4*) and from the distal portion of the deep and superficial flexor tendons of the fore and hind legs of cattle. Tendon collagen was prepared according to a procedure described by Baker, Lampitt, and Brown (*2*).

Gelatin samples were products of Nutritional Biochemicals Corp. and Charles B. Knox Gelatin Co., Inc.

Most absorption spectra and absorbance values were obtained with either a Beckman Model DR spectrophotometer or a Beckman Model DU spectrophotometer. The values in Figure 1 were obtained with a Klett-Summerson photoelectric colorimeter.

RECOMMENDED PROCEDURE

To 1 ml. of sample or standard solution containing from 1 to 15 γ of hydroxyproline are added the following reagents: 1 ml. of 0.01*M* cupric sulfate, 1 ml. of 2.00*N* sodium hydroxide, and 1 ml. of 6% hydrogen peroxide. The resulting solution is shaken first on a rotator for 5 minutes and then in an 80° C. water bath for another 5 minutes. The solution is then cooled in ice water.

For samples of relatively low hydroxyproline content, it is recommended that 0.05*M* cupric sulfate be used (*1*).

After cooling, 4 ml. of 1.5*N* sulfuric acid are added with shaking, followed by 2 ml. of 5% *p*-dimethylaminobenzaldehyde in 1-propanol. The solution is shaken for 3 minutes and then placed in an 80° C. water bath for 30 minutes to develop the color. The absorbance of the resulting color is read at 560 mμ. A blank is used in which 1 ml. of water is substituted for the sample.

The absorbance at 560 mμ is a linear function of the hydroxyproline concentration within the range of 1 to 15 γ per ml. However, at higher concentrations this relationship does not hold.

RESULTS AND DISCUSSION

The effects of sulfuric acid concentration and time on the color produced with 30 γ per ml. of hydroxyproline solution were studied by recording the spectra in the region 450 to 700 mμ (Table I). In all cases, the maximum absorbance occurred at 560 mμ. This study also shows that the color is more intense in 3.0*N* than in 1.5*N* or 9.0*N* sulfuric acid, whereas the color fades more rapidly at high hydrogen ion concentrations. The effect of hydrogen ion concentration on color stability was determined by adjusting the pH after the color was developed and observing the change in absorbance as a function of time (Table II). In this experiment, a precipitate formed in the sample buffered at neutrality. The precipitate was removed by filtration and the absorbance readings reported were taken on the filtrate.

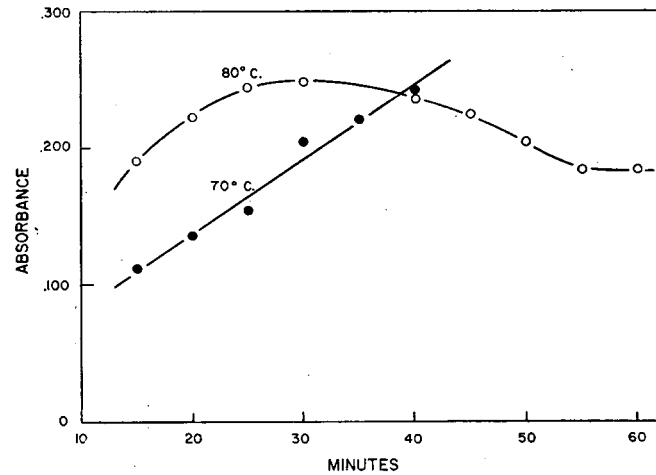

Figure 1. Absorbance of color as a function of heating time at two different temperatures

Table I. Effects of Time and Sulfuric Acid Concentration on Absorbance of Color[a]

Time, Hours	Normality of Sulfuric Acid Added		
	1.5[b]	3.0	9.0
0	0.290	0.492	0.091
2	0.326	0.414	0.070
4	0.342	0.352	0.060
20	0.294	0.096	0.023

[a] Readings obtained at 560 mμ.
[b] Final hydrogen ion concentration is 0.334*N*.

Table II. Effect of Hydrogen Ion Concentration on Absorbance of Color[a]

Time, Minutes	Final Hydrogen Ion Concentration		
	0.000*N* (pH 7.0)	0.223*N*	0.622*N*
0	0.250	0.265	0.265
60	0.242	0.268	0.247
120	0.236	0.264	0.220
180	0.229	0.253	0.197

[a] Readings obtained at 560 mμ.

Table III. Hydroxyproline Content of Some Gelatin and Collagen Samples

Sample	Hydroxyproline, %	Nitrogen, %
Tendon collagen	14.2	16.98
Cattle hide collagen	14.3	17.25
Gelatin (Knox)	15.3	17.21
Gelatin (NBC)	14.3	17.66
Gelatin	14.42 (5)	...
Gelatin (Bacto-Difco)	13.6 (3)	...
Connective tissue	12.39 (3)	...
Connective tissue	13.2 (2)	...
Cattle hide gelatin	13.2 (3)	...

Table IV. Characteristics of Absorption Spectra Obtained with Tyrosine, Tryptophan, and Hydroxyproline

Amino Acids	$\lambda_{max.}$, Mμ	mM Extinction[a], $\lambda_{max.}$	mM Extinction[a], 560 Mμ
Tyrosine	502	96.8	58.7
Tryptophan	470	380	42.9
Hydroxyproline	560	2124	2124

[a] mM extinction = $\frac{\text{absorbance}}{C \cdot x}$, where c is expressed as millimoles of amino acid.

Next, the optimum conditions of heat treatment for maximum color development with 1.5N sulfuric acid were determined. The selection of this acid strength was based on previous findings although 1.5N and 3.0N appear equally good (Table I). In this experiment, absorbance was measured with a Klett colorimeter, using the No. 540 filter. The results given in Figure 1 indicate that optimum heat treatment is 80° C. for 30 minutes.

The procedure given above was applied in a determination of the hydroxyproline content of various gelatin and collagen samples. The results (Table III) were corrected for the degradation of hydroxyproline which occurred during the acid hydrolysis of the sample, using a calibration curve of standard hydroxyproline solutions treated in a similar manner. Knox gelatin was found to have a very high hydroxyproline content (15.3%). The hydroxyproline content of the other samples of gelatin and collagen tested ranged from 14.2 to 14.3%, which compares favorably with the literature values included in the same table for comparison. The average coefficient of variation was found to be 1.33 (100 σ/m) within samples.

This procedure may also be applied to connective tissue determinations in muscle, provided prior extraction is made to reduce the ratio of other amino acids to hydroxyproline.

Because tyrosine and tryptophan are known to react like hydroxyproline, the spectral characteristics of the color produced from tyrosine and tryptophan with this method were determined. Five hundred micrograms per milliliter of tyrosine and 100 γ per ml. of tryptophan were each substituted for hydroxyproline in the analytical procedure described above, and the resulting spectral absorption of the color in the region 425 to 700 mμ was recorded. The results given in Table IV indicate 2.00 and 1.30% interference with hydroxyproline at 560 mμ at equimolar concentrations of tyrosine and tryptophan, respectively. No decrease in absorption maxima of the color from tyrosine and tryptophan was observed during 3 hours.

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LITERATURE CITED

(1) Baker, L. C., Lampitt, L. H., Brown, K. P., *J. Sci. Food Agr.* 4, 165 (1953).
(2) *Ibid.*, 5, 226 (1954).
(3) Neuman, R. E., Logan, M. A., *J. Biol. Chem.* 184, 299 (1950).
(4) Veis, A., Cohen, J., *J. Am. Chem. Soc.* 76, 2476 (1954).
(5) Wierbicki, E., Deatherage, F. E., *J. Agr. Food Chem.* 2, 878 (1954).

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Chromatography of the 2,4-Dinitrophenylhydrazones of Some Aldehydes and Ketones in Tobacco Smoke

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A method of general applicability has been developed for determination of the lower molecular weight aldehydes and ketones in a grossly impure mixture. The procedure was applied to the smoke from bright and burley tobaccos and blends of these, by trapping the smoke at liquid air temperatures and converting the unfractionated raw smoke into 2,4-dinitrophenylhydrazones. Chromatograms on paper treated with *N,N*-dimethylformamide and developed with *n*-hexane effected separation of the 2,4-dinitrophenylhydrazones of furfural, formaldehyde, acetaldehyde, propionaldehyde, acetone, methyl ethyl ketone, diethyl ketone, and butyraldehyde. These compounds were identified by comparative paper chromatography and by their absorption spectra. The free aldehydes and ketones of low molecular weights totaled 3 to 3.5 mg. per cigarette and comprised about 10% of the total weight of the smoke. Acetaldehyde was present in the highest concentration. Only minor variations in content were found in smoke from the types of cigarettes investigated.

THE chemical composition of tobacco smoke has long been a subject of wide interest and qualitative data concerning the identity of some of the more plentiful compounds are available in the literature (*3*, *4*). This list, numbering over 100 different components, is far from complete and there still remain a large number of compounds yet to be identified. Much of the available information was obtained under conditions that amounted essentially to a destructive distillation of the tobacco rather than simulated human smoking. It is not certain that the information thus obtained is applicable, even in a qualitative way, to tobaccos currently in use. In the older chemical literature when quantitative data were reported, the authors often failed to define the conditions of smoking, employed nonstandardized methods, or omitted information about the type of tobacco used. Recently there have appeared quantitative results which were obtained using well-defined standardized conditions (*7*, *9*, *14*). Because important chemical differences undoubtedly occur with differences in tobaccos and with the conditions under which the tobacco is smoked, much of the older information will be of limited value relative to the quantitative composition of smoke from modern American cigarettes.

The aldehyde and ketone fraction of smoke accounts for approximately 10% of the total weight of the smoke constituents. Various components of this fraction have been qualitatively reported (*6–8, 11*), but only very recently have any quantitative data appeared in the literature (*14*). This paper presents quantitative data on this fraction and outlines a new procedure of general applicability for the qualitative and quantitative determination of aldehydes and ketones in a mixture by a paper chromatographic method.

Table I. Absorption Maxima and Minima of 2,4-Dinitrophenylhydrazones of Known Compound and of Tobacco Smoke Constituents

Carbonyl Compound	Wave Length[a] Max.	Wave Length[a] Min.	Chromatographic Band No.	Wave Length Max.	Wave Length Min.
	Standard			From Smoke	
Furfural	385	320	1	385	320
trans form	300	285		295	287
	380	315	2	380	315
cis form	300[b]			300[b]	
Formaldehyde	350	290	3	350	290
Acetaldehyde	354	292	4	354	292
Acetone	358	294	5	358	294
Propionaldehyde	357	294	5A	357	294
Methyl ethyl ketone	362	295	6	362	295
Diethyl ketone	362	295	7	362	295
Butyraldehyde	357	288	8	365	305

[a] In methanol solvent.
[b] Inflection point.

EXPERIMENTAL

Preparation of Sample. Five different types of cigarettes of 70-mm. standard length were supplied through the courtesy of the American Tobacco Co. The kinds of tobaccos and the average weight of each of the cigarettes were the following: uncased burley, 900 mg. ±5%; burley cased with 10.6% dextrose, 900 mg. ±5%; bright, 1200 mg. ±5%; a 50–50 blend of uncased burley and bright, 1050 mg. ±5%; and a 50–50 blend of bright and cased burley, 1050 mg. ±5%.

Previous to use, the cigarettes were stored for 48 hours in an atmosphere of 58% relative humidity obtained by an aqueous, saturated solution of the dihydrate salt of sodium bromide. Fifteen cigarettes of each of the tobaccos were smoked by an automatic smoker kindly furnished on loan by the Liggett and Myers Tobacco Co. A butt length of 2 cm. was obtained by smoking at a 2-second puff of 35-ml. volume at 58-second intervals. The more slowly burning bright tobaccos generally required 11 puffs; the burley, 7 puffs; and the blends of bright and burley, 8 puffs. The smoke was rapidly and completely condensed by passing it through four cold traps, connected in series, kept in liquid air baths. The traps were removed from the surrounding liquid air and the frozen smoke was immediately dissolved in 200 ml. of methanol containing 2 grams of recrystallized 2,4-dinitrophenylhydrazine and 0.5 ml. of 1*N* hydrochloric acid. The resulting dark-colored solutions were heated under reflux for 2 hours. One milliliter of each solution, while still warm, was carefully streaked, at a distance of 5 cm. from the top, across the entire width of a sheet of 20 × 40 cm. Whatman No. 1 chromatographic grade paper. The paper had previously been prepared by immersion for 30 seconds in *N,N*-dimethylformamide, followed by drying at room temperature for approximately 45 minutes in a forced draft hood. The width of the streak was held to less than 1 cm. by slow release of the solution through a small orifice pipet. An ordinary hair dryer provided an amount of warm air sufficient to volatilize the methanol from the paper at a rate such that 1 ml. could be applied in 10 minutes.

