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Papers from the Symposium on Vitamins presented before the joint meeting of the Divisions of Biological and Agricultural and Food Chemistry at the 100th Meeting of the American Chemical Society, Detroit, Mich., pages 209–31.

# Physical and Chemical Determination of Vitamin A

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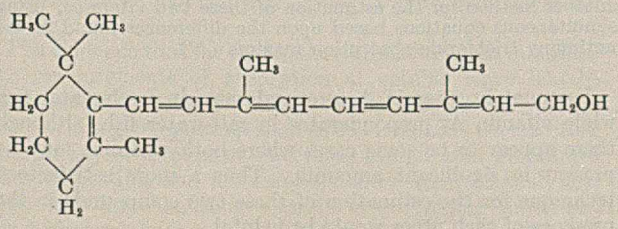
THE existence of vitamin A was biologically established about 25 years ago, and since that time its importance for health and nutrition has become generally recognized. Coincidentally three general methods for its assay have received much attention. One of these, the biological method, is well established and is now recognized by the U. S. Pharmacopoeia as official. However, because of the time involved and the expense associated with the biological method, much effort has been expended upon possible physical and chemical procedures for this vitamin. The literature upon the subject is relatively voluminous and contains many contradictions, but enough work has now been completed to indicate either the causes or the remedies for many of the earlier difficulties. It is a principal purpose of this review to indicate the current status of the physical and chemical determinations of vitamin A.

The accumulated knowledge of the vitamin A determination is largely empirical and not until recently has its structure been determined. A study of this structure may help to determine causes of error in the physical and chemical estimation of this substance. We see by reference to the structural formula that it consists of a beta-ionone ring attached to an unsaturated chain with methyl groups and ending with an OH group.



## Antimony Trichloride Reaction

The earlier efforts toward the estimation of vitamin A dealt largely with chemical reactions. The observation was made that cod liver oils would give color with various types of reagents such as arsenic trichloride (*39*), sulfuric acid (*46*), and antimony trichloride (*49*). As early as 1880 a chemical test for the identity of cod liver oil in the U. S. Pharmacopoeia was based upon the action of the oil with sulfuric acid. Thus it appears that a test for vitamin A was available before the vitamin was discovered biologically.

Carr and Price (*4*) in 1926 studied the analytical possibilities of such a reaction more thoroughly than had been done previously. They established the use of antimony trichloride in chloroform as a quantitative reagent but found that the color resulting from its reaction with vitamin A faded rapidly. When this color was examined spectrophotometrically the 620 and 583 mμ bands were found to predominate in concentrates while 606 and 572 mμ bands were found in unconcentrated oils. The antimony trichloride test has been published as a recommended method for concentrates in the British Pharmacopoeia.

A 20 per cent solution of the oil is prepared at a specified temperature (20°); 0.2 cc. of this solution is dropped into a 1-cm. cell and the oil is placed in a colorimeter or a tintometer. Then 0.2 cc. of the reagent (antimony trichloride) in dry chloroform is added in such a manner as to mix rapidly.

Many other investigators (*2, 3, 7, 17, 18, 21, 30, 32, 33, 34, 36, 37, 43, 45*) have shown that the antimony trichloride reaction is normally susceptible to various factors affecting the developed spectral bands. Changes in these bands may be due to interfering materials, such as inhibitors, or to lack of proper precautions in technique, particularly control of temperature, improper preparation of reagent, or lack of speed in making readings.

For certain classes of materials the numerous investigations indicate that the antimony trichloride reaction with the proper precautions will give results of quantitative significance having about the same accuracy as the ultraviolet spectrophotometric method considered below.

Several attempts to modify the Carr-Price method have been made and significant changes have included the use of catechol (*40*) and guaiacol (*41*). The test performed with guaiacol was claimed to provide greater specificity relative to carotene which might be present and to improve persistence of the color formed. The effect of 20 different modifications of the antimony trichloride reaction has recently been reported by Pacini and Taras (*38*).

Dann (1938) applied the Evelyn (*9*) photoelectric colorimeter to the antimony trichloride reaction. His contribution to the

technique, especially in the use of the photoelectric colorimeter, undoubtedly provides needed objectivity by eliminating errors due to personal judgment. While the dependability of such a method may be established for a certain type of material, the possibility of interfering substances in other products may still be significant. Also, speed and special precautions are still needed, though undoubtedly not so urgently as they were originally.

## Ultraviolet Absorption

Essentially the only physical characteristic of vitamin A utilizable for its determination is its absorption in the ultraviolet at 328 mμ. It appears that the 328 mμ ultraviolet absorption characteristic was first discovered by Takahashi in 1925 in fish liver oil concentrates (*46*). Subsequent British investigators, starting with Morton and Heilbron in 1928, showed a correlation between absorption and biological activity (*7*, *12*, *32*, *35*).

The conclusion from early evidence largely accumulated by the British workers indicated the ultraviolet absorption to be a more reliable index of vitamin A content than antimony trichloride color reaction with its attendant complications. In 1934 the International unit of vitamin A was established by the Health Organization of the League of Nations as 0.6 microgram of beta-carotene. Since that time a large amount of effort both in England and in this country has been directed toward establishing a possible quantitative relationship between biological units and the unit of absorption at 328 mμ which has come to be known as the $E^{1\%}_{1cm.}$ value. This problem has been the subject of individual studies as well as collaborative effort both in this country and in England (*1*, *23*, *26*, *27*). It is now ordinarily assumed that the vitamin A ultraviolet absorption follows Beer's law in concentrations suitable for spectrophotometric examination. Experience in this laboratory, as well as reports and collaborative data from other laboratories, has indicated the validity of this assumption whenever certain precautions are taken, such as the maintenance of purity of the measured radiation. Generally, then, either the use of $E^{1\%}_{1cm.}$ value or a factor for converting $E^{1\%}_{1cm.}$ value into biological units is an accepted practice for comparing results. The factor may be established by direct comparison with biological assay or more generally by the use of a secondary standard whose biological value has been established. Such a secondary standard has been available in the U. S. P. reference cod liver oil biologically standardized against the International standard beta-carotene.

A curious lack of agreement between English workers and American workers as to what the conversion factor should be has persisted until recently. The British workers have found 1600 to be an acceptable factor, while in this country the value most generally used approximates 2000. Differences in biological procedures may possibly explain the discrepancies, although the question of a possible deterioration of improperly stored samples of the U. S. P. reference oil must be considered (*31*). The experienced British workers, Underhill and Coward (*8*), in 1939 deduced from biological studies involving crystalline vitamin A esters and the International standard beta-carotene that the conversion factor should probably be approximately 2000. The crystalline vitamin A prepared by Holmes and Corbet (*24*) was found by one of their collaborators to have a maximum $E^{1\%}_{1cm.}$ value of 2100. With a factor of 2000, this would mean that the pure crystalline vitamin A has a value approximating 4,000,000 units per gram. Biological evidence obtained with solutions of this pure vitamin A indicates that it does in fact have a potency of between 3,500,000 and 4,000,000 International units per gram.

The collaborative investigations have in general not yielded correlations as close as could be desired. The study completed in 1937 by the American Pharmaceutical Association yielded $E^{1\%}_{1cm.}$ values from 1.4 to 1.79 for the U. S. P. reference oil. The study completed last year by the American Drug Manufacturers Association yielded essentially the same values and the same amount of variation when both true spectrophotometers and modified ultraviolet absorption apparatus were jointly considered. However, when only the true spectrophotometers were used, the spread was reduced to ±8 per cent. At the present time another collaborative study by the U. S. Pharmacopoeia committee on the spectrophotometric vitamin A determination is nearly completed. This should afford a more acceptable conversion factor and some additional insight regarding previous difficulties, since this is the first study in which only true spectrophotometers standardized with potassium chromate have been exclusively used.

No doubt the greatest cause for discrepancies in either the ultraviolet absorption method or a colorimetric method lies in the labile nature of the molecule of vitamin A itself. Interrelationships with vitamin $A_2$ and other materials absorbing in the ultraviolet or interfering with a color test furnish additional complications.

Morton, Webster, and Heilbron treated a vitamin A concentrate in alcohol with hydrochloric acid and were able to obtain from the resulting solution the derived product which they identified as 1,6-dimethylnaphthalene. From this it was concluded that vitamin A had undergone cyclization (*13*, *15*, *22*). This change was associated with the development of numerous bands in the ultraviolet region. It appears that this degraded material may be formed in an oil under various normal conditions, especially if fatty acids are present, or it may occur naturally (*29*). The blue values for vitamin A are not changed by cyclization. The cyclized material has considerable ultraviolet absorption with the principal peak shifted to the region of 368 mμ. Some workers in this country have referred to this material as spurious vitamin A, since it has no biological activity.

A compound very similar to vitamin $A_1$ has recently been described and is known as vitamin $A_2$. Lederer and Rosanova (1937) found substances in certain Russian fresh-water fishes in which antimony trichloride bands at 690 and 645 mμ predominate (*29*). This finding was confirmed and elaborated in 1938 by Edisbury, Morton, Simpkins, and Lovern (*14*), Gillam, Heilbron, Jones, and Lederer (*19*), and Lederer and Rathman (*28*). Vitamin A or $A_1$ has a peak around 620 or 605 mμ in oils low in vitamin A and a direct absorption maximum at 328 mμ, while vitamin $A_2$ is responsible for a 690 to 697 mμ antimony trichloride band as well as one at 640 mμ. It was also found to possess direct absorption at the correspondingly longer ultraviolet wave length of 345 to 350 mμ together with another band in the region of 290 mμ. Gillam *et al.* judged that vitamin $A_2$ had the same formula as vitamin $A_1$ except that it had two or more carbon atoms or one more ethylene group. Contradictory evidence has been recently obtained (1939) by Gray to indicate that the differences may be explained by the presence of merely one additional double bond and that vitamins $A_1$ and $A_2$ therefore have the same number of carbon atoms (*20*).

Although vitamins $A_1$ and $A_2$ differ considerably in their ultraviolet absorptions and antimony trichloride values (*19*, *20*), the absorptions of the cyclized compounds have been found by independent investigators to be identical. However, it would appear that these cyclized compounds are not identical, since Embree and Shantz (*16*) have proposed a quantitative determination of each of the constituent vitamins when mixed, based on the differences of their respective adsorptions on alumina. Vitamin $A_2$ is more strongly held by adsorbing agents. Wald has proposed another method for the estimation of these two vitamins, using simultaneous equations based upon the differences found in the antimony trichloride absorption maxima (*47*).

Fortunately vitamin $A_2$ preponderates in fresh-water fish while vitamin $A_1$ preponderates in salt-water fish, although there appear to be some cases where both vitamins may be present in significant amounts. Thus a more satisfactory technique for the estimation of these two compounds in the presence of each other would be helpful.

## Stability of Vitamin A

The stability of vitamin A is influenced principally by oxidation and by light. Vitamin A is destroyed rather easily by oxidation and certain substances, which appear to occur in

natural oils, can inhibit this oxidation. Also, the naturally occurring esters appear to be resistant to attack. It is common observation that an air space in the top of a bottle of fish liver oil can cause a very significant drop in strength in a few weeks, as evidenced by a decrease in the 328 mμ absorption and a corresponding decrease in biological potency even though the oil is stored in the dark in a refrigerator. The oxidative deterioration is distinguished by a decrease in absorption at 328 mμ without the development of new bands as in the case of cyclization.

With reference to the effects of light, some investigations (*6, 10, 11*) have shown that irradiation can partially destroy vitamin A, while others have shown that it can totally destroy it. Consequently, attempts have been made to determine vitamin A by measuring absorption before and after irradiation. The method seems to lack confirmation, although the possibilities in this direction do not appear to be exhausted.