The papers were placed in a 30 × 46 cm. glass cylinder in a position suitable for descending chromatography. To ensure saturation of the entire volume of atmosphere with developing solvent, the cylinder was lined with filter paper which was kept saturated with *n*-hexane by immersion in 2 cm. of this solvent. The sample papers were then allowed to equilibrate overnight at 15° to 18° C. in the saturated *n*-hexane atmosphere. The next morning *n*-hexane, previously saturated with *N,N*-dimethylformamide and cooled to 15° to 18° C., was added to the troughs containing the ends of the sample papers. After 4 hours, the streak at the origin was resolved into eight clearly distinguishable yellow bands spaced throughout the length of paper in widths from 1 to 2 cm. These yellow areas were clearly visible under artificial light, but under ultraviolet the quenching effect even more clearly defined the boundaries. The bands were numbered, cut out, and eluted from the paper with methanol by use of a Soxhlet extractor.

The samples from the extraction were concentrated in vacuo to dryness, redissolved in 0.2 to 0.3 ml. of methanol and rechromatogramed separately in a manner similar to that described above. Band 5 separated into two distinct bands, while all other bands moved down the paper as single narrow areas.

The bands from this second streaking were again cut out, extracted via Soxhlet, and examined critically for homogeneity. This was effected by chromatograming in three different solvent systems selected for their ability to move the spots at different rates relative to each other. The systems used were *n*-hexane on *N,N*-dimethylformamide–treated paper, 50% methanol–50% iso-octane on untreated paper, and 95% water–5% methanol on silane-treated paper. The descending technique in a saturated atmosphere was employed in all cases. The spots were allowed to move down the entire length of paper, thus providing maximum time for any heterogeneity to manifest itself by the appearance of more than one yellow area. All unknowns were found to be homogeneous in the three systems.

Qualitative Identification of 2,4-Dinitrophenylhydrazones. Identification was established by requiring the migration of the unknown 2,4-dinitrophenyl hydrazone to correspond exactly with that of an authentic sample of hydrazone when the knowns and the unknowns were chromatogramed side by side in the three different solvent systems used in the homogeneity test. Table I lists the band number, and the known hydrazone corresponding to it, and also records the wave length of maximum absorption of the hydrazone identified by comparative chromatography with that of the known hydrazone. Both the trans- and cis-stereoisomers of the 2,4-dinitrophenylhydrazone of furfural were detected. The cis isomer was found to be approximately 30% of the concentration of the trans isomer.

Quantitative Determination of 2,4-Dinitrophenylhydrazones. The optimum conditions for conversion of aldehydes and ketones to their 2,4-dinitrophenylhydrazones were first determined using a sample mixture of known amounts of pure furfural, acetone, and methyl ethyl ketone. These three compounds are representative of the carbonyl compounds found in cigarette smoke.

The best procedure investigated involved heating a mixture of the above aldehydes and ketones under reflux in 200 ml. of purified methanol with a tenfold excess of 2,4-dinitrophenylhydrazine reagent in the presence of 0.50 mmole of hydrochloric acid. A paper chromatogram was then run on a small aliquot. The developed bands were eluted as described above and the concentration of the hydrazone was determined as follows. The absorption coefficient of the known 2,4-dinitrophenylhydrazones was calculated at the wave length of maximum absorption. The absorbance of an appropriate aliquot of the chromatographically identified hydrazone was then determined at the same wave length and the amount of hydrazone was calculated.

Table II. Recovery of Furfural, Acetone, and Methyl Ethyl Ketone as Their 2,4-Dinitrophenylhydrazone

Compound	Mg. Added	Mg. Recovered (as Free Aldehyde or Ketone)	Recovery, %
Acetone	9.25	7.86	85.0
Furfuraldehyde	10.0	7.16 (trans form)	
		2.53 (cis form)	96.9
Methyl ethyl ketone	11.6	10.56	91.0

The results obtained on a known mixture by applying such a procedure are given in Table II. In Table III are shown the amounts of the different aldehydes and ketones found in the smoke from the several types of cigarettes when the procedure was followed. The quantities reported are those actually found and are not adjusted by the recovery efficiency shown in Table II.

The 2,4-dinitrophenylhydrazones of acetone and of propionaldehyde migrate down the paper at closely similar rates. The degree of separation under the developing conditions described above was not sufficient to make these two bands clearly dis-

tinguishable. However, by using a separate chromatogram and by allowing the bands to move down almost the entire length of paper they were sufficiently resolved for quantitative determination.

A comparison of the absorption spectrum of the band corresponding to the 2,4-dinitrophenylhydrazone of butyraldehyde with that of an authentic sample showed this band to be a mixture of substances. It is probable that other rapidly moving hydrazones were present; however, because of their mutually high R_f values, it was not possible to separate them further in this system. The total hydrazones present in this band are calculated as the 2,4-dinitrophenylhydrazone of butyraldehyde and are reported as such in Table III.

Table III. 2,4-Dinitrophenylhydrazone and Free Aldehyde and Ketone in Smoke of Cigarettes of Various Tobaccos[a]

	Type of Tobacco Smoked				
Carbonyl Compound	Uncased Burley, mg.	Uncased Bright, mg.	Blend 50–50 Bright-Burley uncased, mg.	Cased Burley, mg.	Blend 50–50 cased Burley and uncased Bright, mg.
Furfuraldehyde	0.084 (0.029)	0.31 (0.11)	0.19 (0.066)	0.13 (0.045)	0.20 (0.070)
Formaldehyde	0.176 (0.026)	0.217 (0.031)	0.288 (0.041)	0.239 (0.034)	0.231 (0.033)
Acetaldehyde	6.65 (1.31)	5.98 (1.17)	5.45 (1.07)	5.47 (1.09)	4.99 (0.98)
Acetone	2.80 (0.68)	2.60 (0.63)	2.60 (0.63)	2.66 (0.63)	3.07 (0.75)
Propionaldehyde	1.20 (0.29)	1.11 (0.27)	1.12 (0.27)	1.16 (0.23)	1.31 (0.32)
Methyl ethyl ketone	1.73 (0.50)	2.26 (0.65)	2.28 (0.65)	1.93 (0.55)	0.98 (0.28)
Diethyl ketone	0.93 (0.30)	0.90 (0.29)	0.72 (0.23)	1.03 (0.33)	0.70 (0.23)
Butyraldehyde[b]	0.81 (0.23)	1.24 (0.35)	...	0.97 (0.28)	...
Total	14.38 (3.36)	14.62 (3.50)	...	13.59 (3.28)	...

[a] Figures in parentheses are milligrams of free aldehyde or ketone per cigarette. All other figures are milligrams of 2,4-dinitrophenylhydrazone per cigarette.
[b] An impure fraction probably composed of several hydrazones which have same rapid migration rate as the 2,4-dinitrophenylhydrazone of butyraldehyde.

The compounds in Table III include only the major constituents of the aldehyde and ketone content of cigarette smoke. This laboratory and others (*6*) have obtained evidence for the presence of carbonyl compounds of higher molecular weight which represent only a small percentage of the total carbonyl fraction. Any hydrazone that remains at the origin—e.g., diacetyl—would, of course, not be detected by this method. Future work is planned in further resolving both the rapidly moving band and the material that is stationary at the origin.

The conditions employed in the formation of the 2,4-dinitrophenylhydrazones differ from the usual method, in that the amount of acid was much less than that commonly used. It was found that a tenfold increase in the hydrochloric acid concentration reduced the recovery of the hydrazones of acetone and of methyl ethyl ketone only slightly. The recovery of the hydrazone of furfural under these conditions, however, was only one third that found for a lower acid content. This observation is consistent with the work of Conant and Bartlett (*1*), who reported a rapid hydrolysis of the semicarbazone of furfural at pH's below 3.0.

Methanol as a solvent for the trapped smoke had a disadvantage, as did most of the solvents available, in that it contained small amounts of aldehydes and ketones as contaminants. The aldehyde impurities were converted into acids by refluxing for 24 hours over alkaline silver nitrate and then distilling from the basic solution. Any carbonyl contaminants which were still present or originated during the solvent treatment were determined by means of blanks on the entire procedure outlined for tobacco smoke. This blank determination showed the presence of small amounts of three hydrazones that moved parallel to the hydrazones of formaldehyde, acetone, and butyraldehyde. In applying a correction for the reagents, the materials found in the blanks were considered to be formaldehyde, acetone, and butyraldehyde, and the extinction coefficients for these substances were used to calculate their respective concentrations. The corrections required in the totals found were 30% for formaldehyde, 10% for acetone, and 20% for butyraldehyde. The values reported in Table III include these corrections.

DISCUSSION

A quantitative determination of the different aldehydes and ketones in smoke requires that the compounds once formed be prevented from reacting either with themselves or with the diverse other compounds present and that they be transformed quantitatively into some derivative which in turn can be easily identified and quantitatively determined. The low temperature provided by the liquid air traps and the formation of the 2,4-dinitrophenylhydrazones of the aldehydes and ketones directly on the thawed raw smoke seemed to fulfill these two requirements.

The solvent systems for the chromatographic separation of the 2,4-dinitrophenylhydrazones of aldehydes and ketones reported in the literature (*5, 10, 12*) were found to be unsatisfactory because of poor resolution of mixtures or excessive streaking, or both. Tarbell (*13*) employed with success an *N,N*-dimethylformamide–treated paper with *n*-hexane as developing solvent in the separation of polycyclic hydrocarbons. When applied to the 2,4-dinitrophenylhydrazones this solvent system gave an excellent separation of a homologous series of aldehydes and ketones and, in the hands of the authors, was superior to any phenylhydrazone chromatographic system reported in the literature. This system had the added advantage of possessing a high capacity for total solids without streaking. Because of this feature, grossly impure fractions could be applied to the paper in quantities large enough to isolate sufficient hydrazone for further characterization. For the best results it was found that the *N,N*-dimethylformamide–dipped papers should be just dry before applying the hydrazones. The chromatograms should be run in a descending manner at a temperature of 15° to 18° C. after equilibration for a minimum of 4 hours. This temperature range is important; if it is too high, the spots move too fast, and streaking and poorly defined spots result. The R_f values for the 2,4-dinitrophenylhydrazones of some known aldehydes and ketones are given in Table IV.

Table IV. R_f Values for 2,4-Dinitrophenylhydrazones in *N,N*-Dimethylformamide–Hexane Paper Chromatography Solvent System

Compound	$R_f \times 100$
Furfuraldehyde	
trans form	11
cis form	19
Formaldehyde	22
Acetaldehyde	30
Crotonaldehyde	36
Acetone	45
Propionaldehyde	48
Methyl ethyl ketone	64
Diethyl ketone	82
Butyraldehyde	89

The methanol–iso-octane solvent system used in the identification of the separated hydrazones was an innovation of the system of Meigh (*5*). It was useful in comparative chromatography because of its ability to give good resolution of the isolated hydrazones once they were obtained in a relatively pure form.

The water-methanol system was satisfactory only by virtue of the inversion of the order of migration of some of the hydrazones when compared with their movement in the other systems. While capable of good resolution, the low capacity and tendency to streak limits the general use of this system.

A tenfold excess of the 2,4-dinitrophenylhydrazine reagent was used in formation of the carbonyl derivative. When the reagent was present in only onefold excess, it was found that the amount of the acetaldehyde derivative was considerably below that found present when a tenfold excess was employed. However, crotonaldehyde, which was detectable in only trace amounts in the presence of a tenfold excess of reagent, was found in significant quantities (30 γ per cigarette) when a onefold excess of reagent was used. These observations seem to indicate that the actual amount of crotonaldehyde in smoke is very low. The higher amounts of crotonaldehyde were probably due to self-condensation of acetaldehyde which could occur under conditions of low hydrazine-aldehyde ratios. The resulting aldol upon dehydration would give rise to the unsaturated aldehyde. Acetaldehyde is known to undergo readily such a reaction under the warm acidic conditions employed in this reaction (*2*).

Table III shows the total aldehyde and ketone content to be between 3 and 3.5 mg. per cigarette. Acetaldehyde was present in the highest concentration and accounted for one third of the total amount. Propionaldehyde and butyraldehyde were present in considerably smaller amounts. Formaldehyde was present in small amounts and represented only approximately 1% of the total carbonyl compounds listed in Table III.

There appear to be no pronounced differences between the amounts of the constituents found in the smoke of the various tobaccos. However, the bright tobacco contained a relatively higher amount of furfural than did the burley.

The presence of furfural in smoke obtained by the destructive distillation of tobacco has been reported (*11*), but sufficient data were not presented at that time to establish conclusively its identity. In addition to the chromatographic matching of the mobility of furfural from smoke with that of an authentic sample, this present report adds further evidence for its presence: both the cis and the trans isomers were isolated; the trans isomer is much redder in color than most of the other 2,4-dinitrophenylhydrazones and this pronounced red color appeared in the band parallel to the authentic trans isomer; and the hydrazones of furfural exhibited spectra which were superimposable upon the spectra of the furfural hydrazones isolated from smoke.

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LITERATURE CITED

(1) Conant, J. B., Bartlett, P. D., *J. Am. Chem. Soc.* **54**, 2881 (1932).
(2) Fieser, L. F., Fieser, M., "Organic Chemistry," 2nd ed., p. 210, Heath, Boston, 1950.
(3) Kissling, R., "Handbuch der Tabakkunde," 3rd ed., Paul Parey, Berlin, 1919.
(4) Kosak, A. I., *Experientia* **10**, 69 (1954).
(5) Meigh, D. F., *Nature* **170**, 579 (1952).
(6) Neuberg, C., Burkard, J., *Biochem. Z.* **243**, 472 (1931).
(7) Osborne, J. S., Adamek, S., Hobbs, M. E., ANAL. CHEM. in press.
(8) Pfyl, B., *Z. Untersuch. Lebensm.* **66**, 501 (1933).
(9) Rayburn, C. H., Harlan, W. R., Hanmer, H. R., ANAL. CHEM **25**, 1419 (1953).
(10) Rice, R. G., Keller, G. J., Kirchner, J. G., *Ibid.*, **23**, 194 (1951).
(11) Roffo, A. H., *Biol. Inst. Med. Exptl. Estud. Cancer.* **15**, 349 (1939).
(12) Sykora, V., Prochazka, Z., *Chem. Listy* **47**, 1674 (1953).
(13) Tarbell, D. S., others, *J. Am. Chem. Soc.* **77**, 767 (1955).
(14) Touey, G. P., ANAL. CHEM. **17**, 1788 (1955).

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CRYSTALLOGRAPHIC DATA

117. Cerium Tetraiodate, $Ce(IO_3)_4$

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CERIUM tetraiodate is prepared by mixing aqueous solutions of cerium disulfate and sodium iodate. The resulting amorphous precipitate was recrystallized from concentrated nitric acid.

CRYSTAL MORPHOLOGY

System and Class. Tetragonal, tetragonal-dipyramidal.

Axial Elements. $a:c$ = 1:0.537 (derived from unit cell dimensions).

Habit. Dipyramidal {111}, usually with {001}; {100} occasionally present.

Partial Powder X-Ray Diffraction Pattern of $Ce(IO_3)_4$

hkl	*d*, A., Calcd.	*d*, A., Measd.[a]	I/I_1[b]
110	7.00	...	..
200	4.95	4.91	5
101	4.69	4.64	<5
111	4.24	...	..
201	3.62	...	..
220	3.50	3.47	35
211, 121	3.40	3.38	100
310, 130	3.13	3.11	15
221	2.92	...	..
301	2.80	2.79	10
311, 131	2.70	...	..
002	2.66	2.65	15
102	2.57	2.52	<5
112	2.487	2.465	25
400	2.475	...	..
321, 231	2.440	2.430	30
202	2.343	2.335	20
330	2.333		
212, 122	2.280	...	..
401	2.244	...	..
420, 240	2.214	2.207	5
411, 141	2.189	...	..
331	2.137	...	..
222	2.118	2.111	10
302	2.071	...	..
421, 241	2.044	...	..
312, 132	2.027	2.020	15
510, 150	1.942	...	..
322, 232	1.911	1.905	5
431, 341, 501	1.856	1.851	15
511, 151	1.824	...	..
402	1.812	1.807	35
412, 142	1.782	...	..
332	1.754	1.752	25
440	1.750		
103	1.746	...	..
521, 251	1.737	1.733	65
113	1.719	...	..
422, 242	1.702	...	..
530, 350	1.698	1.695	15

[a] Philips 114.6-mm.-diameter powder camera, Straumanis mounting; $\lambda(CuK\alpha)$ = 1.5418 A.

[b] Relative peak intensities above background from densitometer measurements.

X-RAY DIFFRACTION DATA

Space Group. $P\,4_2/n$ (C_{4h}^4). The structure of cerium tetraiodate will be described in a forthcoming publication.

Cell Dimensions. a_0 = 9.90 A.; c_0 = 5.32 A. Cell volume 521 A^3.

Formula Weights per Cell. 2.

Formula Weight. 839.81.

Density. 5.35 grams per cc. (x-ray); 5.4 measured by a displacement method.

OPTICAL PROPERTIES

Uniaxial positive.

Refractive Indices (5893 A.). n_O = 2.06; n_E = 2.19; geometric mean 2.102. Molecular refraction 83.6 cc.

Color. Yellow without perceptible pleochroism.

WORK done under the auspices of the Atomic Energy Commission.

118. Lead Diiodate, $Pb(IO_3)_2$

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The University of California, Los Alamos Scientific Laboratory, Los Alamos, N. M.

LEAD diiodate is prepared by adding potassium iodate to a hot aqueous solution of lead nitrate and allowing the resulting solution to cool slowly with agitation.

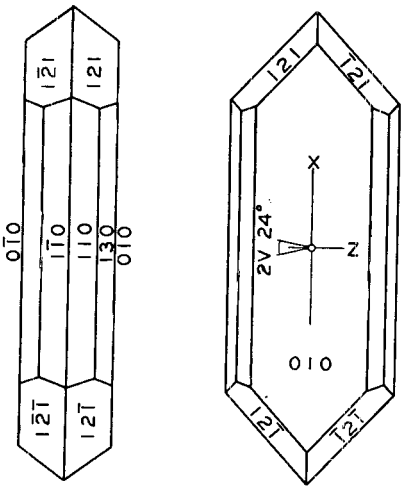

Figure 1. Orthographic projections of a crystal of lead diiodate parallel to *a* and on (010)

Partial Powder X-Ray Diffraction Pattern of $Pb(IO_3)_2$

hkl	*d*, A., Calcd.	*d*, A., Obsd.[a]	I/I_1[b]
020	8.35	8.26	5
131	3.313	3.29	100
002	3.045	3.03	20
012	2.996	2.99	5
060	2.783	2.77	35
151	2.595	2.58	10
122	2.548	2.55	5
062	2.054		
212	2.046	2.047	45
260	1.974	1.964	40
181	1.862		
123	1.860		
310	1.855	1.851	10
242	1.848		
133	1.805		
		1.799	20
		1.687	55
		1.650	30
		1.514	<5
		1.493	5
		1.462	5
		1.421	5
		1.404	5
		1.390	10
		1.350	5
		1.344	35

[a] Philips 114.6-mm.-diameter powder camera, Straumanis mounting; λ (Cu$K\alpha$) = 1.5418 A.
[b] Relative peak intensities above background from densitometer measurements.

CRYSTAL MORPHOLOGY
System and Class. Orthorhombic, dipyramidal.
Axial Elements. $a:b:c$ = 0.336:1:0.370.
Habit. Blades, flattened {010}, elongated [001], with {110}, {130}, and {121}.
Polar Angles. (010) Λ (110) = 73° 0′ 010) Λ (121) = 63° 33′.

X-RAY DIFFRACTION DATA
Space Group. $Pccn$ (D_{2h}^{10})
Cell Dimensions. a_0 = 5.60 A.; b_0 = 16.70 A.; c_0 = 6.09 A.; $a_0:b_0:c_0$ = 0.335:1:0.365. Cell volume 570 A^3.
Formula Weights per Cell. 4.
Formula Weight. 557.05.
Density. 6.50 grams per cc. (x-ray); 6.50 (pycnometer).

OPTICAL PROPERTIES
Refractive Indices (5893 A). n_X = 2.15; n_Y = 2.15; n_Z = 2.18; geometric mean 2.16. Molecular refraction 47.1 cc.
Optic Orientation. $X = c$; $Y = b$; $Z = a$.
Optic Axial Angle (5893 A.). $2V_Z$ = 24° with very strong dispersion $r < v$.
Colorless.

WORK done under the auspices of the Atomic Energy Commission.

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119. Cobalt Trifluoride, CoF_3

EUGENE STARITZKY and R. M. DOUGLASS

The University of California, Lo Alamos Scientific Laboratory, Los Alamos, N. M.

ANHEDRAL crystals of cobalt trifluoride were prepared by L. B. Asprey of this laboratory by sublimation while passing fluorine gas over cobalt dichloride heated to 750° C. Cobalt trifluoride is probably isomorphous with trifluorides of aluminum, iron, palladium, and rhodium (*1*).

CRYSTAL MORPHOLOGY
The crystals examined were anhedral grains exhibiting cleavage or parting on a rhombohedron with an 87° angle and twinned polysynthetically with the same rhombohedron as the twinning plane.

X-RAY DIFFRACTION DATA
Space Group and Cell Dimensions. According to Wyckoff (*1*) the space group is probably $R32$ (D_3^7) and the dimensions of the dimolecular rhombohedral cell are a_0 = 5.31 A., α = 57° 00′. The observed lines of the powder pattern, however, do not require a unit larger than a unimolecular rhombohedral cell with dimensions a_0 = 3.645 A., α = 87.2° [Wyckoff (*1*) gives a_0 = 3.671 A., α = 87° 20′]. The matrix of the transformation from the

Powder X-Ray Diffraction Pattern

hkl[a]	*d*, A., Calcd.	*d*, A., Obsd.[b]	*I*
100	3.637	3.63	vvs
110	2.634	2.63	s
10$\bar{1}$	2.514	2.51	m
111	2.205	2.182	s
11$\bar{1}$	2.068	2.067	w
200	1.818	1.818	s
210	1.658	1.657	vs
20$\bar{1}$	1.597	1.596	ms
211	1.546	1.547	w
21$\bar{1}$	1.473	1.473	m
2$\bar{1}\bar{1}$	1.451	1.450	m
220	1.317	1.317	wm
221	1.266	1.267	wm
202	1.257	1.256	vw
300, 22$\bar{1}$	1.212	1.211	vvw
21$\bar{2}$	1.188	1.187	wm
310	1.1664	1.165	w
30$\bar{1}$	1.1343	1.133	vvw
311	1.1305	1.131	vvw
222	1.1024	1.103	vw
31$\bar{1}$	1.0919	1.085	w
3$\bar{1}\bar{1}$	1.0740	1.073	vw
22$\bar{2}$	1.0339	...	...
320	1.0310	1.0312	w
321	1.0099	1.0099	vvw
30$\bar{2}$	0.9876	0.9875	vvw
32$\bar{1}$	0.9752	0.9749	vvw
31$\bar{2}$	0.9562	0.9559	vw
3$\bar{1}\bar{2}$	0.9501	0.9496	vvw
322	0.9234	0.9240	vw
400	0.9092	0.9092	vvw

[a] Indices for unimolecular rhombohedral cell.
[b] Philips 114.6-mm.-diameter powder camera, Straumanis mounting; λ(Co$K\alpha$) = 1.7902 A.

unimolecular to the dimolecular rhombohedral cell is $\frac{4}{3}\,\frac{1}{3}\,\frac{1}{3}/\frac{1}{3}\,\frac{4}{3}\,\frac{1}{3}/\frac{1}{3}\,\frac{1}{3}\,\frac{4}{3}$. The volume of the unimolecular rhombohedral cell is 48.26 A^3.

Formula Weight. 115.94.

Density. 3.99 grams per cc. (calculated from authors' cell dimensions; weight of unit atomic weight 1.6602×10^{-24} gram).

Optical Properties

Uniaxial positive.

Refractive Indices (5893 A.). $n_O = 1.703 \pm 0.003$, $n_E = 1.726 \pm 0.004$; geometric mean 1.711. Molecular refraction 11.4 cc.

Color. *O* brown, *E* greenish brown.

LITERATURE CITED

(1) Wyckoff, R. W. G., "Crystal Structures," vol. I, chap. V, pp. 12–13, table pp. 15–16, 19, Interscience, New York, 1951.

Work done under the auspices of the Atomic Energy Commission.

120. Diberyllium Carbide, Be_2C

EUGENE STARITZKY, The University of California, Los Alamos Scientific Laboratory, Los Alamos, N. M.

Diberyllium carbide may be prepared by heating beryllium metal with graphite in a neutral atmosphere.

Crystal Morphology

Isometric, hexoctahedral. Preparations examined consisted of anhedral grains and granular aggregates.