Smith (*42, 44*) and associates have reported some interesting studies concerning the stability of vitamin A in solution. They found that decreases in 328 mμ absorption caused by irradiation were partially reversible, indicating isomeric equilibrium affected by light. They also reported similar reversible changes by specific solvent action. For example, in one experiment a vitamin A–containing material was first made to volume in chloroform, the absorption was measured, the chloroform was removed with a vacuum, and the material was again made to volume with diethyl ether. In this experiment the latter ether solution was found to have an appreciably higher absorption value than the original chloroform solution. Such results have been attributed to isomerism. It would appear expedient to have in mind the possibility of such phenomena occurring inadvertently before or during a spectroscopic examination.

In the preparation of vitamin A solutions sufficiently pure for light-absorption measurements, it is ordinarily necessary to use extraction and saponification methods (*43, 48*). (A fish liver oil, for example, might be saponified for 20 minutes with alcoholic potash, extracted four times with diethyl ether, then washed to remove soaps. The ether would then be evaporated almost to dryness but never to dryness, and made to volume with a solvent more suitable for spectrophotometric use.)

These methods have received considerable study and although not completely satisfactory at the present time, they are usually dependable for most materials in the hands of experienced workers. The fact that maximum correlation in collaborative work has not been attained with the nonsaponifiable and extracted portions of oils suggested that further improvement in these methods is desirable. In the separation of carotene from vitamin A in solutions to be used for light-absorption measurements, both the selective action of solvents and the adsorption of carotene on charcoal have been reported to be satisfactory. Attempts have also been made to separate vitamin A by means of selective adsorption. In general, however, this may be a dubious practice since there is some evidence that chromotographic separation results in uncertain molecular changes in the vitamin component (*5, 25*).

The evidence thus far accumulated indicates that quantitative measurements of absorption at specific wave lengths either before or after chemical reaction can be fairly satisfactory for a given product such as cod liver oil. On the other hand, it is just as evident that more complicated procedures are both possible and necessary for more accurate analytical work with miscellaneous vitamin A–containing materials. For such a program a more rapid and accurate definition of entire spectral curves throughout the ultraviolet and visible regions is regarded as fundamental. The author is therefore at present actively concerned with apparatus, and while his work in this direction is probably far from complete, he feels that progress is being made along this line. It is expected that such apparatus will be the subject of subsequent publication.

## Literature Cited


(1) Barthen, C. L., Berg, F. F., Carter, E. B., Copley, D. M., Fosbinder, R. J., Tewes, T., and Taylor, F. O., "A Collaborative Investigation of the Spectrophotometric Assay for Vitamin A", presented at 28th Annual Meeting of American Drug Manufacturers Association, Hot Springs, Va. (May 1, 1939).
(2) Brode, W. R., and McGill, M. A., *J. Biol. Chem.*, 92, 89–98 (1931).
(3) Carr, F. H., and Jewell, W., *Biochem. J.*, 28, 1702–11 (1934).
(4) Carr, F. H., and Price, E. A., *Ibid.*, 20, 497–501 (1926).
(5) Castle, D. C., Gillam, A. E., Heilbron, I. M., and Thompson, H. W., *Ibid.*, 28, 1702–11 (1934).
(6) Chevallier, Andre, *Z. Vitaminforsch.*, 7, 10–16 (1938).
(7) Coward, K. H., Dyer, F. J., Morton, R. A., and Gaddam, J. H., *Biochem. J.*, 25, 1102–20 (1931).
(8) Coward, K. H., and Underhill, S. W. F., *Ibid.*, 33, No. 4, 589–600 (1939).
(9) Dann, W. J., and Evelyn, K. A., *Ibid.*, 32, 1008–17 (1938).
(10) De, N. K., *Indian J. Med. Research*, 23, 505–14 (1935).
(11) *Ibid.*, 24, 737–49 (1937).
(12) Drummond, J. C., and Morton, R. A., *Biochem. J.*, 23, 785–802, No. 4 (1929).
(13) Edisbury, J. R., Gillam, E. A., Heilbron, J. M., and Morton, A., *Ibid.*, 26, 1164–73 (1932).
(14) Edisbury, J. R., Morton, R. A., Simpkins, G. W., and Lovern, J. A., *Ibid.*, 32, 118–40 (1938).
(15) Embree, N. D., *J. Biol. Chem.*, 127, 187–98 (1939).
(16) Embree, N. D., and Shantz, E. M., *Ibid.*, 132, No. 2, 619–26 (1940).
(17) Emmerie, A., Eckelen, M. V., and Wolf, L. K., *Nature*, 128, 495–6 (1931).
(18) Gillam, A. E., *Biochem. J.*, 28, 79–83 (1934).
(19) Gillam, A. E., Heilbron, I. M., Jones, Wm. E., and Lederer, E. A., *Ibid.*, 32, 405–16 (1938).
(20) Gray, E. LeB., *J. Biol. Chem.*, 131, 317–26 (1939).
(21) Heilbron, I. M., Gillam, A. E., and Morton, R. A., *Biochem. J.*, 25, 1352–66 (1931).
(22) Heilbron, I. M., Morton, R. A., and Webster, E. T., *Ibid.*, 26, 1194–6 (1932).
(23) Holmes, A. D., Black, A., Eckler, A. D., Emmett, A. D., Heyl, F. W., Nelsen, C., and Quinn, E. J., *J. Am. Pharm. Assoc.*, 26, 525–40 (1937).
(24) Holmes, H. N., and Corbet, R. E., *J. Am. Chem. Soc.*, 59, 2042–7 (1937).
(25) Holmes, H. N., and Corbet, R. E., *J. Biol. Chem.*, 127, 449–56 (1939).
(26) Hume, E. M., *Nature*, 143, 22–33 (1939).
(27) Hume, E. M., and Chick, Harriette, Med. Research Council, *Special Rept. Series* 202, London, His Majesty's Stationery Office, 1935.
(28) Lederer, E. A., and Rathman, F. H., *Biochem. J.*, 32, 1252–61 (1938).
(29) Lederer, E. A., and Rosanova, V. A., *Biokhimiya*, 2, 293–303 (1937).
(30) Lovern, J. A., Creed, R. H., and Morton, R. A., *Biochem. J.*, 25, 1341–45 (1931).
(31) McFarlan, R. L., "Vitamin A Determination", Spectroscopy Conference, Mass. Institute of Technology, Cambridge, Mass., 1940.
(32) MacWalter, R. J., *Biochem. J.*, 28, No. 2, 472–5 (1934).
(33) Morgan, R. S., Edisbury, J. R., and Morton, R. A., *Ibid.*, 30, 1645–60 (1936).
(34) Morton, R. A., *Ibid.*, 26, No. 4, 1197–201 (1932).
(35) Morton, R. A., and Heilbron, I. M., *Ibid.*, 22, No. 4, 988–96 (1928).
(36) Norris, E. R., and Church, A. E., *J. Biol. Chem.*, 85, 477–89 (1930).
(37) Notevarp, O., and Weedon, H. W., *Biochem. J.*, 32, 1054–63 (1938).
(38) Pacini, A. E., and Taras, M. H., *J. Am. Pharm. Assoc.*, 26, 721–3 (1937).
(39) Rosenheim, O., and Drummond, J. C., *Biochem. J.*, 19, No. 4, 753–6 (1925).
(40) Rosenthal, E., and Erdelyi, J., *Biochem. Z.*, 267, 119–23 (1933).
(41) Rosenthal, E., and Szilard C., *Biochem. J.*, 29, 1039–42 (1935).
(42) Smith, E. L., *Ibid.*, 33, No. 2, 201–6 (1939).
(43) Smith, E. L., and Hazley, V., *Ibid.*, 24, 1942–51 (1930).
(44) Smith, E. L., Robinson, F. A., Stern, E. B., and Young, F. O., *Ibid.*, 33, 207–12 (1939).
(45) Smith, J. H. C., *J. Biol. Chem.*, 90, 597–605 (1931).
(46) Takahashi, K., *et al.*, *Sci. Papers Inst. Phys. Chem. Research* (Tokyo), 3, 81–148 (1925).
(47) Wald, George, *J. Gen. Physiol.* 22, No. 3, 391–415 (1939).
(48) Wilkie, J. B., *J. Assoc. Official Agr. Chem.*, 23, 336–41 (1940).
(49) Willimott, S. G., Moore, T., and Wokes, F., *Biochem. J.*, 20, 1292–8 (1926).

# Recent Developments in Methods for Determining Carotene

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MOST procedures that have been devised for the determination of carotene and other carotenoid pigments have been based upon the discovery of Borodin (*3*) in 1883 that the carotenoid pigments could be separated into alcohol-soluble and petroleum ether–soluble fractions. Early methods for the quantitative determination were also reported by Arnaud (*1*) and by Monteverde and Lumbimenko (*20*), but the method which has been used most widely as a starting point in the development of new techniques is that of Willstätter and Stoll (*36*).

Lest it be assumed that the latter method, which was published in 1913, is only of historic significance scientifically it should be pointed out that in most essentials later extraction methods have offered no strikingly new contributions. The extent to which this is true is shown by the following brief summary of the Willstätter and Stoll method.

The fresh plant material is ground finely in a mortar with quartz sand and 40 per cent acetone. The ground material is then filtered and washed with 30 per cent acetone until the filtrate comes through clear. The extracted material is finally washed with pure acetone, removed from the filter, macerated once again under pure acetone, and filtered a second time. The combined acetone extract is then treated with ether and the acetone saponified with methyl alcoholic potash, which is removed from the ether solution by washing with water. The ether extract is evaporated to dryness with vacuum, the residue taken up in petroleum ether, and the extract poured into a separatory funnel. Xanthophyll is then removed from the carotene by washing first with 85 per cent methanol, then 90 per cent, and finally 92 per cent methanol until the washings are colorless. The xanthophyll which is present in the alcohol phase is then brought into ether solution. Both the carotene and xanthophyll solutions are washed free of methanol with water, dried and brought to volume and the concentrations are determined colorimetrically, using a 0.2 per cent solution of potassium dichromate as the colorimetric standard.

Numerous modifications of the Willstätter and Stoll method have appeared (*5*, *11*, *15*, *28*, *29*, *30*, *32*). Of these, the Guilbert method (*11*), which was first presented in 1934, has been most widely used as a starting point for recent modifications. In this method, ethyl ether is used as an extraction solvent from which the pigments are transferred to petroleum ether. Rapid and effective saponification and extraction are accomplished simultaneously by boiling the sample for 0.5 hour with saturated alcoholic potash.

More recently Peterson, Hughes, and Freeman (*26*) introduced a modification of the Guilbert method which is considerably shorter, eliminates several possibilities of carotene loss in manipulation, and gives results which are readily reproducible. The original ether extraction of the Guilbert method has been eliminated entirely in their modification. Instead, petroleum ether (boiling point 40° to 60°) is used. This obviates the necessity of carrying on a single solvent evaporation during the course of the determination, and excludes the possibility of carotene decomposition which might occur during the ether evaporation required in the original method. The method is considerably shortened, inasmuch as the chlorophyllins, flavones, alkali, and xanthophyll can be removed directly from the petroleum ether exactly as Guilbert describes their removal from ether and petroleum ether, respectively.

Since the latter modification was published a number of changes have been made in the method. Some of these changes were a development resulting from experience gained in the carotene analyses of several hundred samples yearly. Other changes were suggested by published modifications.

Early in the author's investigations it was found, for example, that the original method, which was highly effective in the extraction of carotene in dry feeds which had been run through the Wiley mill, extracted only 50 to 80 per cent of the total carotene from fresh tissues such as pasture plants, grasses, silages, etc. With these materials it was found advisable to grind the residue from the original digest under alcoholic potash with sea sand in a mortar and to reflux again for an additional 15 minutes to remove all the carotene. If the residue is only ground under alcohol but not redigested, 95 per cent of the carotene is removed. If, instead of grinding after the original digestion, the sample is ground before digestion the extraction is not so complete, usually 90 to 95 per cent of the carotene being extracted. It is always a safe practice in the carotene analysis of strange materials to repeat the digestion of the thoroughly macerated residue to determine whether all the carotene has been removed by one extraction. This technique has also proved valuable in testing the efficacy of other extraction techniques which do not involve the use of alcoholic potash as an extraction and saponification medium.