Powder X-Ray Diffraction Pattern of Be_2C

hkl	*d*, A., Calcd.	*d*, A., Obsd.[a]	I/I_1[b]
111	2.5069	2.502	98
200	2.1710	2.171	1
220	1.5351	1.535	100
311	1.3092	1.309	16
222	1.2534	....	..
400	1.0855	1.0860	15
331	0.9961	0.9959	7
420	0.9709	0.9709	2
422	0.8863	0.8863	41
333, 511	0.8356	0.8356	11

[a] Philips 114.6-mm.-diameter powder camera, Straumanis mounting; $\lambda(CuK\alpha) = 1.5418$ A.; $\lambda(CuK\alpha_1) = 1.54050$ A.; $\lambda(CuK\alpha_2) = 1.54434$ A.
[b] Relative peak intensities above background from densitometer measurements.

X-Ray Diffraction Data

The structure of beryllium carbide has been determined by Stackelberg and Quatram (*1*) to be of the antifluorite type. Space group $Fm3m$ (O_h^5).

Cell Dimension. $a_0 = 4.342_0 \pm 0.0005$ A. Cell volume 81.86 A^3. Stackelberg (*1*) reported $a_0 = 4.34$ A.

Formula Weights per Cell. 4.

Formula Weight. 30.036.

Density. 2.437 grams per cc. (x-ray).

Optical Properties

Isotropic.

Refractive Index (6640 A.). 2.635 ± 0.010. Molecular refraction 8.19 cc.

Colorless.

LITERATURE CITED

(1) Stackelberg, M. von, Quatram, F., *Z. phys. Chem.* **(B) 27**, 50–2 (1934).

Work done under the auspices of the Atomic Energy Commission.

121. Beryllium Sulfide, BeS

EUGENE STARITZKY, The University of California, Los Alamos Scientific Laboratory, Los Alamos, N. M.

Beryllium sulfide is prepared by the reaction of beryllium metal with hydrogen sulfide at 900° C.

Crystal Morphology

Isometric, hextetrahedral. Preparations examined consisted of anhedral grains and granular aggregates.

X-Ray Diffraction Data

The structure of beryllium sulfide has been determined by Zachariasen (*1*) to be of the sphalerite type. Space group $F\bar{4}3m$ (T_d^2).

Cell Dimension. $a_0 = 4.8624 \pm 0.0005$ A. Cell volume 114.96 A^3. Zachariasen (*1*) reported $a_0 = 4.86$ A.

Formula Weights per Cell. 4.

Formula Weight. 41.079.

Density. 2.373 grams per cc. (x-ray).

Powder X-Ray Diffraction Pattern of BeS

hkl	*d*, A., Calcd.	*d*, A., Obsd.[a]	I/I_1[b]
111	2.8073	2.807	100
200	2.4312	2.432	31
220	1.7191	1.718	39
311	1.4661	1.466	32
222	1.4037	1.404	10
400	1.2156	1.216	5
331	1.1155	1.115	13
420	1.0873	1.087	10
422	0.9925	0.9924	13
333, 511	0.9358	0.9357	12
440	0.8596	0.8595	7
531	0.8219	0.8219	18
600, 442	0.8104	0.8104	13

[a] Philips 114.6-mm.-diameter powder camera, Straumanis mounting; $\lambda(CuK\alpha) = 1.5418$ A.; $\lambda(CuK\alpha_1) = 1.54050$ A.; $\lambda(CuK\alpha_2) = 1.54434$ A.
[b] Relative peak intensities above background from densitometer measurements.

Optical Properties

Isotropic.

Refractive Index (5893 A.). 2.275 ± 0.010. Molecular refraction 10.07 cc.

Colorless.

LITERATURE CITED

(1) Zachariasen, W., *Z. phys. Chem.* 119, 201–13 (1926).

Work done under the auspices of the Atomic Energy Commission.

122. Dipotassium Platinum Tetrachloride, K_2PtCl_4

EUGENE STARITZKY, The University of California, Los Alamos Scientific Laboratory, Los Alamos, N. M.

Crystal Morphology

System and Class. Tetragonal, ditetragonal-dipyramidal.

Axial Elements. The author's observations were in agreement with the axial ratio determined by Nordenskiold (*3*). $a:c = 1:0.4161$. Transformed by the matrix $(110/\bar{1}10/002)$ to correspond to the orientation of the primitive unit cell, this becomes $a:c = 1:0.5885$.

Habit. {100} prisms terminated by {001} and {101}.

Polar Angle. (100) Λ (101) = 59° 31′.

Partial Powder X-Ray Diffraction Pattern of K_2PtCl_4

hkl	*d*, A., Calcd.	*d*, A., Obsd.[a]	I/I_1[b]	*hkl*	*d*, A., Calcd.	*d*, A., Obsd.[a]	I/I_1[b]
100	7.017	6.94	100	311	1.9548	1.945	40
110	4.962	4.93	15	320	1.9462		
001	4.131	4.12	30	112	1.9069	1.904	5
101	3.560	3.55	50	202	1.7799	1.776	5
200	3.508	3.49	5	321	1.7606	1.755	30
111	3.175	3.16	65	400	1.7543		
210	3.138	3.12	15	212	1.7253	1.722	10
201	2.674	2.67	<5	410	1.7019	1.698	5
211	2.499	2.489	30	330	1.6539	1.651	<5
220	2.481	2.474	30	401	1.6147	1.610	5
300	2.339	2.333	5	222	1.5873	1.585	15
310	2.219	2.212	5	411	1.5736	1.570	10
221	2.127	2.119	20	420	1.5690		
002	2.065	2.060	10	302	1.5482	1.547	<5
301	2.035	2.031	5	331	1.5354	1.532	5
102	1.9814	1.978	10	312	1.5119	1.510	<5

[a] Philips 114.6-mm.-diameter powder camera, Straumanis mounting: $\lambda(CuK\alpha)$ = 1.5418 A.

[b] Relative peak intensities above background from densitometer measurements.

X-Ray Diffraction Data

The structure of dipotassium platinum tetrachloride has been determined by Dickinson (*2*). The space group is $P\ 4/mmm$ (D_{4h}^1).

Cell Dimensions. a_0 = 7.017 ± 0.003 A.; c_0 = 4.131 ± 0.003 A.; $c_0:a_0$ = 0.5887. Cell volume 203.4 A.^{3} Dickinson (*3*) reported a_0 = 7.00 A.; c_0 = 4.14 A.

Formula Weights per Cell. 1.

Formula Weight. 415.25.

Density. 3.389 grams per cc. (x-ray).

Optical Properties

Uniaxial negative.

Refractive Indices (5893 A.). n_O = 1.6815; n_E = 1.5535; geometric mean 1.6377. Molecular refraction 44.01 cc.

Color. Red with absorption $O > E$.

These differ from properties listed by Winchell (*4*). Winchell's data were derived from Bolland's (*1*) description of a "potassium platinum chloride" of unstated composition and evidently refer to a different compound.

LITERATURE CITED

(1) Bolland, A., *Monatsh.* 31, 387–419 (1910).
(2) Dickinson, R. G., *J. Am. Chem. Soc.* 44, 2404–11 (1922).
(3) Groth, P., "Chemische Krystallographie," Bd. I, pp. 351–2, Engelmann, Leipzig, 1906.
(4) Winchell, A. N., "Microscopic Characters of Artificial Inorganic Solid Substances or Artificial Minerals," p. 173, Wiley, New York, 1931.

Contributions of crystallographic data for this section should be sent to Walter C. McCrone, 3140 South Michigan Ave., Chicago 16, Ill. Work done under the auspices of the Atomic Energy Commission.

CORRESPONDENCE

Coulometric Determination of Aromatic Amines in Acetonitrile

Sir: Under certain conditions aromatic amines may be titrated coulometrically in essentially nonaqueous solution. Aliphatic and heterocyclic amines have been determined by this technique (*4*). When attempts were made to determine aromatic amines in this manner, nonquantitative results were obtained. The solutions became colored when the amines were added, and poor breaks were observed in indicator potential. This behavior may well be due to the ease of oxidation exhibited by most aromatic amines. Oxygen is generated in the solution at the anode concurrently with hydrogen ion, according to the equation

$$2H_2O \longrightarrow 4H^+ + O_2 + 4e^-$$

and this oxygen could oxidize the amines to nontitratable products. Evidently, the primary mode of the amine oxidation is through the electrolytically produced oxygen, since addition of an amine to the pretitrated solution, even when current is not flowing, produces color (*4*).

The addition of an antioxidant to the solution should obviate this difficulty and permit quantitative determination of the aromatic amines. Hydroquinone, H_2Q, shows good antioxidant properties and should combine with the oxygen produced at the anode to yield quinone, Q.

$$H_2Q + {}^1/_2O_2 \longrightarrow Q + H_2O$$

Neither hydroquinone nor quinone is titratable. Furthermore, if the hydroquinone, itself, should undergo electrolytic oxidation, hydrogen ion should be produced with no loss of current efficiency.

$$H_2Q \longrightarrow Q + 2H^+ + 2e^-$$

The hydroquinone could then serve as a source of hydrogen ion in place of water.

Because the solvent contains both water and hydroquinone, it is not known which of the above equations represents the principal mechanism of hydrogen ion production.

PROCEDURE

Apparatus. The generating system was essentially the same as that previously described (*1*, *4*), but a platinum wire spiral was substituted as the anode in place of the gauze electrode formerly used. The spiral proved as efficient as the gauze and appeared to eliminate time lags when low currents were used. These lags may have been due to hydrogen ion entrapped in the platinum gauze, which requires several seconds for the establishment of equilibrium in the solution. Titrations were timed with a Standard Electric Co. Type S-10 electric clock reading to 0.1 second, included in the generator circuit.

The detection system consisted of a Model G Beckman pH meter, a glass indicating electrode, and a silver–silver chloride reference electrode. The reference electrode was made by coating a silver wire with silver chloride and immersing it in acetonitrile saturated with lithium and silver chlorides. The solution was contained in the outer shell of a Leeds & Northrup Type 1199-31 calomel electrode.

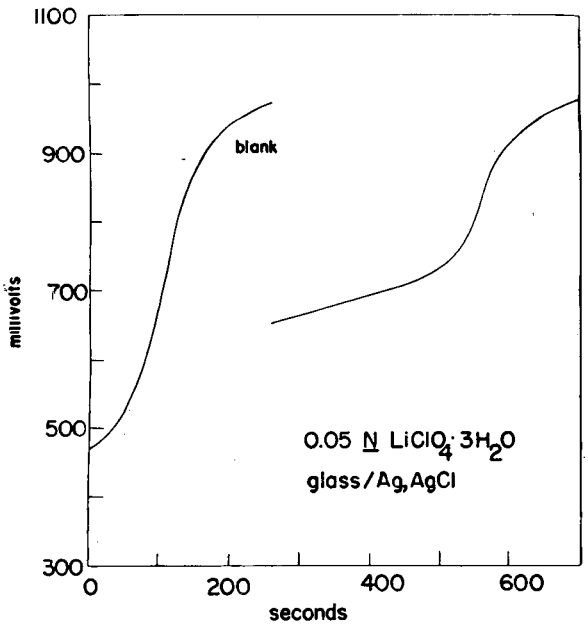

Figure 1. Coulometric titration of pyridine in acetonitrile

This electrode shows greatly improved stability over the usual fiber-type saturated calomel electrode first tried. A sleeve-type calomel electrode cannot be used in the coulometric determinations, as it leaks chloride into the main body of solution and this ion is oxidized at the anode to chlorine.

The silver wire indicating electrode of Khalifa and Issa (*3*) was also investigated, but was found to have too low a rate of change of potential with hydrogen ion concentration to be useful.

The lithium perchlorate concentration of the agar bridge between anode and cathode compartments was 2%.

Reagents. The solvent was commercial grade acetonitrile and contained 8 grams of lithium perchlorate trihydrate per liter (0.05*N*). The salt was obtained from the G. Frederick Smith Chemical Co.; 0.1 gram of hydroquinone was added to the solvent as noted.

The compounds studied included *p*-toluidine, *o*-toluidine, *o*-phenylenediamine, *o*-aminophenol, *p*-aminophenol, and 1-naphthylamine. The purity of these compounds was determined by potentiometric titration in glacial acetic acid with perchloric acid in acetonitrile (*2*).

Titration of *p*-toluidine with a dioxane solution of perchloric acid resulted in a discolored solution, which may have been due to reaction of the base with aldehyde in the dioxane. No discoloration was observed when acetonitrile solutions of acid were used.

Method. The modified apparatus was first tested using pyridine. Before a titration was performed, a plot of indicator potential *vs.* generating time was made (Figure 1). Hydrogen ion was generated in the solvent past the point of inflection, potential readings being taken at suitable intervals. Then a sample of the base to be titrated was added (in 2 ml. of solvent) and hydrogen ion was generated again until the point of inflection was again passed. From this second part of the curve a suitable end-point potential was chosen. Theoretically, this is the potential at the point of inflection in the titration curve of the sample. However, it has been noted that the potential tends to shift upwards during a series of titrations, apparently from heating and buffering effects. Therefore, a potential about 20 mv. above the inflection point is selected. The actual value chosen is not critical, as an error of ±25 to 50 mv. does not have an appreciable effect on the accuracy.

In the actual titration, hydrogen ion is generated until the solution reaches the selected end-point potential, the clock zeroed, a sample added, and generation of hydrogen ion continued until the end-point potential is again established. About half way through the titration the current is determined by measuring the potential drop across the precision resistor. During long titrations—i.e., longer than 500 seconds—the potential drop is measured several times and an average is taken. Even here the current fluctuation is less than ±0.3%, while for shorter titrations current fluctuations are about ±0.1%. A number of titrations, from three to ten, were run consecutively in each batch of solvent solution.

The aromatic amines were determined in the same manner, except that in some of the runs 0.1 gram of hydroquinone was added to the solvent immediately before titration.

RESULTS

Results of the various coulometric titrations, given in Tables I and II, indicate that the method is reasonably accurate down to microgram quantities of base. The revised apparatus was used for the pyridine determinations reported in Table I.

In Table II results in each case were obtained by consecutive titrations in the same solution. The "wt. taken" figures have been corrected for purity as determined by previous potentiometric titrations. During the course of the titrations in the absence of hydroquinone, solutions exhibited colors with all compounds except *p*-toluidine. These colors varied with the amine being titrated. As *p*-toluidine gave neither significantly low results nor any color change in the absence of hydroquinone, titration of this base was not run with the antioxidant.

It is apparent from these data that the use of hydroquinone leads to better results in most cases. Accuracy and precision of determination were improved for every amine tested when hydroquinone was added to the solution, and only in the case of 1-naphthylamine are results significantly low even under these conditions. Average error is less than 2% in most cases, although it may exceed 10% when the hydroquinone is absent, as in the case of *o*-aminophenol.

Without hydroquinone, results in any series of determinations tend to decrease as more samples are added and oxygen and amine concentrations increase. With hydroquinone this tendency is not observed and precision is increased.

Table I. Summary of Pyridine Determinations

Pyridine Taken, Mg.	Pyridine Found (Av.), Mg.	Standard Deviation, Mg.	No. of Samples
0.475	0.468	0.024	3
0.6188	0.6252	0.0032	7
0.2475	0.2495	0.0026	10
0.0619	0.0596	0.0032	10
0.0124	0.0131	0.0007	10
0.0062	0.0060	0.0003	10

Table II. Effect of Hydroquinone on Coulometric Determination of Aromatic Amines

Amine	Wt. Taken, Mg.	Av. Wt. Found without H_2Q, Mg.	Av. Error, Mg.	Av. Wt. Found with H_2Q, Mg.	Av. Error, Mg.
p-Toluidine	0.680	0.680	0.000	...	...
o-Toluidine	0.401	0.405	+0.004	0.403	+0.002
o-Phenylenediamine	0.521	0 489	−0 032	0.530	+0.009
p-Aminophenol	0.388	0.379	−0.009	0.394	+0.006
o-Aminophenol	0.431	0.387	−0.044	0.425	−0.006
1-Naphthylamine	0.623	0.581	−0.042	0.598	−0.025

LITERATURE CITED

(1) Cooke, W. D., "Coulometric Methods" in "Organic Analysis," vol. II, p. 186, Interscience, New York, 1954.
(2) Fritz, J. S., "Acid-Base Titrations in Nonaqueous Solvents," G. Frederick Smith Chemical Co., Columbus, Ohio, 1952.
(3) Khalifa, H., Issa, I. M., *J. Indian Chem. Soc.* 31, 426 (1954).
(4) Streuli, C. A., ANAL. CHEM. 28, 130 (1956).

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RECEIVED December 8, 1955.

Division of Refining, American Petroleum Institute

THE 21st Midyear Meeting of the Division of Refining, American Petroleum Institute, will be held in Montreal May 14 to 17. Papers to be presented include the following.

Other sessions include papers on instrumentation and computers.

Trace Analysis Symposium

Spectrophotometric Determination of Chloride, Bromide, and Iodide. F. W. CHAPMAN, JR., AND R. M. SHERWOOD, Atlantic Refining Co., Philadelphia, Pa.

The petroleum refiner has known for years that trace amounts of metals in petroleum are harmful to various catalysts. More recently it has been found that certain nonmetallic elements such as chlorine can also inhibit the performance of catalysts. Unfortunately, there has been available no accurate and simple method by which very minute quantities of chlorine can be determined in petroleum products. As a result, needless losses of higher quality products have been encountered in the refinery. The method presented here can determine less than 1 part of chlorine, bromine, or iodine in 1,000,000 parts of gasoline. Such a determination will enable a refiner to keep his catalysts from contact with this harmful material.

Determination of Trace Amounts of Arsenic in Petroleum Distillates. N. C. MARANOWSKI, R. E. SNYDER, AND R. O. CLARK, Gulf Research and Development Co., Pittsburgh, Pa.

Arsenic poisons platinum-containing reforming catalysts. Numerous methods have been devised for refining control in the selection of charge stocks for reforming from the standpoint of their arsenic content. Nearly all of these procedures employ prior treatment of the sample to concentrate the arsenic in a form so that the well known Gutzeit test can be employed. Some of these methods gave results that did not agree with the known arsenic content of standards prepared synthetically from triphenylarsine and arsenic-free naphtha. Other procedures appeared to lack sufficient sensitivity to be used in pilot plant work in which it was desired to discriminate between two or more stocks whose arsenic contents were nearly the same.

A method which overcomes these shortcomings is based upon destructive digestion of the sample with acids and subsequent isolation of the arsenic as the colored complex with mercuric bromide in a paper disk. The intensity of the resulting color is measured with a spectrophotometer, using a reflectance attachment. The reading is converted to a value for arsenic by a means of a previously prepared calibration curve. The test can be performed routinely by nontechnical personnel and is applicable to stocks containing arsenic of the order of 1 p.p.m. It has been in use for nearly 2 years and has satisfied the requirements of reliability, discrimination between stocks of nearly the same arsenic content, and speed.

Determination of Trace Amounts of Total Nitrogen in Petroleum Distillates. G. R. BOND, JR., AND C. G. HARRIZ, Houdry Process Corp., Marcus Hook, Pa.

The rapidly increasing interest in methods for determining low concentrations of nitrogen in petroleum charge stocks used in catalytic conversion processes is clearly shown by numerous recent publications. However, very few satisfactory procedures have been developed to cover samples containing less than 30 p.p.m. of nitrogen. The authors have developed a unique method whereby it is possible to concentrate the nitrogenous impurities from large samples onto a small column of silica gel. This gel is then treated by customary Kjeldahl analytical procedures to determine the amount of such nitrogenous impurities present. No unusual laboratory equipment is required.

X-Ray Absorption Edge Spectroscopy as an Analytical Tool. Determination of Molybdenum and Zinc. ROBERT E. BARIEUA, California Research Corp., Richmond, Calif.

An old elemental analysis scheme combined with modern instrumentation gives a convenient, accurate method of analysis. Using the continuous x-ray radiation from a copper x-ray diffraction tube, in combination with a modern commercial x-ray spectrometer, it is possible to make x-ray absorption measurements on materials as a continuous function of monochromatic wave length. By making measurements near and on each side of an absorption edge, a specific analysis for the element characterized by the edge is possible. At the higher concentrations, the method is preferred to x-ray fluorescence because of the absence of a matrix effect. It is not so sensitive as fluorescence at the trace level. Calibration curves and method of measurement are presented for the determination of molybdenum and zinc. The calibration measurements can be used to analyze liquid hydrocarbons or catalysts. The method is applicable to elements above titanium in the periodic table.

Determination of Trace Amounts of Lead in Gasolines and Naphthas. M. E. GRIFFING, Ethyl Corp., Detroit, Mich., AND ADELE ROZEK, L. J. SNYDER, AND S. R. HENDERSON, Ethyl Corp., Baton Rouge, La.

The poisoning effect of heavy metal ions on platforming catalysts is well known. The increasing use of these catalysts for upgrading gasoline and naphtha has resulted in a need for a rapid and accurate method for the determination of 10 to 100 parts per billion of lead. Because the same pipelines, trucks, and storage tanks may be used for both leaded and nonleaded fuels, it is necessary to analyze each shipment and plant feed stream before charging the catalyst. The method used must determine both inorganic lead and tetraethyllead.

Two analytical methods have been developed, both of which are modifications of dithizone colorimetric procedures. One is a two-color method involving the use of a spectrophotometer, and the other is essentially a one-color method involving the use of a color comparator. The former is more suitable for use by laboratories with specialized equipment for the simultaneous analysis of a number of samples; the latter may be applied in a refinery or field situation using very simple equipment. The accuracy of each method is 10 parts per billion.

Determination of Water in Methane and Ethanethiol. G. MATSUYAMA, Union Oil Co. of California, Brea, Calif.

Mercaptans are a vile smelling nuisance in petroleum. To prevent pollution of air in refining operations, oil companies isolate these materials and dispose of them. One way of disposal is by selling these materials, which are used as odorants in domestic fuel gases and as starting materials in making other useful chemicals. In order to sell mercaptans, however, it is necessary to know their purity. Chemists at the Union Oil Co. of California have devised a simple, rapid method for determining the water content of methyl and ethyl mercaptan.

Determination of Trace Amounts of Carbon Monoxide in Gaseous Hydrocarbons. K. H. NELSON, M. D. GRIMES, D. E. SMITH, AND B. J. HEINRICH, Phillips Petroleum Co., Bartlesville, Okla.

Information as to the amounts of carbon monoxide present in gaseous hydrocarbons can be of great importance. The yield of products and the reactivity of catalysts in many petrochemical processes may be materially affected by trace quantities of this contaminant. Suitable analytical procedures do not exist for the determination of a few parts per million of carbon monoxide in gaseous hydrocarbons. Generally, the methods lack sufficient sensitivity, or the hydrocarbons react with the reagents.

A distillation-spectrophotometric method has been successfully applied to the determination of 0 to 100 p.p.m. of carbon monoxide in hydrocarbon gases, with an accuracy of ± 1 to 2 p.p.m. In this procedure, the carbon monoxide is separated from the interfering hydrocarbons by a low temperature fractional distillation after the addition of methane. The carbon monoxide is then measured using an iodine pentoxide-spectrophotometric technique.

General Session on Analytical Research

Application of Vapor-Phase Chromatography to the Analysis of Liquid Petroleum Fractions. D. H. Desty and B. H. F. Whyman, British Petroleum Co., Ltd., Sunbury-on-Thames, Middlesex, England.

In this day of atomic reactors, electronic brains, and other advanced technology, we seldom hear of scientists turning in their more complicated tools for simpler ones. However, a new technique for analyzing gases and liquids, called "vapor-phase chromatography," may prove to be the exception. Though no one intends junking his multi-thousand-dollar equipment for doing such things, analytical chemists are looking toward vapor-phase chromatography as a rapid inexpensive method for determining the composition of natural gas, gasolines, and other products—even of smog. The paper presents an example of what vapor-phase chromatography will do. The authors report successful separation and analysis of hitherto unidentified trace impurities present in nearly pure materials such as octane number reference fuels.

Quantitative Analysis of Organic Disulfides. R. L. Hubbard, W. E. Haines, and J. S. Ball, Petroleum and Oil-Shale Experiment Station, U. S. Bureau of Mines, Laramie, Wyo.

A new, fast, and simple method for determining disulfides in petroleum products was developed. Disulfide determinations are important in product control, particularly in "sweetening" processes. The new method was tested, together with three older methods, for its reactivity toward disulfides of various structures. It was found to be superior to older methods when applied to disulfides in the gasoline boiling range.

Determination of Fuel Oil Stability to Optical Density Measurements. W. H. Armstrong, D. Milsom, H. P. Hebert, and A. R. Rescorla, Cities Service Research and Development Co., New York, N. Y.

For the past number of years a considerable amount of research has been conducted to develop a satisfactory method for measuring the storage stability of distillate fuel oils. Several methods employ filter pad discoloration, filterability, and actual insoluble determinations by gravimetric procedures.

A method for determining the insoluble content of an oxidized fuel oil by use of absorbance consists of measurements employing a 1-cm. light path and 500 mμ on oxidized oil samples before and after filtration through a fine sintered-glass filter (4.5–5 microns). Correlations have been established for determining the insoluble content with an accuracy equivalent to the gravimetric procedure from the difference in absorbance on the filtered and unfiltered samples.