In the original method of Peterson, Hughes, and Freeman, it was customary, after digestion of the sample was complete, to decant the liquid extract from the sample residue into a separatory funnel. The residue was re-extracted several times with petroleum ether and the solvent similarly removed. This operation, from a routine standpoint, was slow, and with finely ground meals stopcocks of the separatory funnels were frequently clogged. Sintered-glass filter funnels were found to be excellently suited to this step in the procedure, since the residue can be repeatedly and conveniently washed and stirred on the funnel plate until the solvent comes through clear. Alcohol and petroleum ether, used alternately in small portions, are particularly effective. The use of Büchner funnels fitted with the appropriate paper is also an improvement over the older decantation technique.

At the suggestion of Buxton and Dombrow (*4*) it was found that chlorophyllins, flavones, alkali, and xanthophylls could be removed from the original petroleum extract effectively by direct extraction with 90 per cent methanol. It is not necessary to remove first the water-soluble constituents by preliminary washings with water. This also reduced appreciably the number of washings with 90 per cent methanol, since it appears that xanthophylls are more soluble in the presence of small amounts of alkali. In the extraction of xanthophylls, no advantage can be found in extracting the petroleum layer with 85 per cent methanol preliminary to washing with 90 per cent methanol.

Since most petroleum ethers ordinarily used in the extraction of carotene are partially soluble in 90 per cent methanol, considerable difficulty is frequently encountered in the serious reduction of volume in the petroleum phase during the removal of the xanthophylls. Skellysolve B, a widely used commercial grade of petroleum ether, has a solubility of 12 cc. per 100 cc. of 90 per cent methanol. Where this mutual solubility of the two solvents is troublesome it is advisable to saturate the methanol with petroleum before use.

Revised procedures may now be presented for the extraction of carotene from dry and fresh plant tissues.

## Extraction of Carotene from Dry Plant Tissue

Weigh out the samples (1 to 5 grams, more or less, depending on the relative potency), transfer to a 250-cc. Erlenmeyer flask, and add 100 cc. of freshly prepared, 10 per cent ethanolic potassium hydroxide. Fit the flasks with reflux condensers, and boil the contents on a steam bath or hot plate for 30 minutes. If portions of the sample collect on the sides of the flask, wash down

with alcohol from a wash bottle. Cool the contents of the flask, then pour them into a sintered-glass filter funnel, applying a vacuum only until most of the solvent has come through. Then wash the residue alternately with 25-cc. portions of Skellysolve B and absolute alcohol until the filtrate comes through clear. The suction should at no time be applied unless the sediment is partially covered by solvent. After the addition of each wash portion of solvent, more complete extraction may sometimes be obtained by stirring the sediment on the funnel plate with a stirring rod before applying suction. Transfer the filtrate to a 500-cc. separatory funnel.

Pour gently about 100 cc. of distilled water through the alcohol-Skellysolve solution in the separatory funnel. Draw off the alkaline alcohol-water solution from the bottom of the funnel, and re-extract three times by shaking gently with 30-cc. portions of Skellysolve B, using two other separatory funnels. Combine the Skellysolve extracts and wash free from chlorophyllins, flavones, alkali, and xanthophylls by shaking thoroughly with 30-cc. portions of 90 per cent methanol (five washes are generally sufficient) and re-extract the first methanol portion with 50 cc. of Skellysolve B. Wash the Skellysolve B solution once with 50 cc. of distilled water to remove the alcohol and filter into a volumetric flask through filter paper upon which is placed a small amount of anhydrous sodium sulfate. After making the carotene solution up to a definite volume, determine the concentration by the spectrophotometer, photoelectric colorimeter, or colorimeter by comparison with 0.1 per cent or 0.036 per cent potassium dichromate.

## Extraction of Carotene from Fresh Plant Tissue

Cut fresh plant tissue finely with shears and mix as thoroughly as possible. Weigh samples of 4 to 10 grams into a 250-cc. Erlenmeyer flask and proceed with digestion and washing as for dry materials. When the residue on the filter plate has been thoroughly washed, transfer it into a deep mortar with a well-defined lip, add 10 cc. of a saturated solution of potassium hydroxide in ethanol, and macerate with the pestle. Add quartz sand and grind. From time to time wash down the sides of the mortar with a stream of alcohol from a wash bottle and grind until the tissue is fine. Transfer the contents of the mortar to the Erlenmeyer flask used in the original digestion, using more alcoholic potassium hydroxide to effect the transfer. Add alcoholic potassium hydroxide until the total volume is 100 cc., digest for 15 minutes, and proceed as indicated for dry tissue. Combine filtrates from both digestions and washings before extraction of xanthophylls.

## Determination of Carotene Concentration

Of the three methods available for the determination of carotene concentration, colorimetric, spectrophotometric, and photoelectric photometric, the colorimetric method has probably been used most extensively (*1*, *11*, *21*, *26*, *27*, *28*). Accurate results have been obtained by this method. The spectrophotometer, however, has an additional advantage in that no standard solution is needed for comparison. One needs only determine the absorption coefficient for pure carotene in the solvent to be used at some convenient wave length, usually in the region of one or both of the absorption maxima.

In laboratories of this station, as in many others, the photoelectric colorimeter (*31*) has almost completely replaced the colorimeter and spectrophotometer in the determination of pigment concentration.

The spectrophotometer, however, has not lost its value in the study of many aspects of the carotene problem. It is useful in the determination of related chromogens which frequently are found in carotene extracts, and which may or may not possess vitamin A potency, and may also be used to advantage in the calibration of the photoelectric photometer. It is in the latter use of the instrument that some significant observations regarding carotene solutions have been made.

The photoelectric photometer is usually calibrated for the determination of carotene by the preparation of a curve of transmission *vs.* carotene concentration in which the carotene concentration of a number of solutions has been determined or checked by means of the spectrophotometer. Crystalline $\beta$-carotene, which gives a normal absorption spectrum, is usually the standard. Rarely, however, does one find in practice a carotene extract which has absorption maxima and a minimum identical with that of true $\beta$-carotene, nor are the ratios of the optical densities at various wave lengths correct. The value of $\log \frac{I_0}{I}$ at 4500 Å. is frequently too large with respect to that at 4700 Å. (*27*) while $\log \frac{I_0}{I}$ at 4800 Å. is usually less than that at 4700 Å. In Table I are shown the relative spectral absorptions of a number of carotene extracts from different sources expressed in percentages of absorption at wave length 4700 Å.

Table I. Relative Spectral Absorption of Carotene Extracts

(Expressed in percentages of absorption at wave length 4700 Å.)

| No. of Samples | Sample | 4500 Å. | 4700 Å. | 4800 Å. |
|---|---|---|---|---|
| 5 | $\beta$-Carotene | 118 | 100 | 105 |
| 20 | Spring grasses | 117 | 100 | 103 |
| 21 | Summer grasses | 119 | 100 | 103 |
| 11 | Fall grasses | 129 | 100 | 106 |
| 6 | Butter | 114 | 100 | 104 |
| 42 | Egg yolk | 117 | 100 | 92 |
| 46 | Dehydrated alfalfa | 119 | 100 | 105 |
| 16 | Prairie hay | 138 | 100 | 103 |
| 3 | Sorghum silage | 123 | 100 | 100 |
| 6 | Yellow corn | 116 | 100 | 87 |
| 27 | Miscellaneous commercial feeds | 120 | 100 | 96 |

It is apparent from an inspection of Table I that a carotene concentration obtained by means of a photoelectric photometer, calibrated with true $\beta$-carotene, may agree with that obtained by the spectrophotometer at one wave length, and not at all at some other wave length.

Reasons for these peculiar changes in the absorption spectrum of $\beta$-carotene are not readily explained. On all adsorbents the main pigment fraction adsorbs in the same position as $\beta$-carotene and cannot be distinguished from it when the two are adsorbed simultaneously. Within the usual limits of experimental error in the rat assay for vitamin A potency it appears that the main pigment fraction has a vitamin A potency equivalent to that of true $\beta$-carotene. The main pigment fraction on removal from an adsorption column possesses an abnormal absorption spectrum approaching more nearly that of $\alpha$-carotene rather than $\beta$-carotene. Zechmeister and Tuzson (*38*) have made the observation that carotenoids readily undergo certain isomerization processes in certain solvents, particularly when heated. These changes are said to be accompanied by a movement of the extinction maxima toward the shorter wave lengths. It is claimed that this phenomenon is not induced by the adsorption process as described by Gillam and co-workers (*10*). All attempts to separate petroleum fractions from a number of the materials listed in Table I under conditions known to prevent the solvent, heat, and adsorption effects described resulted in no improvement in the absorption spectra. It seems clear that the abnormal absorption spectra are an intrinsic property of the pigments as found in the original material previous to extraction.

No discussion of methods for the determination of carotene would be complete without some consideration of techniques suggested for the separation of "true" $\beta$-carotene from the noncarotene chromogens found in most petroleum extracts of feeds and silages.

Hegsted, Porter, and Peterson (*12*) have described a method involving the extraction of the petroleum extract with aqueous diacetone.

This technique, which can be applied successfully in the separation of acid-modified carotenoids present in silages, has not proved generally applicable with many other forages. Attempts to use this solvent in the extraction of cryptoxanthin from petroleum extracts of yellow corn have also proved unsuccessful. It is unfortunate that selective solvents have not been more successful in the separation of these impurities from petroleum ether solutions, since this would offer a convenient and rapid method of solving this troublesome problem. In view of the similar solubilities it seems very unlikely that absolute separation of such closely related pigments will ever be accomplished by phase separations.

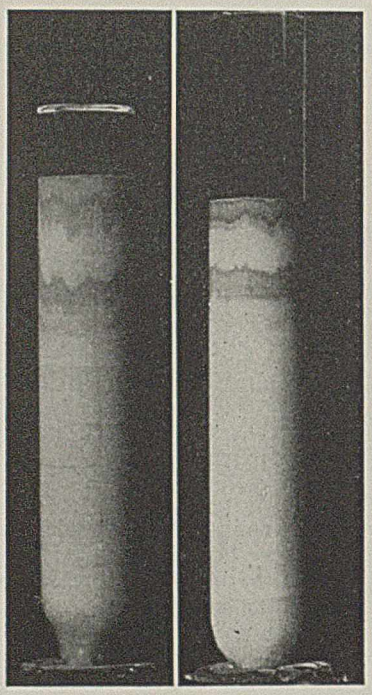

FIGURE 1. CHROMATOGRAPH OF PETROLEUM-PHASIC FRACTION OF CANADIAN YELLOW CORN ON A MAGNESIA COLUMN

Left. Improperly washed tube
Right. Properly washed tube

Adsorption methods have proved by far the most successful in the purification of carotene solutions. Fraps and associates (*6–9*) have recently studied extensively methods of selective adsorption.

Their technique involves shaking the impure carotene solution with a given amount of specially activated adsorbent (magnesium hydroxide or carbonate). These authors have also described an "adsorbent X" which adsorbs xanthophyll but does not absorb carotene, and an "adsorbent L" which removes large quantities of impurities in such foods as watermelon, ripe tomatoes, and apricots. The latter impurities are not removed by "adsorbent X". In a recent contribution, Fraps, Kemmerer, and Greenberg (*9*) have presented a new modification of the modified A. O. A. C. method (*21*) in which xanthophylls and impurities are adsorbed directly instead of first washing with methyl alcohol and then using the adsorbent.

With the idea of providing suggestions for further improvements in this direction, the following points may be presented in criticism of the present methods of Fraps *et al.*: (1) special techniques are required in the preparation of the adsorbents; (2) proper activation of the adsorbent must be frequently checked to determine whether or not it will adsorb the desired impurities and not β-carotene; (3) completeness of adsorption must be checked photocolorimetrically from time to time throughout the determination until no more color is removed; (4) no provision is made for determining the quantity or number of pigments adsorbed or for studying their absorption spectra and other properties; (5) the colored impurities associated with carotene may not be identical in all feeds or in different samples of the same feed, thus involving the preparation of an adsorbent of a different degree of activation; and (6) the vitamin A potency of these impurities has not been extensively investigated.

## Chromatographic Adsorption

There probably is no development which has been so helpful in the separation and identification of the closely related carotenoids as has chromatographic adsorption, or the separation of pigments by their adsorption on a column of adsorbent suited to the purpose.

Since the early work of Tswett (*34*) adsorption methods have been used to advantage and improved techniques have been developed by a great many workers in the field, notably Palmer (*22*, *23*), Palmer and Eckles (*24*), Vegezzi (*35*), Lipmaa (*18*), Kuhn and Lederer (*16*), Karrer and Walker (*13*), Kuhn, Winterstein, and Lederer (*17*), and Kuhn and Brockmann (*14*, *15*).

Especially noteworthy is the work of Strain (*33*) who, in the study of numerous adsorbents for specific purposes, has found that a special brand of magnesium oxide possesses the most desirable qualities for the resolution of different carotenes.

Precautions and conditions which must be observed in the preparation and effective use of adsorption columns have been described by Miller (*19*) and by Zechmeister (*37*).

Though chromatographic adsorption techniques have been very useful in the qualitative separation of the fractions present in a pigment mixture and have permitted their identification, attempts to apply adsorption methods on a strictly quantitative basis have been only partially successful. An adsorbent which is sufficiently activated to accomplish a sharp and clear-cut separation of the component fractions of a pigment mixture may hold the pigments so tenaciously as to make their removal from the adsorbent difficult. Oxidative effects are also likely to occur. On the other hand, an adsorbent which is less activated, and permits a more nearly quantitative removal of adsorbed pigments, is apt to have lost much of the power of selective adsorption frequently necessary in the separation of very closely related carotenes.

Chromatographic adsorption studies covering a period of three years on the petroleum-phasic fractions of a large variety and number of feeds and biological materials using the Strain technique have given an average recovery of only 84 per cent.

COLUMN PREPARATION. Further refinements in the technique of column preparation have been developed.

Unless more than the usual care was taken in assuring the cleanliness of the tube before introducing the adsorbent, a washing or seepage of pigment away from the main pigment zones along the inner glass surface occurred. This could be prevented by preliminary treatment of the tube with dichromate cleaning solution. In Figure 1 is shown a chromatographic separation of β-carotene and cryptoxanthin in Canadian yellow corn, on a column in which the tube had not been treated with cleaning solution before packing, and the same separation in a cleaned tube.

A new technique was developed in the preparation of adsorption columns as a result of attempts to obtain acceptable photographs. In all earlier attempts the chromatographs showed marked "packing lines" where separate portions of added adsorbent had come together in the packing of the column. Apparently the lower part of each disk of adsorbent was less densely packed than that part which had come in direct contact with the packing tamper. Thus, in the development of a chromatograph that region immediately above a packing line adsorbed considerably less pigment than that region immediately below, making identification of the true pigment zones difficult. In Figure 2 is shown a chromatograph of the xanthophylls of a molasses–oat grass silage, illustrating to advantage the confusing effects of packing lines.

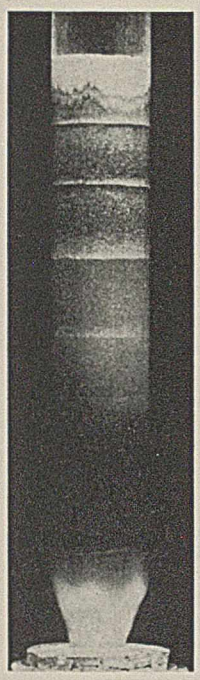

FIGURE 2 (*Left.*) CHROMATOGRAPH OF XANTHOPHYLLS OF MOLASSES–OAT GRASS SILAGE

From dichloroethane solution of pigments on magnesia columns, showing packing lines due to improper preparation of column

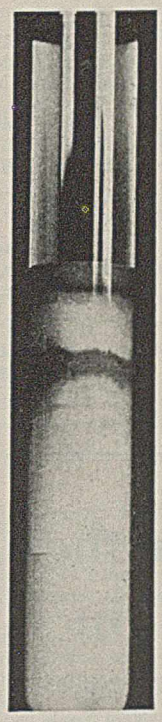

FIGURE 3 (*Right*). CHROMATOGRAPH OF PETROLEUM-PHASIC FRACTION OF ARGENTINE YELLOW CORN ON MAGNESIA COLUMN PROPERLY PREPARED

Though it proved difficult to prepare an adsorption column which did not show slight evidences of packing lines it was possible greatly to minimize the effects of these lines on the proper development of a chromatograph. This was accomplished by packing only small portions (0.63 to 1.25 cm., 0.25 to 0.5 inch) of adsorbent at a time in building up the column, and, after the uniform packing of each disk, loosening the upper, tightly packed portion (0.156 to 0.31 cm., 0.063 to 0.125 inch) by means of a square-ended spatula before packing the next portion. A column, so prepared, is shown in Figure 3.

## Petering-Wolman-Hibbard Procedure for Determining Carotene

Of recent methods for the determination of carotene, one of the most interesting and promising is the novel method of Petering, Wolman, and Hibbard (*25*), designed primarily for fresh tissue.

The finely cut tissue is ground with quartz sand and acetone repeatedly with mortar and pestle until the solvent comes off clear. The solvent is brought to volume and an aliquot is boiled with a small amount of activated barium hydroxide or barium hydroxide octahydrate. The solution is filtered by suction, the barium hydroxide retaining the chlorophyll by adsorption. The xanthophylls and flavones are separated from carotene by the usual methods.

If the proper precautions are taken, excellent results may be obtained by this method. The barium hydroxide must be free of carbonate and must be finely dispersed. A highly active preparation may be obtained by filtering 15 cc. of a hot saturated aqueous solution of barium hydroxide directly into the acetone extract immediately before digestion. This obviates the necessity for keeping on hand a barium hydroxide strictly free of carbonate. The aliquot must also be sufficiently small that the chlorophyll it contains can be completely adsorbed by the amount of barium hydroxide recommended.

Benne, Wolman, Hibbard, and Miller (*2*) have recently published the results of a comparison of the above method and two modifications thereof with the technique of Peterson, Hughes, and Freeman (*26*). A large variety of plant tissues were analyzed, and the results were comparable throughout, except with certain samples of bluegrass taken late in the season. Similar comparisons in this laboratory have shown that the greatest discrepancy lies in the inefficacy of the grinding method for removing all the carotene from fresh tissue. Excellent checks were always obtained by the two methods when aliquots of the filtrate were used as the starting point. There is evidence that grinding until the solvent appears clear does not always ensure complete extraction. It is the opinion of the author that the alcoholic potash digestion method can effectively replace much of the manual labor of grinding in the disruption of tissue.

Though it is not believed that the barium hydroxide technique offers many advantages with regard to the time required in effecting a complete analysis, it does have several valuable features. For example, when chlorophyll is once removed, all the remaining carotenoids can be transferred quickly and completely to the petroleum phase. This is not the case in the older method, since xanthophylls are but slowly removed from alcoholic potash, and, to the uninitiated, it is difficult to determine when one is removing carotene and xanthophyll, or only xanthophyll from the alcoholic potash mixture. This method should have advantages in the determination of cryptoxanthin and many other carotenoids which are less readily removed from alcoholic potash solutions by means of petroleum ether.

## Summary

Improvements in methods for the extraction and quantitative determination of $\beta$-carotene in dry and fresh plant tissue are described.

Solvent and adsorption methods for the separation of $\beta$-carotene from accompanying petroleum-soluble carotenoids are discussed in the light of new developments.

The Petering-Wolman-Hibbard method has excellent potentialities in the development of methods for the quantitative determination of cryptoxanthin and other carotenoid pigments, as well as carotene.

## Acknowledgments

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## Literature Cited

(1) Arnaud, A., *Compt. rend.*, 104, 1293 (1887).
(2) Benne, E. J., Wolman, W., Hibbard, R. P., and Miller, E. J., *J. Assoc. Official Agr. Chem.*, 23, 709 (1940).
(3) Borodin, L., *Bull. Acad. Imp. Sci. St. Petersburg*, 9, 512; *Bot. Ztg.*, 41 577–9 (1883).
(4) Buxton, L. O., and Dombrow, B. A., IND. ENG. CHEM., Anal. Ed., 10, 262 (1938)
(5) Coward, K. H., *Biochem. J.*, 18, 1114 (1924).
(6) Fraps, G. S., and Kemmerer, A. R., *J. Assoc. Official Agr. Chem.*, 22, 190 (1939).
(7) Fraps, G. S., Kemmerer, A. R., and Greenberg, S. M., IND. ENG. CHEM., Anal. Ed., 12, 16 (1940).
(8) Fraps, G. S., Kemmerer, A. R., and Greenberg, S. M., *J. Assoc. Official Agr. Chem.*, 23, 422 (1940).
(9) *Ibid.*, 23, 659 (1940).
(10) Gillam, A. E., Ridi, M. S. E., and Kon, S. K., *Biochem. J.*, 31, 1605 (1937).
(11) Guilbert, H. R., IND. ENG. CHEM., Anal. Ed., 6, 452 (1934).
(12) Hegsted, D. M., Porter, J. W., and Peterson, W. H., *Ibid.*, 11, 256 (1939).
(13) Karrer, P., and Walker, O., *Helv. Chim. Acta*, 16, 641 (1933).
(14) Kuhn, R., and Brockmann, H., *Z. physiol. Chem.*, 200, 255 (1931).
(15) *Ibid.*, 206, 41 (1932).

(16) Kuhn, R., and Lederer, E., *Naturwissenschaften*, 19, 306 (1931).
(17) Kuhn, R., Winterstein, A., and Lederer, *Z. physiol. Chem.*, 197, 141 (1931).
(18) Lipmaa, Th., *Schr. Naturwiss. Ges. Dorpat*, 24, 83 (1925).
(19) Miller, E. S., *Botan. Gaz.*, 96, 447 (1935).
(20) Monteverde, N. A., and Lumbimenko, V. N., *Bull. Acad. Sci. St. Petersburg*, 1913, 1007–28.
(21) Munsey, V. E., *J. Assoc. Official Agr. Chem.*, 20, 459 (1937).
(22) Palmer, L. S., dissertation, University of Missouri, 1913.
(23) Palmer, L. S., *J. Biol. Chem.*, 113, 89 (1936).
(24) Palmer, L. S., and Eckles, C. H., *Ibid.*, 17, 191, 211, 223, 237, 245 (1914).
(25) Petering, H. G., Wolman, W., and Hibbard, R. P., IND. ENG. CHEM., Anal. Ed., 12, 148 (1940).
(26) Peterson, W. J., Hughes, J. S., and Freeman, H. F., *Ibid.*, 9, 71 (1937).
(27) Peterson, W. J., Hughes, J. S., and Payne, L. F., Kansas Agr. Expt. Sta., *Bull.* 46 (1939).
(28) Russell, W. C., Taylor, M. W., and Chichester, D. F., *Plant Physiol.*, 10, 325 (1935).
(29) Schertz, F. M., *J. Agr. Research*, 26, 383 (1923).
(30) Schertz, F. M., *Plant Physiol.*, 3, 211 (1928).
(31) Shrewsbury, C. L., Kraybill, H. R., and Withrow, R. B., IND. ENG. CHEM., Anal. Ed., 10, 253 (1938).
(32) Smith, J. H. C., and Smith, O., *Plant Physiol.*, 6, 265 (1933).
(33) Strain, H. H., *J. Biol. Chem.*, 105, 523 (1934).
(34) Tswett, M., *Ber. deut. Botan. Ges.*, 24, 384 (1906).
(35) Vegezzi, G., dissertation, Fribourg, 1916.
(36) Willstätter, R., and Stoll, A., "Untersuchungen über Chlorophyll", Berlin, Julius Springer, 1913.
(37) Zechmeister, L., "Carotenoide", Berlin, Julius Springer, 1934.
(38) Zechmeister, L., and Tuzson, P., *Biochem. J.*, 32, 1305 (1938).