The correlation established between absorbance and insoluble content has been developed, using oils from varying types of crudes and oxidized over a range of temperatures. Additional correlations permit the establishment of predicted storage time at ambient storage.

Rapid, Precise Micro Vapor Pressure Method. A. Y. Mottlau, Products Research Division, Esso Research and Engineering Co., Linden, N. J.

To manufacture motor gasolines of high quality, the refiner must build into them many important characteristics. Of these characteristics, vapor pressure is one of the most critical from the consumer's standpoint. There is a desirable vapor pressure for each season of the year, for each geographical location. If the vapor pressure is too high for the air temperature, the customer's car may vapor-lock. If the vapor pressure is too low for the air temperature, the car will be difficult to start and warm up. Vapor pressure is also an important property of many other fuels.

The precise adjustment of fuel vapor pressure will be made much easier by a newly developed device for measuring it. The new vapor-pressure method has five times the accuracy of the old method, greater speed, and an improved sample handling procedure. This means improved performance for the consumer's equipment and a savings in money to the refiner.

Another big advantage of the new vapor-pressure measuring apparatus is the small amount of sample required to make a measurement. Only 1 cc., now used about 0.034 ounce, is needed. The apparatus requires over 130 times this amount. This will be a great help to the researcher who oftentimes would like to measure the vapor pressure of very small samples obtained from laboratory scale counterparts of the huge refinery stills and cracking units.

Combustion and EDTA Titrimetric Determination of Total Sulfur in Petroleum Products. O. N. Hinsvark and F. J. O'Hara, The Girdler Co., Louisville, Ky.

An improved method, applicable to control procedures, for the determination of sulfur in petroleum samples permits determination of all types of sulfur which might be present in a wide variety of sample materials ranging from low boiling naphthas to high boiling coker gas oils. In general, the sample is aspirated into an oxy-hydrogen flame of a Beckman atomizer burner and burned. As products of combustion are formed, they are swept by air into a hydrogen peroxide scrubber, where oxides of sulfur are converted to sulfate. The sulfate formed in the scrubber is then measured by adding an excess of barium acetate and determining the excess barium by an EDTA titration. Samples covering a wide range of concentration from 100 to several thousand parts per million of sulfur have been successfully analyzed without danger of interference from halides or nitrogen in the sample. An error of ±16 p.p.m. is expected at the lower level with a slightly greater value at a higher level. By combining the combustion procedure and the highly selective titrimetric determination, a single reliable value can be obtained in about 45 minutes.

Fluorescent X-Ray Spectral Analysis of Powdered Solids by Matrix Dilution. E. L. Gunn, Humble Oil and Refining Co., Baytown, Tex.

Present-day petroleum technology requires the fastest and most precise analytical tools available for determining elements in varied substances encountered in the industry. The determination and control of elements are important because in some materials the presence of certain elements is highly detrimental, whereas in other materials elements occurring as specific compounds are very useful for enhancing the manufacture or final quality of a finished product. In recent years a number of rapid precise instrumental techniques have become available for inspection of the materials of petroleum refining. The x-ray fluorescent spectrograph is in the forefront of new instruments for elemental analysis of such materials.

Basically, the x-ray fluorescence spectrograph consists of a high-power x-ray tube, the beam of which causes the elements in a specimen to emit their own characteristic x-rays or to fluoresce, a diffracting device to separate the x-rays into their respective unique wave lengths, and a device to detect and measure each wave length. The measurement of an element is independent of the manner of chemical combination with other elements but not of their mutual presence in the substance. The elements influence each other in such a way that the intensity of x-rays emitted by a given element is not a direct measure of its concentration. This paper describes a technique for lessening or eliminating this effect so that a number of elements coexisting in a powdered solid can be measured with reasonable accuracy.

Determination of Calcium or Zinc Additives in Lubricating Oils and Concentrates by an EDTA Titration Method. P. B. Gerhardt and E. R. Hartmann, Products Research Division, Esso Research and Engineering Co., Linden, N. J.

In many chemical and petroleum processes it is important to know when a particular unit operation is completed or when to proceed to the next operational phase. The stepwise following of a process to establish the quality of an intermediate or end product is commonly referred to as plant control. Although most chemical methods of analysis are time-consuming, they nevertheless can be used to control a batch process. How can these methods be used to control a continuous flow operation? As more manufacturing procedures are converted from batch to continuous flow operations, this question assumes greater importance. The most satisfactory solution to the problem is to develop rapid methods applicable to control of a continuous process.

Chemists at the Esso Research and Engineering Co. have developed a rapid method for determining either calcium or zinc. This can be used to control additive manufacturing plants or the blending of lubricating oils. The procedure consists of a direct titration of the metal in an acetone solution of oil with an organic complexing agent. With this procedure it is possible to complete an analysis in about 20 minutes, as compared to 4 hours by conventional methods.

The method has been used successfully to control the manufacture of various additives and the blending of many lubricating oils with additives containing calcium or zinc.

Society for Analytical Chemistry

At the meeting of Microchemistry Group, held January 27 in London, the following papers were presented and discussed:

Microchemical Methods in the Art Gallery and Museum. A. E. Werner, Research Laboratory, British Museum.

Microanalytical techniques are indispensable in the scientific examination of paintings and other works of art, since the very nature of the objects permits the taking of only minute samples. In the case of paintings, microchemical methods have been devised for the identification of pigments and media. Modern specific organic reagents for the detection of metals have been applied to the detection of pigments using Feigl's spot test technique. This has certain advantages over the previously used microscopical precipitation reactions—for example, it can be carried out on individual layers of a paint sample mounted in a synthetic resin as a cross section. On occasion, spectrographical and spectrophotometrical methods are used to supplement microchemical methods.

The technique of partition chromatography has recently been used for the examination of paint media, both tempera (protein) and oleoresinous. For the latter a special reversed phase system has been devised in the National Gallery Laboratory.

A scheme for the quantitative analysis of bronzes and other metallic museum objects has been worked out using microdeposition supplemented by photometric methods using organic reagents. Finally, micro spot tests have been applied for specific purposes in museum work—e.g., the iodine-azide reagent for the identification of niello and a phosphate test for classifying early English porcelains.

Ring-Oven Technique and Its Application in Archeology. H. Weisz, Department of Chemistry, University of Birmingham.

The ring-oven technique is a simple method for separating ions, or groups of ions, in a single drop. An apparatus called the ring oven is designed to wash soluble materials out from a spot on a filter paper and to concentrate them in a sharply bounded circular ring zone, where they can be detected. Some other pieces of equipment have been developed for this purpose. With the aid of this method an analytical scheme for 14 commoner ions has been worked out; 1 drop of about 1.5 μl. is sufficient for the analysis. The method has also been employed for ring colorimetric analysis.

In the examination of valuable museum specimens, microsamples must be taken, so that the minimum damage is done to the specimens. The available methods for the qualitative examination of such samples are laborious or require expensive apparatus. The ring oven is, however, particularly suited to this type of analysis. A description was given of the analysis of eight Egyptian bronzes of archeological interest.

A meeting of the Midlands Section was held February 7 in Birmingham, at which the following papers were presented and discussed:

Analytical Chemistry of Germanium. H. J. Cluley, G.E.C., Wembley.

Methods of separation and determination of germanium were reviewed. Recent work, stimulated by the use of germanium as a semiconductor and the consequent search for new sources of the element, has mainly concerned the development of colorimetric methods; these methods have greatly facilitated the determination of germanium in coal, flue dust, minerals, etc. On the other hand, gravimetric and volumetric methods have received little attention in recent years and few of the existing methods possess both accuracy and selectivity.

Analytical Chemistry of Gallium. G. W. C. Milner, A.E.R.E., Harwell.

Important improvements have taken place in gravimetric and volumetric methods for the determination of milligram amounts of gallium. Similarly absorptiometric methods for the determination of microgram amounts of this constituent have been reported recently. These developments were considered in some detail, together with improvements for the separation of gallium from other elements by solvent extraction. Examples were given in alloy analysis of volumetric procedures with EDTA which avoid the need for the preliminary separation of the gallium.

The Physical Methods Groups met February 14 in London for a presentation of papers on polarography.

Comparison of Three Highly Sensitive Polarographs. D. J. Ferret, G. W. C. Milner, H. I. Shalgosky, and L. J. Slee, U. K. Atomic Energy Establishment, Harwell.

The new sensitive polarographs include the cathode ray polarograph, the square wave polarograph, and the Cambridge univector unit. Experiments have been carried out to obtain information on the relative merits of the three instruments, and details of the results of these tests were described. The instruments have been tested for sensitivity for reversible and irreversible reductions at the dropping mercury electrode, resolution for elements with $E_{1/2}$ values very close together, effects of the reduction of a major constituent at more positive potentials on the determination of a minor constituent, and speed of application, reproducibility, and usefulness in analytical chemistry.

Polarography of the Dithionite (Hydrosulfite) Anion and Some Related Oxy Acids of Sulfur. W. Furness, Brotherton & Co., Ltd., Leeds.

In the polarography of anions three kinds of reactions at the dropping mercury electrode are distinguishable. First, if the anion is oxidized it yields an anodic wave. Secondly, a reducible anion yields a cathodic wave. Thirdly, in the presence of certain anions, the mercury of the dropping electrode may itself be oxidized and then an anodic wave results.

In the analysis of commercial dithionites and their decomposition products, at least six of the oxy acids of sulfur are encountered. Polarography provides a useful method for the determination of dithionite in such mixtures. The principal wave is anodic and the electrode reaction is of the first kind. Mathematical examination of the polarogram suggests the electrochemical reaction $S_2O_{4aq} \rightarrow 2SO_{2aq} + 2e-$, and gives a value for its standard potential from which the free energy of sulfur dioxide in aqueous solution can be calculated. In certain supporting electrolytes polarograms of the dithionite ion show a small cathodic wave.

In slightly acidic solution one of the principal decomposition products of dithionite, though not formerly recognized as such, is the trithionate ion. At the dropping mercury electrode this ion undergoes a reaction of the second kind and the cathodic wave is useful for analytical purposes.

The polarography of the thiosulfate ion, a well known decomposition product of dithionite, provides an example of a reaction of the third kind.

Polarographic Determination of Uranium in Ores. H. I. Shalgosky, U. K. Atomic Energy Establishment, Royal Arsenal, Woolwich, S.E. 18.

The method developed at the Chemical Research Laboratory, D.S.I.R., for the polarographic determination of uranium in an acid tartrate medium has been examined. Under the conditions used to achieve maximum sensitivity, the method gives low results. This is shown to be due to the effect of heating uranium in concentrated sulfuric acid solution. Satisfactory results are obtained when perchloric acid is used in place of sulfuric acid for the destruction of organic matter.

The factors which affect sensitivity were discussed and the sensitivity of the final method was shown to be similar to that of other polarographic methods.

At a meeting of the Midlands Section on March 6, C. J. Van Nieuwenburg, Delft, Holland, spoke on "Modern Qualitative Analysis and Industrial Practice."

After a short historical survey, the essential features of modern qualitative analysis were given: microscopic reactions and spot tests as identification reactions superimposed on traditional separations, and the trend towards microtechniques. The desirability of adhering to separations and the importance of trace analysis for biochemical and metallurgical practice were discussed, then the possibility of an analysis in situ, without destroying the structure of the sample, and the growing importance of the less common elements. Finally, the competition which must be expected from chromatography and spectrography, and the use of inorganic reagents for groups in organic molecules, came up for discussion.

The following officers have been elected for 1956:

Microchemistry Section. Chairman, G. F. Hodsman; vice chairman, D. F. Phillips; secretary, D. W. Wilson, Sir John Cass College, Jewry St., Aldgate, London E. C. 3; treasurer, G. Ingram.

Midlands Section. Chairman, J. R. Leech; vice chairman, R. Belcher; secretary, G. W. Cherry, 48 George Frederick Road, Sutton Coldfield, Warwicks; treasurer, F. C. J. Poulton.

North of England Section. Chairman, J. R. Walmsley; vice chairman, A. N. Leather; secretary-treasurer, A. C. Wiggins, J. Lyons & Co., Ltd., 5 Laurel Road, Liverpool 7.

Scottish Section. Chairman, F. J. Elliott; vice chairman, Magnus Pyke; secretary-treasurer, J. A. Eggleston, Boots Pure Drug Co., Ltd., Airdrie Works, Airdrie, Lanarkshire.

Microchemical Symposium and Exhibition

The Eleventh Annual Microchemical Symposium and Exhibition, sponsored by the Metropolitan Microchemical Society, was held March 23 and 24 in New York. The following papers were presented:

Improvements in Gravimetric Determination of Sulfur in Organic Compounds. Joseph Grodsky, Ortho Research Foundation, Raritan, N. J.