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# Chemical Methods for the Determination of Vitamin $B_1$

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THE importance of rapid and accurate methods of analysis for vitamin $B_1$ needs no emphasizing. The biological methods have unavoidable limitations to which the chemical methods are not subject. The nonbiological methods for the determination of vitamin $B_1$ are based on the measurement of the concentration of substances produced from the vitamin in standardized procedures. Although the percentage conversion of thiamin into the measured product is fairly constant in the concentration range used in these methods, in no case is the vitamin known to be so converted either completely or exclusively.

The methods of Kinnersley and Peters (*15*, *16*), of Willstaedt and Barany (*40*), and of Melnick and Field (*20–24*, *26*), the last-named using a reagent proposed by Prebluda and McCollum (*28*, *29*), all depend on the formation of a pigment, presumably formed by the coupling of the vitamin with a diazotized aromatic amine. The measurement, by colorimetric or photometric means, of the amount of pigment produced becomes a measure of the amount of vitamin $B_1$. The aromatic amines employed in these methods by these investigators are sulfanilic acid, 2,4-dichloroaniline, and *p*-aminoacetophenone, respectively. Recently, Emmett, Peacock, and Brown (*4*) have simplified the Melnick and Field procedure in its application to certain materials having a fairly high potency.

A method of vitamin $B_1$ assay based on a type of reaction and measurement different from those described above is proposed by Jansen (*14*). Thiamin is oxidized in alkaline medium by ferricyanide and thus converted into thiochrome. When irradiated by light in the near ultraviolet region of the spectrum, this substance shows a strong blue-violet fluorescence, the intensity of which is proportional to the concentration of the thiochrome. This, in turn, is proportional to the amount of thiamin from which it was formed by oxidation. Extraction of the thiochrome from the oxidation medium by isobutanol increases the intensity of the thiochrome fluorescence, while at the same time the aqueous layer retains most of the interfering materials. A blank determination for the measurement of preformed nonspecific fluorescence is conducted with the omission of the ferricyanide. Adsorption on Franconite with subsequent elution and oxidation is suggested for urine.

This general procedure has been used with or without modification by many investigators. The more important earlier references are given by Hennessy and Cerecedo (*10*). Many later publications have appeared, some describing modification of the method in its details or application to different materials. The method has been applied to the analysis of blood by Jansen (*13*), Widenbauer (*36*), Widenbauer *et al.* (*37*), and Ritsert (*31*, *32*). Thiamin has been determined in urine by several investigators (*3*, *6*, *11*, *18*, *26*, *30*, *33*, *35*, *38*). More general application of the method is described by Hennessy and Cerecedo (*10*), Wiegand (*39*), Bertagni (*2*), and Flavier and Genevois (*7*). Some investigators are critical of the procedure as employed with various modifications by others. In particular, Wang and Harris (*35*), Hills (*11*), Mallinckrodt-Haupt (*19*), Otto and Rühmekorb (*27*), Huhn (*12*), Baucke (*1*), and Gahlen (*8*) have suggested deficiencies, usually as a prelude to offering some alternative.

The first problem in any of the chemical methods is to obtain the vitamin in solution, and this in many cases requires an extraction procedure. Whether or not complete extraction may be expected with all types of materials is not definitely known. Refluxing and heating in a boiling water bath with 0.1 *N* sulfuric acid have been practical means of extraction. A single extraction suffices, especially if the ratio of the volume of extracting liquid to the weight of sample is maintained as high as is feasible in the light of the minimum concentration of thiamin required in the aliquot of extract to be used in the subsequent step of the assay procedure. A ratio greater than 50 cc. of extracting liquid per gram of sample is generally not necessary, while if this ratio be less than 15 to 1, a lowered extraction efficiency may result. Incubation of the residues of a properly conducted extraction with various digestive enzymes has not shown any significant quantities of vitamin $B_1$ remaining therein. General agreement of the results of chemical assays of extracts with the results of biological assays of the corresponding samples would indicate that the extraction is as efficient as the process taking place in the digestive tract of the animal. In the literature (*4*, *10*) this is shown to be the case with many materials. When a chemical and biological assay of a sample of some material agree with each other, this agreement may be expected to persist for other samples of the same material.

The occurrence of thiamin, in some sources, in the form of its phosphoric esters necessitates an enzymatic hydrolysis, which usually follows immediately after the extraction. Takadiastase, mylase, clarase, diastase-Merck, kidney phos-

phatase, and yeast phosphatase have been used to bring about this hydrolysis. The first four of these, being diastatic enzymes, are particularly suitable for use in the preparation of extracts of starchy materials. Takadiastase has been most widely employed. Maintenance by a buffer of the pH at the optimum for the phosphatase activity is essential during the entire incubation period if the hydrolysis is to be completed within the allotted time. The need for the conversion of the thiamin esters into thiamin may be found in the consideration of their different behavior in the purification step, or in the solvent extraction step, both of which are used in the two types of chemical methods. In fact, thiamin in the form of its phosphoric esters may be completely lost in these steps.

Any chemical method of analysis of a substance occurring in its source in 1 to 2000 parts per 10,000,000, unless it be highly specific, must allow considerable purification without losses before the actual colorimetric or fluorometric procedure is begun.

The most successful single step which can bring about the desired purification is that proposed by Hennessy and Cerecedo (*9*) for use with the thiochrome method. Melnick and Field (*24*) used a similar step in their colorimetric method. It consists of passing the impure extract which contains the vitamin through a column of the synthetic zeolite, Decalso. The vitamin is retained on the zeolite, from which it can later be removed by treatment of the zeolite column with potassium chloride solution. This process is not essential for the thiochrome procedure when materials of fairly high potency are being assayed. The variable and incomplete recoveries, the high blanks, and the need for varying the ferricyanide concentration in the thiochrome method, described by the European investigators who used Franconite or similar clays for purification, are not experienced when the base-exchanging zeolite is employed. Melnick and Field report that the use of clay is not satisfactory as a method of purification in their procedure. Ritsert and Wang and Harris have omitted the use of Franconite in determining vitamin $B_1$ in urine by the thiochrome method, as they believe that the benefits accruing from the use of the adsorption step are outweighed by the disadvantages.

An analysis of the sensitivity of the colorimetric and the fluorometric methods shows that the advantage lies with the latter, particularly in the assay of low-potency materials.

The amount of thiamin in the aliquot, which is used to produce the pigment in the colorimetric methods, should be more than 5 micrograms, the preferable range being 20 to 100 micrograms. For the fluorometric thiochrome procedure, the aliquot need contain only 0.1 microgram with the preferred range between 0.5 and 2.5 micrograms of thiamin. For general practicability, it is essential that a fluorometer, if used, be of sufficient sensitivity to provide a galvanometer deflection of at least five scale divisions for 0.1 microgram of thiamin chloride. The Pfaltz & Bauer fluorophotometer (*25*) meets this requirement. The usual type of colorimetric or photometric instruments may be used as the means of measurement of the pigment produced in the Melnick and Field procedure. It is only necessary that they be adaptable to a 2-cc. volume.

The reproducibility of the methods is of the same order. In a series of determinations, one may expect an average deviation of less than 5 per cent.

Of the colorimetric procedures, that of Melnick and Field has had its specificity best established, although the exact chemical basis of this specificity is not agreed upon by Prebluda and McCollum (*29*) and Melnick and Field (*20*). The chemical composition and structure of the pigment are not known. A well-established fact, which as yet has no explanation, is the effect of phenol and alcohol on the reaction between thiamin and the Prebluda-McCollum reagent. Melnick and Field add these materials to the reaction mixture in order to increase sensitivity, stabilize the vitamin, eliminate variation in apparent recovery, eliminate the effect of trace sensitizers, and permit the use of a single standard. The reaction is allowed to proceed overnight to give maximum color production. There is a possibility of a shorter reaction time, since a high percentage of the final color intensity is produced in less than 0.5 hour.

The results obtained by Melnick and Field and by Emmett and co-workers on materials of relatively high potency have shown good agreement with the results of biological assay. The Melnick and Field procedure for the assay of urine (*23*) has given results in substantial agreement with the results of the thiochrome assay on the same samples. The results of the thiochrome method were on the average 15 per cent higher than those of the Melnick and Field procedure on six samples of urine ranging in potency from 50 to 1300 micrograms per 24-hour sample (*5*). This is reassuring, since the two procedures are based on entirely different reactions. The less sensitive colorimetric procedure requires a 4- to 8-hour sample (75 to 300 cc.), while the fluorometric method needs only 10 to 20 cc. of urine. With the larger quantity of urine used in the colorimetric procedure, removal of salts by a benzyl alcohol treatment is needed to ensure nearly complete recovery of thiamin at the zeolite purification stage (*23*).

## Thiochrome Method

The specificity of the thiochrome method is based on extensive comparison with the results of bioassay on a variety of materials of the entire range of potency (*10*). The chemical basis is well known, since the chemical structure of thiochrome has been established (*17, 34*). The oxidation of thiamin in alkaline ferricyanide solution does not seem to result in the production of an equivalent amount of thiochrome, but whether this is due to the establishment of an equilibrium in the oxidation process or to side reactions is not definitely known. Nevertheless, under the conditions of the method, when interfering materials are efficiently removed, the production of thiochrome proceeds uniformly. The phosphoric esters of thiamin, when oxidized, form what are probably the phosphoric esters of thiochrome. Measured in the aqueous alkaline oxidation medium, the fluorescent intensities of the three thiochromes are in the ratio 12 to 15 to 19 on an equivalent basis in the order of increasing phosphoric acid content. This precludes any direct measurement of mixtures of the esters by fluorometry on the aqueous phase following isobutanol extraction of thiochrome.

In the thiochrome method, a blank determination is made for the measurement of preformed, nonspecific fluorescence by omitting the potassium ferricyanide. In most cases, the zeolite purification eliminates or reduces to insignificance the materials responsible for this fluorescence. There is the possibility that these materials, if present, may be partly destroyed when the potassium ferricyanide is used for the oxidation of thiamin to thiochrome. The difference between the readings obtained with and without oxidation would be erroneously low in this case. This consideration is of importance only if the nonoxidized reading be larger for the sample than for the standard thiamin chloride by an amount of 10 per cent or more of the difference between the oxidized and nonoxidized readings for the sample. In such cases the same nonspecific fluorescence which is destroyed by the ferricyanide also largely disappears merely on standing for 5 minutes with alkali and isobutanol (*5*).

The thiochrome method is being used routinely in this country with considerable success and satisfaction in many industrial, clinical, and university laboratories. The procedure now in general use is essentially that of Hennessy and Cerecedo (*10*) with subsequent changes reported by Hennessy at the Baltimore, Boston, and Detroit meetings of the

American Chemical Society. The main purpose of these changes has been to develop a more suitable routine procedure.

Preparation of Extracts. The extraction is now accomplished in a single treatment of the sample with 0.1 *N* sulfuric acid. The pH of 4.0 to 4.5 required for the enzymatic hydrolysis and for the base exchange is attained by the addition of 0.1 volume of 1.2 *M* sodium acetate solution to the extraction mixture.

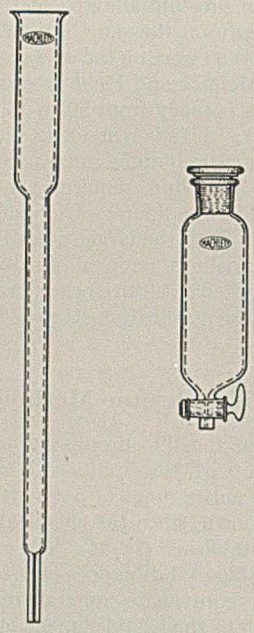

Figure 1. Separatory-Centrifuge and Exchange Tubes

Method I. All preparations, including the standard thiamin chloride, should be in 20 to 25 per cent potassium chloride solution at the oxidation step, to keep the volume of isobutanol constant in the extraction. It has been found advantageous to mix the ferricyanide and alkali before adding them to the sample. A separatory-centrifuge tube (obtainable from E. Machlett & Son, New York, N. Y.) has been devised (Figure 1, right), so that oxidation, extraction, separation, and clarification may be carried out in this single vessel with uniform timing throughout. The shaking time for the isobutanol extraction seems best at 1.5 minutes, followed by centrifuging at 500 r. p. m. for 0.75 minute.

Method II. *Exchange Tube.* The tubes for the exchange (Figure 1, left) are 15 × 0.7 cm. with a 25-cc. reservoir at the upper and a 3.0 × 0.03 cm. capillary at the lower end. A 6- to 7-cm. column of Decalso is held in the tube by a small plug of glass wool at the lower end.

*The Exchange.* The base exchange is conducted at room temperature, using 60- to 80-mesh Decalso prepared in bulk by stirring or shaking with four 10-volume portions of 3 per cent acetic acid for 10 minutes each. Between the second and third acid washing a 15-minute treatment with 5 volumes of 25 per cent potassium chloride solution is introduced. Thorough washing with water finishes the preparation of the Decalso. The eluting mixture is 25 per cent potassium chloride in 0.1 *N* hydrochloric acid. Complete removal of thiamin from the zeolite is accomplished by passing 21 cc. of this mixture at room temperature at a rate not exceeding 0.9 cc. per minute. This allows for four 5-cc. aliquots. As many as sixteen of these tubes may be run in parallel and eight samples carried through this step in duplicate in 70 minutes. Although the Decalso may be used repeatedly, it is safer practice to use a fresh charge for each sample.

Method III. *Enzymatic Hydrolysis.* The enzymatic hydrolysis is brought about in 2 hours at 50° C. using 0.5 per cent takadiastase at pH 4.0 to 4.5. This hydrolysis, if carried out on extraction mixtures, is done before any separation of the still insoluble portions is attempted.

*Fluorometry.* The use of quinine sulfate is to be preferred to the use of the rear photocell of the fluorophotometer for setting the galvanometer deflection per microgram of thiamin chloride. The quinine sulfate fluorescence should be determined immediately before that of the unknown.

The thiochrome method with the modifications described above has been applied to almost all the samples whose assay was reported by Hennessy and Cerecedo (*10*). The results are the same, but are obtained with less effort and in a shorter time. Other materials of all types have been assayed, the results being, with few exceptions, in good agreement with the results of biological assay.

Between eight and twenty samples may be assayed in duplicate in a single day by the thiochrome method.

## Summary

Accurate and rapid chemical methods are available for the determination of vitamin $B_1$.

The Prebluda-McCollum reagent used in the manner prescribed by Melnick and Field is practical for such determinations on materials of moderate and high potency. The thiochrome method, having considerably greater sensitivity than the colorimetric methods, may also be used for the assay of materials of low potency. The use of the synthetic zeolite, Decalso, as a preliminary step in both procedures effectively eliminates interfering materials. Recent modifications of the thiochrome method have made it suitable for routine analysis.

## Literature Cited

(1) Baucke, J., *Klin. Wochschr.*, 18, 1128 (1939).
(2) Bertagni, P., *Boll. soc. ital. biol. sper.*, 14, 244 (1939).
(3) Borson, H. J., *Ann. Internal Med.*, 14, 1 (1940).
(4) Emmett, A. D., Peacock, G., and Brown, R. A., *J. Biol. Chem.*, 135, 131 (1940).
(5) Ferrebee, J. W., personal communication.
(6) Ferrebee, J. W., and Carden, G. A., *J. Lab. Clin. Med.*, 25, 1320 (1940).
(7) Flavier, H., and Genevois, L., *Compt. rend. soc. biol.*, 130, 497 (1939).
(8) Gahlen, W., *Klin. Wochschr.*, 18, 1446 (1939).
(9) Hennessy, D. J., and Cerecedo, L. R., abstracts of Dallas Meeting Papers, American Chemical Society, April, 1938.
(10) Hennessy, D. J., and Cerecedo, L. R., *J. Am. Chem. Soc.*, 61, 179 (1939).
(11) Hills, G. M., *Biochem. J.*, 33, 1966 (1939).
(12) Huhn, O., *Klin. Wochschr.*, 18, 1094 (1939).
(13) Jansen, B. C. P., *Acta Brev. Neerland. Physiol. Pharmacol. Microbiol.*, 8, 119 (1938).
(14) Jansen, B. C. P., *Rec. trav. chim.*, 55, 1046 (1936).
(15) Kinnersley, H. W., and Peters, R. A., *Biochem. J.*, 28, 637 (1934).
(16) *Ibid.*, 32, 1516 (1938).
(17) Kuhn, R., and Vetter, H., *Ber.*, 68, 2375 (1935).
(18) Magyar, I., *Klin. Wochschr.*, 18, 1157 (1939).
(19) Mallinckrodt-Haupt, A. St. v., *Ibid.*, 18, 467 (1939).
(20) Melnick, D., and Field, H., *J. Biol. Chem.*, 127, 505 (1939).
(21) *Ibid.*, 127, 515 (1939).
(22) *Ibid.*, 127, 531 (1939).
(23) *Ibid.*, 130, 97 (1939).
(24) Melnick, D., and Field, H., *Proc. Soc. Exptl. Biol. Med.*, 38, 723 (1938).
(25) Müller, R. H., Ind. Eng. Chem., Anal. Ed., 12, 600 (1940).
(26) Mukherji, A., *J. Indian Chem. Soc.*, 16, 273 (1939).
(27) Otto, H., and Rühmekorb, F., *Klin. Wochschr.*, 18, 645 (1939).
(28) Prebluda, H. J., and McCollum, E. V., *J. Biol. Chem.*, 127, 495 (1939).
(29) Prebluda, H. J., and McCollum, E. V., *Science*, 84, 488 (1936).
(30) Ritsert, K., *Deut. Med. Wochschr.*, 64, 481 (1939).
(31) Ritsert, K., *Klin. Wochschr.*, 18, 852 (1939).
(32) *Ibid.*, 18, 1370 (1939).
(33) Schneider, E., and Burger, A., *Ibid.*, 17, 905 (1938).
(34) Todd, A. R., Bergel, F., Fraenkel-Conrat, H. L., and Jacob, A., *J. Chem. Soc.*, 1936, 1601.
(35) Wang, Y. L., and Harris, L. J., *Biochem. J.*, 33, 1356 (1939).
(36) Widenbauer, F., *Klin. Wochschr.*, 18, 1392 (1939).
(37) Widenbauer, F., Huhn, O., and Disselhoff, V., *Zentr. inn. Med.*, 60, 113 (1939).
(38) Widenbauer, F., Huhn, O., and Ellinger, R., *Z. ges. exptl. Med.*, 105, 138 (1939).
(39) Wiegand, J. A., *Arch. neerland. physiol.*, 23, 281 (1938).
(40) Willstaedt, H., and Barany, F., *Enzymologia*, 2, 316 (1938).

# Determination of Vitamin $B_2$ (Riboflavin)

## Comparison of Bioassay, Microbiological, and Fluorometric Methods

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THE increased interest in the possible sources as well as the biological properties and uses of vitamin $B_2$ (riboflavin) has stimulated a desire for a more rapid method of assay than the biological rat growth procedure which is generally followed. At the present time, while there is no official biological method of assay, the one of choice is basically that of Bourquin and Sherman (*1*). This has been modified by different workers from time to time with the intent chiefly of improving upon the supplemental factors and thereby making it more specific. One of the chief objections to the method is the time it requires—at least 28 days—which not only entails the factor of cost but interferes markedly with the execution of many problems of practical interest.

In contrast to the rat growth method, two comparatively rapid procedures have been used to a limited degree for determining flavin. The first measures the degree of fluorescence given by flavin in violet light. This can be done with suitable filters using either a visual procedure with the Fluoray or similar type of instrument as described by Supplee and others (*7*), or by photoelectric fluorescence measurement as developed by Cohen (*2*), and Euler (*3*) and adapted by Hand (*4*). The second method, the microbiological procedure of Snell and Strong (*6*), measures the influence of flavin on both the cell growth and the acid production of *Lactobacillus casei* ϵ grown on a synthetic medium free of flavin.

The present study is a report of progress with these methods as applied concurrently to several samples varying in type, composition, and potency.

### Methods

BIOLOGICAL METHOD. The ration consisted of purified casein 18 per cent, sucrose 68 per cent, Crisco 9 per cent, McCollum salt mixture No. 185 4 per cent, and cod liver oil 1 per cent. This was supplemented with daily treatment of 16 micrograms of thiamine hydrochloride and 0.08 gram of Labco rice polish extract freed from flavin with fuller's earth. The rats at the start were 22 to 25 days of age and weighed 40 to 50 grams. The depletion period ranged from 2 to 3 weeks. The test period was 4 weeks. Concurrently with the unknown samples, fed at different levels, 3 groups of rats were treated daily with 2.5 , 3.75, and 5.0 micrograms of riboflavin, respectively. These groups served as standard controls and permitted one to calculate the potency in terms of riboflavin.

FLUORESCENCE METHODS. (*a*) In cases where the riboflavin might have been in partial combination, the sample was digested as follows: five grams were suspended in 100 cc. of water, about 0.1 gram each of papain and of standard takadiastase were added, and the mixture was digested at 37° to 40° C. for 2 hours. Then 0.2 cc. of glacial acetic acid was added. The whole was heated to 80° C. to coagulate the protein and then filtered or centrifuged. The readings were made on the clear solution.

(*b*) If the preparation was too dark, decolorizing was carried out by the method of Kemmerer (*5*) which is a modification of that of Van Eekelen and Emmerie (*8*). This consists of making a methanol extract, adding a few cubic centimeters of glacial acetic acid, filtering if necessary, adding a few drops of saturated solution potassium permanganate, and then a little fresh hydrogen peroxide. The fluorescence was measured on this decolorized final solution.

In some cases it was necessary to introduce adsorption and elution steps, making the determination of the riboflavin on the eluate.

(*c*) For the visual reading the Fluoray Eveready carbon lamp was employed, fitted with a red-purple Corning filter No. 587 and a Corning filter No. 351 to screen out interfering blue fluorescence.

In assaying an unknown, at least three dilutions were made, preferably in the range of 0.1 to 0.2 microgram of riboflavin per cc. These were read against suitable standards and the potency was computed from the average values.

(*d*) The photoelectric fluorescence measurements were made with the Pfaltz & Bauer fluorophotometer. In this instrument emission from a mercury capillary arc is filtered (Corning glasses 038 and 511) and directed through a solution of the riboflavin. The fluorescent light strikes a photocell at the side of the cuvette and is transformed into electrical energy, the current of which is measured by a galvanometer. This photocell is protected from the incident intensity by Zeiss filter No. OG1. With a constant incident intensity, there is a linear relationship between the deflection of the galvanometer and the concentration of the riboflavin.