A procedure was described for the combustion of organic compounds using the Grote apparatus. The sulfate formed is determined as barium sulfate after precipitation in the presence of picric acid. A technique was described for handling the precipitate, using a syringe with a special glass "needle" to transfer the precipitate to a Neubauer platinum crucible.

Method of Analyzing Small Amounts of Materials by Means of the Spectrograph. R. Pomatti, The Texas Co., Beacon, N. Y.

Spectrographic techniques were described which make possible the analysis of extremely small amounts of materials—a few milligrams of solids or a few drops of liquids—for their metallic constituents and phosphorus, silicon, and boron. Techniques include the porous cup electrode, whereby a liquid is analyzed directly by means of a porous graphite cup and spark excitation. Another technique makes use of a revolving graphite platform on which a few drops of a liquid have been vaporized. This is then subjected to arc or spark excitation.

Small amounts of solids can be analyzed with the graphite powder bed method. A few milligrams of the sample are weighed directly on a bed of graphite powder containing a buffer and internal standard, mixed, and subjected to d. c. arc excitation. Some materials can be handled best by dissolving in a small amount of acid; the solution is then evaporated on flat electrodes and arced or sparked. Thin coatings, platings, or deposits on metal samples are identified by playing a low-powered spark discharge on the surface of the specimen.

Polarography of Osmium(VIII) in Alkaline Solutions. Rapid Coulometric Assay of Osmium Tetroxide. Louis Meites, Polytechnic Institute of Brooklyn, Brooklyn, N. Y.

The reduction of osmium(VIII) from alkaline solutions at a dropping mercury electrode proceeds via the +6 and +4 states to osmium(II). Reduction to metallic osmium could not be secured in the presence of alkali metal ions. A curious anomaly in the height of the third wave is due to the interaction of osmium(II) and osmium(VIII) to give a precipitate of hydrous osmium(IV) oxide in the diffusion layer. On the basis of these results a rapid procedure has been devised for the assay of osmium tetroxide (or for the standardization of perperosmate solutions) by coulometry at controlled potential.

Micro Identification of Trace Components of Fruit Juices. Nicholas D. Cheronis and Stamos Eleftheriou, Brooklyn College, Brooklyn, N. Y.

The older literature about this type of natural products (fruit juices) is concerned primarily with the determination and estimation of the major constituents: water, total solids, ash, total sugars, proteins, fats, and a few individual compounds such as ethanol, and such acids as citric, malic, and tartaric. The methods are well described in the "Official Methods of Analysis" of the Association of Official Agricultural Chemists.

Since the advent of micromethods (column and paper chromatography, ion exchange procedures, etc.) there have been references in the literature to the characterization of milligram and microgram quantities of hydroxy and carbonyl compounds. The paper gave a systematic approach for the fractionation and characterization of individual organic compounds from fruit juices and other natural products. Besides the fractionation of amino acids and hydroxy and carbonyl compounds, a procedure for carboxylic acids was presented.

Derivatization of Small Quantities of Organic Compounds and Lower Limits of Organic Reactions. Nicholas D. Cheronis, Herman Stein, and Victor Levey, Brooklyn College, Brooklyn, N. Y.

A brief review was given of the factors which are important in the preparation of derivatives on the milligram scale: purity of derivatizing agent, effect of molar concentration of reactants, and purification procedure. The paper dealt with the development of exact procedures in the microderivatization of carbonyl compounds. The derivatives being investigated are 2,4-dinitrophenylhydrazones, semicarbazones, and methones. An apparatus was described for the derivatization and fractionation of a few micrograms of organic compounds.

The systematic study of the conditions under which 2,4-dinitrophenylhydrazones are formed indicates that the important factors which determine the formation and separation of the desired derivatives are: temperature, solubility, and the molar concentration of the reactants. The reaction of acetaldehyde and acetone with 2,4-dinitrophenylhydrazine was investigated, using melting point—composition diagrams of the pure derivative and the hydrazine hydrochloride (reagent). At $0.1M$ and above the reaction is complete within a few minutes; at a concentration of $5 \times 10^{-2}M$ the rate reaches a plateau at about 70% completion within 15 minutes; and at $5 \times 10^{-3}M$ the rate is so slow that even after several days the reaction does not proceed above the 50% level. Some generalizations as to the lower limits of organic reactions were proposed.

Techniques and Instrumentation for Microanalysis by Gas Chromatography. L. V. Guild, Burrell Corp., Pittsburgh, Pa.

The method and necessary instrumentation were briefly described. The discussion included specific applications and the analytical results achieved for liquid and gas phase samples. Slides were shown of the actual analysis curves obtained.

Microdetermination of Carbonyl Groups by Hydrazone Formation. John Logun, Peter Mazzela, and T. S. Ma, Brooklyn College, Brooklyn, N. Y.

A procedure was described for the microdetermination of carbonyl compounds, based on the condensation of the carbonyl compound with 2,4-dinitrophenylhydrazine reagent, forming an insoluble hydrazone. The hydrazone formation with the above reagent is frequently used in the isolation and characterization of the carbonyl compound under analysis. Shortcomings in the various known gravimetric procedures have been encountered, particularly with respect to the varying accuracies obtained in the analytical results. The technique and procedures are somewhat long and tedious. The kinetics and the mechanisms in the formation of the hydrazone have indicated that the condensation is a balanced reaction. Any deviations of the experimental factors limit the formation of the hydrazone derivative of the carbonyl compound completely. Experimental conditions are being investigated which fulfill the requirements for a practical microdetermination of the carbonyl compound.

The new modification involves the use of oxalic acid in the preparation of the 2,4-dinitrophenylhydrazine solution instead of hydrochloric acid. The oxalic acid, being of an organic nature, lends itself to the preparation of the above reagent solution in various types of solvents other than methanol. The carbonyl compound under analysis is solubilized with methanol and condensed with a saturated methanolic solution of 2,4-dinitrophenylhydrazine and oxalic acid. The hydrazone thus formed, being insoluble, is filtered in the conventional manner, dried, and weighed.

Automatic Weighing. J. Kertzman, Nopco Chemical Co., Harrison, N. J.

The recent trend toward more automation in various industries is also used in the laboratory in the design of laboratory apparatus. Automation has not only improved the quality and quantity of the results, but has cut costs.

Several automatic instruments are everyday laboratory tools: spectrophotometers, temperature recorders and controllers, automatic titrators, etc. Each instrument uses a transducer as a sensing element. The transducers are thermocouple, thermistor, or resistance wire for temperature, photocell and phototubes for spectrophotometers, and glass or metal electrodes for automatic titrators.

To build an automatic balance one of several transducers may be employed: differential transformers, linear or rotary potentiometers, resistance wire strain gage, magnetic strain gage, and piezocrystal electric strain gage. The differential transformer was described as the transducer in our automatic balance. The elements, circuitry, linearity, and other characteristics of the transformers were given. The differential transformer may be adapted either to present analytical balances or to a balance based on the design of the displacement of a cantilever beam. Both instruments were described.

Automatic Control for Fluoride Distillation

Adrian C. Kuyper, Department of Physiological Chemistry, Wayne University College of Medicine, Detroit, Mich.

THE most generally used procedure for the determination of fluoride [Godfrey, P. R., Shrewsbury, C. L., *J. Assoc. Offic. Agr. Chemists* 28, 335 (1945)] requires a preliminary ashing of the sample, steam distillation, and titration of the distillate. The most tedious part of this procedure is the distillation, which must be performed at 135°±3° C. and requires the constant attention of the operator for 1 or 2 hours. The author has devised a simple temperature control which regulates the amount of gas delivered to the burner and maintains the desired temperature automatically.

The thermoregulator is designed to have a small temperature-sensitive area, not much larger than that of a thermometer, which may be inserted into the narrow neck of a flask and immersed in a small volume of solution (Figure 1). The capillary tubing extends through a stopper on the distillation flask and the electrical contacts are made outside of the flask. The regulator owes its sensitivity to the rapid increase in the vapor pressure of a liquid as its boiling temperature is approached. The ethylene glycol–water mixture and the mercury may be introduced into the regulator by placing small amounts in a very large test tube, inverting the regulator and placing it in the test tube, evacuating the test tube with a water aspirator, and then allowing air to reenter the tube. About 0.05 ml. of air should be allowed to remain in the bulb. Mercury rises in the capillary only a few millimeters over the first 80° rise in temperature, but then more rapidly until at 135° it rises a few millimeters for each 1° rise in temperature. The variation in temperature during distillation is within 0.5° as measured with a thermometer. If the upper third of the bulb of the regulator extends above the resting solution, the temperature during distillation is elevated about 1°. Differences in atmospheric pressure cause insignificant changes in the distillation temperature.

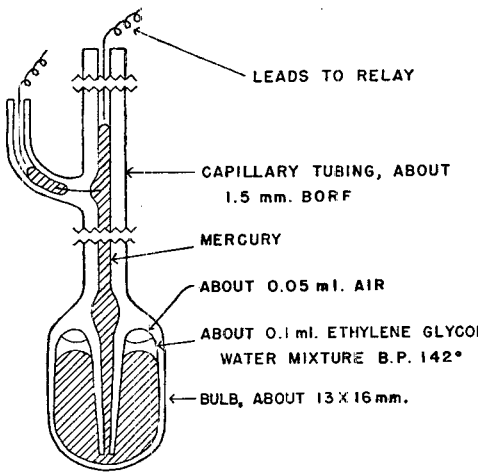


Figure 1. Thermoregulator for constant temperature steam distillation

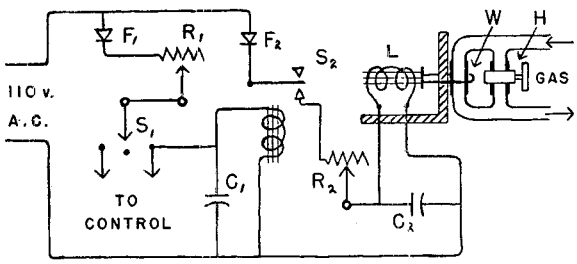


Figure 2. Control circuit for thermoregulator

C_1. 10-μfd. electrolytic capacitor
C_2. 20-μfd. electrolytic capacitor
F_1. 35-ma. selenium rectifier
F_2. 200-ma. selenium rectifier
H. Hoffman clamp
L. Solenoid, Guardian 33073, 110-volt, a.c. continuous duty, 15-ounce lift. A solenoid valve operating on 110-volt a.c. may be purchased from Skinner Electric Valve Division, Skinner Chuck Co., New Britain, Conn.
R_1. 5000-ohm 4-watt industrial control
R_2. 250-ohm 50-watt adjustable resistor
S_1. SPDT toggle switch, center off
S_2. 10,000-ohm SPDT plate circuit relay
W. Stout wire clamp for closing rubber tubing

With the exception of the thermistor type of regulator, which requires an expensive resistance-operated electronic relay and adaptation for use with a solenoid valve, commercially available regulators have too large a heat-sensitive area and are usually not sufficiently long for use in the control of this distillation.

In the control circuit (Figure 2) current passes from the 110-volt line through a rectifier, a variable resistance, the thermoregulator, and a vacuum tube plate relay. The use of pulsating current of low amperage minimizes sparking at the thermoregulator contacts to the extent that it is rarely discernible. The resistance should be set so that there is a time delay of a few tenths of a second in the closing of the relay. This delay, together with the time delay on opening the relay, effectively prevents chattering. Closure of the relay causes closure of the solenoid valve, which clamps a rubber tube and stops the flow of gas to the burner. The solenoid is operated on rectified current in order to avoid the excessive chattering that occurs when it is operated on alternating current. A small amount of gas is allowed to bleed past the solenoid valve in order to keep the burner lighted when the valve is closed. If it is desired to control the distillation by introduction of cold water, connection to the solenoid is made through the opposite arm of the double-throw relay. The control circuitry may be easily mounted in a 6 × 9 × 5 inch cabinet.

This equipment was developed for use in research supported in part by the National Institutes of Health.

Antivibration Table for a Semimicro Balance

D. G. Gage and Patrick Sullivan, Naval Research Establishment, Dartmouth, Nova Scotia, Canada

THE elimination of vibration in tables for analytical balances has often created problems, particularly in industrial laboratories or in buildings in which heavy machinery is used. A table devised in this laboratory has very good vibration-damping qualities when used with the Mettler semimicro Gram-atic balance.

The damping material is 0.5-inch heavy felt of the type commonly used in mounting lathes and milling machines in machine shops. In order to take full advantage of its damping qualities, it must be heavily weighted; the table top, therefore, is a granite plate weighing 165 pounds. In this case, an instrument maker's layout plate was used, but any flat plate of similar weight and dimensions should suffice.