In preparation of the sample, a 10-cc. portion of the riboflavin solution was diluted with twice the volume (20 cc.) of acetone as suggested by Hand (*4*). The solution was agitated for a few seconds. If a precipitate formed the solution was filtered, the first part of the filtrate being passed again through the filter.

After suitable dilution, the fluorescence was then read directly in the cuvette, and the concentration of the riboflavin determined by reference to a standard curve prepared from dilutions of pure riboflavin.

MICROBIOLOGICAL METHOD. This procedure was carried out according to the method of Snell and Strong (*6*). The growth response of the bacteria was measured in terms of culture turbidity expressed as galvanometer extinction by means of the Pfaltz & Bauer fluorophotometer; and acidimetry, measuring the amount of acid produced in 72 hours' incubation. In this connection an innovation was introduced. The cultures were grown in carefully standardized 16 × 150 mm. test tubes which could be inserted directly into the light path of the fluorophotometer for extinction measurements at the end of 21 to 24 hours' incubation. Following this, the cultures were returned to the incubator to complete a 72-hour incubation, when the contents of each tube were removed and titrated against standard alkali. In this way both determinations were made on one and the same culture tube.

Samples were prepared for assay by simple water dilution except in the case of dry yeasts. These were digested with the enzymes as outlined above. The standard riboflavin solution was run for each assay in duplicate at five levels ranging from 0.05 to 0.15 microgram per 10-cc. culture tube. The unknowns were run in groups of 5 tubes covering this same range of dilutions. Response curves for the standard riboflavin were constructed from the turbidity values and from the acidimetric values. The unknowns were evaluated in terms of these and expressed as micrograms of flavin per gram.

### Discussion

In Table I the results are given as micrograms of riboflavin per gram of sample for the four procedures—biological rat growth, microbiological, fluorophotometric, and Fluoray. The type of samples begins at the top with relatively low-potency extracts of grain which are rich in sugars, followed by samples of dried yeast and extracts of the same; by liver extracts, plain and fortified with other vitamins; by high concentrates; and finally by blends of the more potent types with the low ones, high in sugar.

Observing these data for the various groups, the respective assay values for the four methods show a general tendency toward agreement. The largest differences occur, as one might expect, in the samples that are lowest in potency. This is brought out by comparing the values by the respective methods with the average of data by all the methods, given in the last column. If the average deviations from the mean are calculated for the different methods, that for the biological

TABLE I. COMPARATIVE POTENCIES, DETERMINED BY DIFFERENT METHODS

(Expressed in micrograms of riboflavin per gram)

| Sample Number | Description | Biological | Microbiological | Fluorophotometric | Fluoray | Average of All Methods |
|---|---|---|---|---|---|---|
| 38,540 | Wheat germ extract | 4.5 | 3.2 | 2.7 | 3.7 | 3.5 |
| 40,480 | Rice polish extract | 9 | 7.1 | 10.9 | 6.4 | 8.4 |
| 39,590 | Rice bran extract | 10 | 8.5 | 9 | 6.5 | 8.5 |
| 35,120 | Yeast, dried | 62 | 46 | 51 | 65 | 56 |
| 36,950 | Yeast, dried | 57 | 50.5 | 54 | 48 | 52.4 |
| 34,900 | Yeast concentrate | 200 | 171 | 192 | 195 | 189.5 |
| 38,790 | Liver extract, dried | 208 | 195 | 246 | 230 | 220 |
| 33,509 | Liver extract, dried | 230 | 204 | 234 | 225 | 223 |
| 39,140 | Liver extract, dried | 285 | 207 | 226 | 300 | 254.5 |
| 33,139 | Liver extract, dried | 500 | 501 | 507 | 537 | 511 |
| 20,879 | Liver extract, fortified | 250 | 277 | 302 | 244 | 268 |
| 23,659 | Liver extract, fortified | 250 | 241 | 269 | 216 | 244 |
| 40,360 | Liver extract, fortified | 682 | 670 | 685 | 727 | 691 |
| 34,880 | Special concentrate | 4690 | 4610 | 4800 | 4900 | 4750 |
| 37,630 | Special concentrate | 4685 | 5260 | 5470 | 4680 | 5024 |
| 40,160 | Special concentrate with wheat germ | 942 | 940 | 965 | 934 | 945 |
| 40,170 | Special concentrate with wheat germ | 317 | 312 | 347 | 340 | 329 |
| 40,420 | Yeast concentrate with wheat germ | 102 | 88 | 85 | 89 | 91 |
| 40,430 | Yeast concentrate with wheat germ | 135 | 114 | 109 | 134 | 123 |

assay is lowest, about 8 per cent. The other three are very similar, 10 to 12 per cent from the mean.

In Table II the microbiological, fluorophotometric, and Fluoray results in Table I are expressed as per cent difference (plus or minus) from the corresponding biological values. While the biological rat growth method is generally considered the standard criterion, such results in themselves are not necessarily correct to within 10 to 15 per cent. Nevertheless it is of interest to make the comparison. It is seen that there are some rather wide variations which in general occur with the low-potency samples. The averages of the per cent differences (including sign) are, respectively, −10.7, +5.3, and −7.0 by the microbiological, fluorophotometric, and Fluoray methods.

The question then arises as to what advantage, if any, one of these three methods has over the other, granting that they all require much less time than the biological method. In general, with the fluorometric measurement, it is often difficult to know how and when to treat the samples so as to obtain the optimum value. As a result, there is entailed considerable preliminary experimentation by trial and error. In addition, with the visual (Fluoray) procedure there is apt to be a large personal error unless the operator has had much experience. With the photoelectric procedure, there are also objections due to possible solarizing or cell fatigue. This entails a frequent careful checkup and restandardization of the instrument before each series of runs.

Since the microbiological method of Snell and Strong seemed to possess certain advantages, it was tested out further as to comparison of results obtained by turbidity extinction with those by acidimetry, reproducibility, and specificity in relation to the influence of other water-soluble vitamins.

The data for the comparison of extinction and acidimetric determinations are given in Table III for the same samples that were reported on in Tables I and II. As stated previously, the extinction values represent the culture turbidity formed in 24 hours' incubation and the acidimetric values represent acidity produced during 72 hours' incubation. The two sets of data show remarkable agreement throughout, irrespective of type or degree of potency of the samples. This fact is brought out clearly in the last column, giving the ratio of the two corresponding values, the average (19 samples) being 0.981. This shows that under the conditions as outlined, either procedure gives the same end results.

The reproducibility of the method was tried on a liver extract (sample 39,140). In all, fifteen separate runs were made. The data are given in Table IV and express extinction values. The micrograms of riboflavin per gram (column 2) ran very close, averaging 207, with an over-all range of 8.3 per cent and an average difference from the mean of only 2.5

TABLE II. DEVIATION FROM BIOASSAY VALUES

| Sample Number | Description | Bioassay | Difference from Bioassay Value: Microbiological | Fluorophotometer | Fluoray |
|---|---|---|---|---|---|
| | | μg./g. | % | % | % |
| 38,540 | Wheat germ extract | 4.5 | −28.9 | −40.0 | −17.8 |
| 40,480 | Rice polish extract | 9.0 | −21.2 | +21.1 | −28.9 |
| 39,590 | Rice bran extract | 10 | −15.0 | −10.0 | −35.0 |
| 35,120 | Yeast, dried | 62 | −25.8 | −17.8 | + 4.8 |
| 36,950 | Yeast, dried | 57 | −11.4 | − 5.3 | −15.7 |
| 34,900 | Yeast concentrate | 200 | −14.5 | − 4.0 | − 2.5 |
| 38,790 | Liver extract, dried | 208 | − 6.3 | +18.0 | +10.6 |
| 33,509 | Liver extract, dried | 230 | −11.2 | + 1.5 | − 2.0 |
| 39,140 | Liver extract, dried | 285 | −29.8 | −20.7 | + 5.3 |
| 33,130 | Liver extract, dried | 500 | + 0.2 | + 1.4 | + 7.5 |
| 20,879 | Liver extract, fortified | 250 | +10.8 | +20.8 | − 2.4 |
| 23,659 | Liver extract, fortified | 250 | − 3.6 | + 6.5 | −13.6 |
| 40,360 | Liver extract, fortified | 682 | − 1.8 | + 0.5 | + 6.6 |
| 34,880 | Special concentrate | 4690 | − 1.9 | + 2.4 | + 4.5 |
| 37,630 | Special concentrate | 4685 | +12.3 | +16.7 | − 0.2 |
| 40,160 | Special concentrate with wheat germ | 942 | − 0.3 | + 2.4 | − 0.8 |
| 40,170 | Special concentrate with wheat germ | 317 | − 1.6 | + 9.4 | + 7.3 |
| 40,420 | Yeast concentrate with wheat germ | 102 | −13.8 | −16.7 | −12.8 |
| 40,430 | Yeast concentrate with wheat germ | 135 | −15.6 | −19.9 | − 0.7 |
| | Average of 19, including sign | | −10.7 | + 5.3 | − 7.0 |

per cent. These values indicate that the method gives good reproducibility.

In respect to specificity, Snell and Strong (*6*) reported that such substances as copper, manganese, iron, zinc, barium, pyridine, and alcohol did not inhibit the growth of the *Lactobacillus casei*. The question arose as to the specificity of the organism for riboflavin in the presence of the other water-soluble vitamins—that is, whether they might act as accelerators or inhibitors. The findings are given in Table

TABLE III. COMPARISON OF EXTINCTION AND ACIDIMETRIC RESULTS OF MICROBIOLOGICAL METHOD

| Sample Number | Description | Extinction, I | Acidimetry, II | Ratio II/I |
|---|---|---|---|---|
| | | *Micrograms per gram* | | |
| 38,540 | Wheat germ extract | 3.2 | 3.3 | 1.03 |
| 40,480 | Rice polish extract | 7.1 | 7.1 | 1.00 |
| 39,590 | Rice bran extract | 8.5 | 8.2 | 0.96 |
| 35,120 | Yeast, dried | 46 | 44 | 0.96 |
| 36,950 | Yeast, dried | 50.5 | 50 | 0.99 |
| 34,900 | Yeast concentrate | 171 | 162 | 0.95 |
| 38,790 | Liver extract, dried | 195 | 194 | 1.00 |
| 33,509 | Liver extract, dried | 204 | 183 | 0.90 |
| 39,140 | Liver extract, dried | 207 | 210 | 1.02 |
| 33,139 | Liver extract, dried | 501 | 431 | 0.86 |
| 20,879 | Liver extract, fortified | 277 | 275 | 0.99 |
| 23,659 | Liver extract, fortified | 241 | 239 | 0.99 |
| 40,360 | Liver extract, fortified | 670 | 673 | 1.00 |
| 34,880 | Special concentrate | 4610 | 4520 | 0.98 |
| 37,630 | Special concentrate | 5260 | 5270 | 1.00 |
| 40,160 | Special concentrate with wheat germ | 940 | 954 | 1.02 |
| 40,170 | Special concentrate with wheat germ | 312 | 310 | 0.99 |
| 40,420 | Yeast concentrate with wheat germ | 88 | 90 | 1.02 |
| 40,430 | Yeast concentrate with wheat germ | 114 | 113 | 0.99 |
| | | | Av. | 0.981 |

TABLE IV. REPRODUCIBILITY OF MICROBIOLOGICAL METHOD FOR RIBOFLAVIN

(Liver extract 39,140)

| Test | | Micrograms per Gram | Difference from Mean | Per Cent Difference |
|---|---|---|---|---|
| 1 | | 207 | 0 | 0.0 |
| 2 | | 212 | +5 | 2.4 |
| 3 | | 215 | +8 | 3.9 |
| 4 | | 206 | −1 | 0.5 |
| 5 | | 209 | +2 | 1.0 |
| 6 | | 213 | +6 | 2.9 |
| 7 | | 200 | −7 | 3.4 |
| 8 | | 214 | +7 | 3.0 |
| 9 | | 207 | 0 | 0.0 |
| 10 | | 198 | −9 | 4.4 |
| 11 | | 199 | −8 | 3.9 |
| 12 | | 207 | 0 | 0.0 |
| 13 | | 206 | −1 | 0.5 |
| 14 | | 211 | +4 | 1.9 |
| 15 | | 202 | −5 | 2.4 |
| | Av. (15) | 207 | | 2.5 |

V as galvanometer extinctions for 0.1 microgram of riboflavin per 10-cc. tube, first alone as control, and successively when combined with varying concentrations of thiamine, pyridoxine, nicotinic acid, pantothenic acid, ascorbic acid, and finally a combination of all six vitamins. The values represent extinction readings for individual tubes. Since they do not vary in any case more than ±10 per cent from the readings for the tubes containing riboflavin alone, it is evident that the method measured only riboflavin and that the supplemental vitamins had no stimulating or retarding effect.