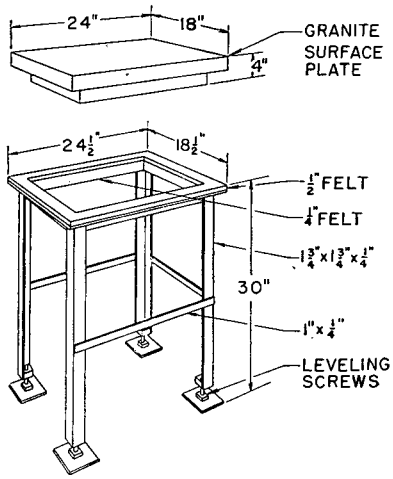
The dimensions of the plate are as shown in the diagram, and there is a 2-inch ledge on all sides. Dimensions of the frame are dependent on those of the top plate. The frame and legs of the table are constructed of angle iron welded at all joints. The legs are braced on three sides with 1-inch strapping; the remaining side is left open as knee space. Leveling screws are provided on each leg.

Some evidence of the vibration-damping qualities of this table was recently provided during alterations and new construction in the building in which it is housed. Weighings could be made on a semimicrobalance on this table while riveting and compressed air drillings were going on. An ordinary Gram-atic balance in the same room was mounted on a commercial pattern balance table consisting of a Transite plate weighted with a steel pendulum and damped with soft rubber. The scale of this balance

Table I. Zero Readings of Balances on Commercial and Antivibration Tables[a]

Commercial Table, Mg.	Antivibration Table, Mg.
Semimicro Gram-atic Balance	
0.56	−0.4
0.10	0
−0.48	0.25
−0.20	0
−0.06	0
−0.14	−0.10
0.04	0
Gram-atic Balance	
−0.2	−0.4
−0.2	0
0.5	0.2
0	0
0.1	0
−0.2	0
−0.1	0.1

[a] Balances adjusted to zero after each reading.



could not be read while the activities mentioned were being carried out.

A series of zero readings was made over a period of several days on each of the above balances on both mountings. (Table I shows that the described mounting is less subject to tilt than the commercial pattern table; the tilt appears easily corrected by manipulation of the zero adjustment of the balance.)

Concentration Cell for Following Ion Exchange Separation of Halides

Lewis P. Larson and Harry C. Becker, Beacon Laboratories, The Texas Co., Beacon, N. Y.

With ion exchange studies it is highly desirable to use a device that will continuously record some property of the effluent which is dependent on the composition of the solution. Various procedures have been reported in which the effluent is allowed to flow through an arrangement for determining continuously the refractive index (*2*), light absorption (*4*), conductivity (*6*), pH value (*5*), or radioactivity (*1*). In this laboratory a simple silver–silver chloride concentration cell has been used for following the ion exchange separation of halides.

The cells for this technique were designed and arranged so that the eluent would flow through one half of the cell and the eluate through the other, as shown in Figure 1. The cell was simply a silver wire cemented in 8-mm. glass tubing with Apiezon cement. The electrodes were given a silver chloride coating by electrolyzing for a few minutes with a current of approximately 6 ma. in a dilute potassium chloride solution, to which a few drops of hydrochloric acid had been added. The cells were connected with a salt bridge of potassium nitrate in agar. Satisfactory potential measurements could be made with either a recording potentiometer or an electronic voltmeter.

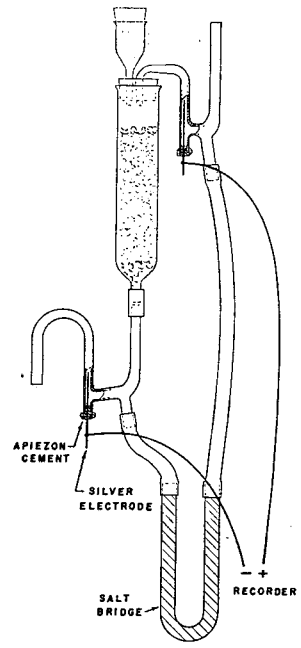


Figure 1. Ion exchange apparatus

The technique has been used to study the separation of chloride, bromide, and iodide, such as in the method of De Geiso, Rieman, and Lindenbaum (*3*), in addition to gradient elution procedures. Fluoride could not be detected, nor did it interfere in the method. Although the potential from the cell in the halide mixtures gave only a semiquantitative measure of the halides, it was extremely sensitive and was found adequate for the study of such factors as resin type, eluent concentrations and pH, flow rates, and column geometry. The use of this technique required considerably less time and effort than previous studies that involved the collection and analysis of numerous fractions. The type of data obtained is illustrated in Figure 2. Here the cell potential *vs.* effluent volume has been recorded for a gradient elution separation. This separation was made using approximately 0.25 meq. of each of the halides, a 19 mm. (inner diameter) × 7.5 cm. column of Dowex 1-X10 (100 to 200 mesh), 0.1 to 6*N* sodium nitrate (pH 10) as the eluent, and a flow rate of 2.4 ml. per sq. cm. per minute. Cut points for the quantitative analysis of individual halides, if desired, may be easily selected.

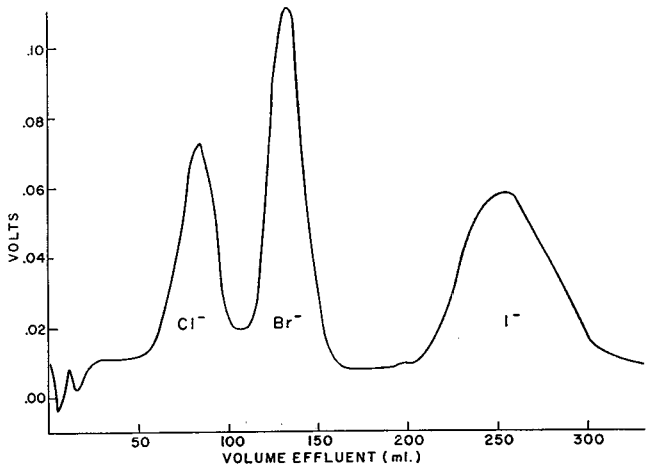


Figure 2. Gradient elution separation of halides on ion exchange column

LITERATURE CITED

(1) Atteberry, R. W., Boyd, G. E., *J. Am. Chem. Soc.* **72**, 4805 (1950).
(2) Claesson, S., *Arkiv Kemi Mineral. Geol.* **A23**, No. 1 (1946).
(3) DeGeiso, R. C., Rieman, Wm., III, Lindenbaum, Siegfried, ANAL. CHEM. **26**, 1840–1 (1954).
(4) Deutsch, Alfred, Zuckerman, Richard, Dunn, M. S., *Ibid.*, **24**, 1763 (1952).
(5) Partridge, S. M., Westall, R. G., *Biochem. J.* (*London*) **44**, 418 (1949).
(6) Wickbold, R., *Z. anal. Chem.* **132**, 401 (1951).

Inexpensive Micro Vacuum Desiccator

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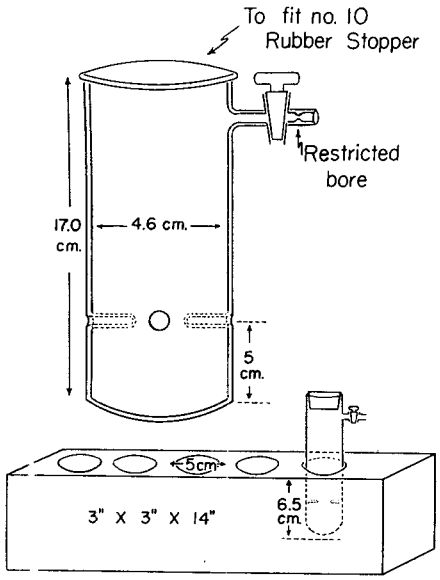
THE efficient removal of excess solvent from recrystallized solids requires a different type of desiccant for each class of solvent. For example, it is convenient to have available a vacuum desiccator containing sulfuric acid for pyridine recrystallizations, phosphorus pentoxide for water recrystallizations, and paraffin for petroleum ether recrystallizations.



Having available the usual type of large desiccator containing these desiccants not only is expensive, but uses a good amount of shelf or desk space. Since, in a research laboratory, the amount of material to be dried is often only a few grams, the author has been using a very simple form of vacuum desiccator, which is inexpensive and occupies relatively little space. It was designed to hold small sintered-glass crucibles or sample bottles. The desiccant is normally placed in the lower section, below the indented tips, and separated from the upper section (containing the sample bottle or crucible) by a small pad of glass wool. A tightly fitted rubber stopper makes an adequate seal for most purposes and, in contrast to ground-glass stoppers, does not "freeze" on prolonged storage.

A rack of five desiccators placed in a 3 × 3 × 14 inch wooden block, containing appropriately bored holes, takes up only a small amount of desk space and yet makes available five separate desiccants—e.g., phosphorus pentoxide, sulfuric acid, potassium hydroxide, paraffin, and calcium sulfate—for immediate use. Where compounds must be stored under refrigeration in an anhydrous state, these small desiccators utilize relatively little room in a refrigerator or deep-freeze.

Vial Testing with Manometric Apparatus

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AMPOULE testing with the Van Slyke manometric blood gas apparatus has already been described [Steyermark, A., IND. ENG. CHEM., ANAL. ED. 17, 191 (1945); Steyermark, A., "Quantitative Organic Microanalysis," p. 280, Blakiston, Philadelphia, 1951] and used extensively in the laboratories of this company as a means of obtaining quantitative data for stability studies in which carbon dioxide is a decomposition product. The problem presented itself of performing similar tests on materials for parenteral injections stored in rubber-capped vials. Modification of the vessel used for ampoule testing proved satisfactory in the case of vials. Instead of using the plunger to break an ampoule, the device was modified so that the depression of the plunger caused a hypodermic needle to pierce the rubber cap of the vial, thus connecting its contents with the manometric system. Otherwise, the procedure is identical to that used for ampoule testing. Figure 1 gives the details of construction.

ACKNOWLEDGMENT

The authors are indebted to Henry Mianecki for preparation of Figure 1.

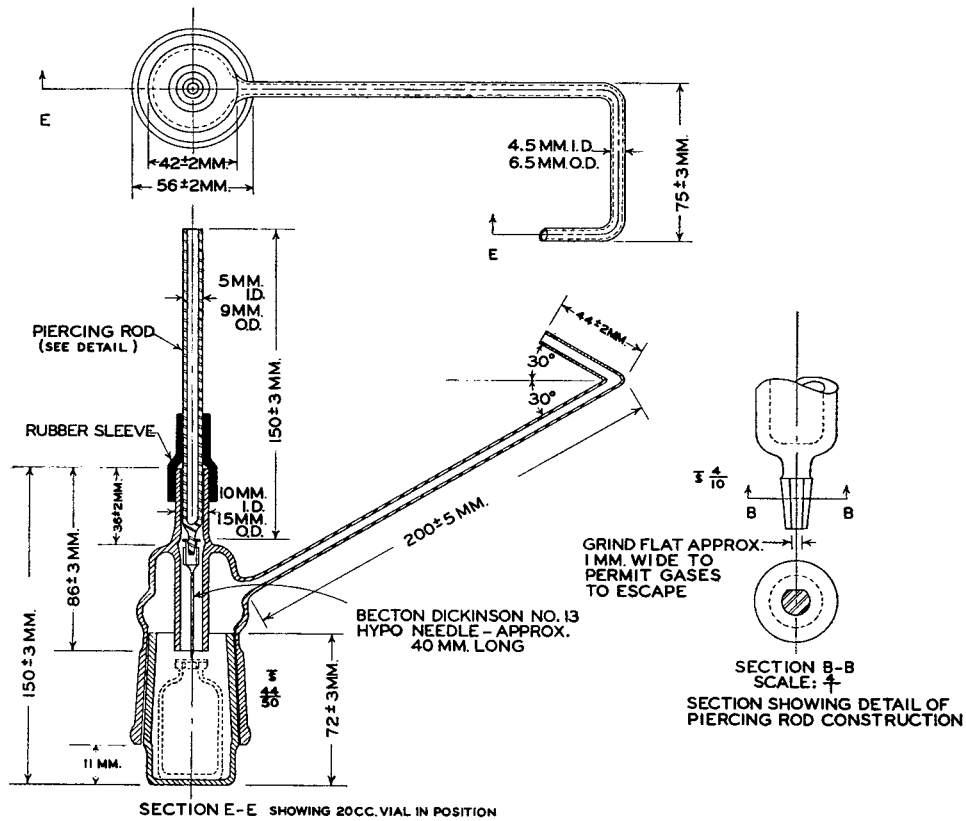

Figure 1. Details of manometric apparatus