## Summary

A comparison has been made of four methods for determining vitamin $B_2$ (riboflavin): biological rat growth; visual fluorescence; photoelectric fluorescence; and microbiological, by both culture turbidity and acidimetry. The four methods gave similar results; the greatest differences were with the low-potency samples.

In a further study the microbiological method showed excellent specificity and reproducibility. The results were almost identical whether measured as culture turbidity at the end of 24 hours' or acidimetry at the end of 72 hours' incubation.

TABLE V. SPECIFICITY OF MICROBIOLOGICAL METHOD FOR RIBOFLAVIN

(Influence of other vitamins on galvanometer extinction)

| Added per 10-Cc. Tube | 0.1 μg. of Riboflavin Alone | Factor Added: Thiamine | Pyridoxine | Nicotinic acid | Pantothenic acid | Ascorbic acid | All combined |
|---|---|---|---|---|---|---|---|
| μg. | | | | | | | |
| 0.1 | 0.52 | 0.55 | 0.53 | 0.52 | 0.54 | 0.54 | 0.53 |
| 1 | 0.52 | 0.55 | 0.51 | 0.53 | 0.51 | 0.52 | 0.50 |
| 10 | 0.53 | 0.54 | 0.53 | 0.51 | 0.53 | 0.49 | 0.53 |
| 100 | 0.52 | 0.53 | 0.53 | 0.53 | 0.53 | 0.54 | 0.50 |
| 1000 | 0.53 | 0.53 | 0.52 | 0.53 | 0.52 | 0.57 | 0.56 |

## Literature Cited

(1) Bourquin, A., and Sherman, H. C., *J. Am. Chem. Soc.*, 53, 3501 (1931).
(2) Cohen, F. H., *Rec. trav. chim.*, 54, 133 (1935).
(3) Euler, H. von, and Adler, E., *Z. physiol Chem.*, 223, 105 (1934).
(4) Hand, D. B., IND. ENG. CHEM., Anal. Ed., 11, 306 (1939).
(5) Kemmerer, A. R., *J. Assoc. Official Agr. Chem.*, 23, 346 (1940).
(6) Snell, E. E., and Strong, F. M., IND. ENG. CHEM., Anal. Ed., 11, 347 (1939); Strong, personal communication.
(7) Supplee, G. C., *et al.*, *J. Dairy Sci.*, 19, 215 (1936); IND. ENG. CHEM., Anal. Ed., 11, 495 (1939).
(8) Van Eekelen and Emmerie, *Acta Brevia Neerland. Physiol. Pharmacol. Microbiol.*, 6, No. 9–10, 136 (1936).

# Chemical Estimation of Nicotinic Acid and Vitamin $B_6$

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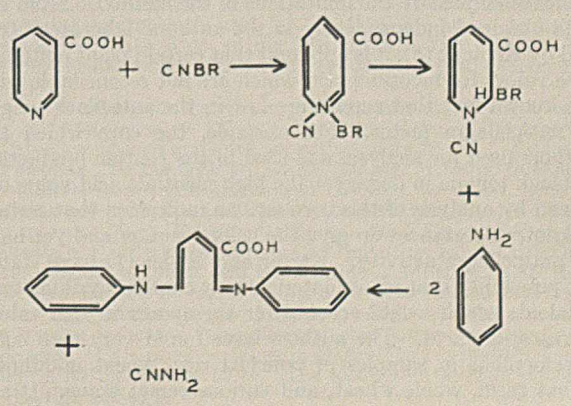
THE chemical methods now available for the determination of nicotinic acid depend upon the pyridine ring structure. It is well known that the pyridine ring is more reactive than the benzene ring and this greater reactivity is due to the nitrogen in the pyridine ring. The proposed methods depend on the breakdown of the pyridine nucleus with the liberation of nitrogen, thus opening the ring, followed by conjugation of the carbon chain with some aromatic amine.

A number of compounds have been used to react with the tertiary nitrogen for the purpose of splitting the pyridine ring. Vongerichten (*22*) and Reitzenstein (*15*) first proposed 2,4-dinitrochlorobenzene as the compound to add to the nitrogen. König (*9*) used cyanogen bromide, phosphorus trichloride, and other compounds. Once the nitrogen has its greatest valence satisfied by the addition of any one of these compounds, the ring is more susceptible to decomposition. The ring is easily broken with the liberation of the nitrogen in the form of a tertiary amine, thus yielding the carbon skeleton that combines with the amine to give a stable colored compound. The skeleton of the ring is a derivative of glutaconic aldehyde, which readily enolizes to give a reactive hydroxyl which can then combine with the organic base. The di-anil reaction illustrated



is with cyanogen bromide, but dinitrochlorobenzene and phosphorus trichloride will also give this reaction.

The aromatic amine plays an important role in the development of the color. Aniline, the simplest aromatic amine, was first used with cyanogen bromide, while later other organic bases were used which included β-naphthylamine, sulfanilic acid, *p*-phenetidin, cumidene, xylidine, etc. Bandier and Hald (*3*) used sodium sulfanilate, *p*-aminophenol, and sulfanilamide and found that none of these gave as consistent results as did *p*-methylaminophenol under the conditions employed. The authors have used aniline with satisfactory results. Shaw and McDonald (*19*), Melnick and Field (*12*), Pearson (*13*), and Swaminathan (*20*) used this amine to advantage. von Euler *et al.* (*4*) claimed better results with β-naphthylamine. Harris and Raymond (*6*) and Kodicek (*8*) used *p*-amino acetophenone and Arnold, Schreffler, and Lipsius (*1*) obtained good results with this amine.

## Specificity

A most important consideration in any colorimetric method is the specificity of the reaction. Natural materials often contain closely related compounds which give similar chemical reaction but dissimilar biological activity. One known vitamin other than nicotinic acid that contains the pyridine ring is vitamin $B_6$. This substance does not give the cyanogen bromide color reaction. It has been stated by some workers that the cyanogen bromide reaction is specific for pyridine compounds having the trivalent nitrogen. This is erroneous, since good evidence supports the theory that the alpha position must be unsubstituted for the complete cyanogen bromide color development. Since vitamin $B_6$ has a methyl group in the alpha position, it is to be expected that no color will develop. Bandier (*2*, *3*) found that if the analysis is carried out with *p*-aminophenol sulfate in the presence of potassium dihydrogen phosphate, small amounts of pyridine will not give the color. Picolinic acid, α-picoline, trigonelline, and methylpyridinium chloride do not give the color under the conditions stated.

A great number of compounds have been tested by the cyanogen bromide reaction, and many investigators have found that all the amino acids, a variety of purines, pyrimidines, certain metabolites, and carbohydrates will not give the yellow color. The extensive list indicates that a great many chemically important cell constituents are inactive in this test. However, compounds such as β-picoline, α-aminopyridine, and nepecotic acid have given positive tests. α-Aminopyridine probably gives the test since the amino group enters into the di-anil reaction. It is fortunate that these compounds must be present in high amounts to give a fair color, and more important still is the fact that these compounds probably occur in very small amounts if at all in biological materials.

Although the reaction is highly specific, certain pieces of evidence emphasize the limitations of the method. From the data obtained independently in the authors' laboratory (*23*) and by Kodicek (*8*), it is apparent that certain plant materials give values for nicotinic acid which are not reconcilable with the known fact that cereals are low in the anti-black-tongue or antipellagra factor. For example, the corn which the authors used for analysis was used in the routine production of black tongue in dogs, yet the high nicotinic acid value obtained by analysis of this corn was an indication that certain unknown substances do give the color reaction and yet have no antipellagra activity. Karrer and Keller (*7*) have stated the possibility that plant materials may contain pyridine type alkaloids which would account for the apparent high values for nicotinic acid. The authors have found very high color development in samples of polished rice, wheat middlings, wheat germ, whole wheat, and various cereal brans. It appears that the chromogen responsible for the color is tied up and is liberated only on hydrolysis. Kodicek (*8*) and Waisman, Henderson, and Elvehjem (*23*) have found that a water extract of corn will remove the nicotinic acid but leave the accompanying chromogen behind. The values obtained by this extraction procedure are more in keeping with the known facts.

## Conditions of Reaction

Colored Complex. The colored derivative formed from nicotinic acid, cyanogen bromide, and aniline is the simplest reaction product. It is soluble in water, but as amines of higher molecular weight are used the solubility of the colored complex decreases. Various workers have attempted to extract the colored complex into suitable solvents in order to stabilize the color by removing it from the excess reagents. Swaminathan (*20*) used amyl alcohol, Ritsert (*16*) and Perlzweig, Levy, and Sarett (*14*) used butanol, but in the authors' laboratory (*23*) these solvents proved unsatisfactory for color comparison. Arnold and co-workers (*1*) employed ethyl acetate for extraction of the color formed when *p*-amino acetophenone was used.

Absorption Maxima. In general, the majority of investigators have used photometers, tintometers, or photoelectric colorimeters for reading the color developed. The readings are made with filters that cut out all wave lengths other than those between 420 and 440 millimicrons. There have been reported slight variations from this maxima. Perlzweig and co-workers (*14*) claim that the true absorption maximum lies at 400 millimicrons. The authors have found that reliable readings are obtained with 400, 420, or 440 millimicron filters.

Time of Reaction. The time at which the colorimetric readings are made varies, depending upon the particular amine used and the conditions under which the color is developed. Various workers have used widely differing procedures and when aniline is used the readings can be made within 5 minutes. It would seem advisable to plot a time curve for a particular procedure that is to be used by the investigator. When amines other than aniline are used the color is developed more slowly.

H-Ion Concentration. The hydrogen-ion concentration of the test solution has a definite effect on color intensity. A number of workers have adjusted the solution to be tested to pH 7 before the development of color, while others have shown that there is a definite decrease in intensity with a decrease in pH. Using β-naphthylamine, von Euler and coworkers (*4*) found the color intensity to decrease on either side of the optimum pH. From the observations of many workers it seems that the optimum pH range in which there is a nonvarying color maximum is between 5.5 and 7.5.

Influence of Salts on Color. The detrimental influence of some salts on the development of color has been a difficulty to most workers who have attempted to perfect a chemical method for the determination of nicotinic acid. This difficulty has been one reason for extracting the color with organic solvents.

Bandier and Hald (*3*) found sulfate and nitrate ions to cause no change in the tint, but claimed that acetate ions interfered. Sodium chloride, ammonium chloride, and potassium dihydrogen phosphate have no effect on the color. von Euler *et al.* (*4*) found acetates and sulfates to interfere but alkali chlorides had no effect. These two workers used amines other than aniline, which might account for the divergent observation of Kringstad and Naess (*10*) that alkali chlorides in low concentrations gave increased intensities. Kodicek (*8*) found bicarbonate to cause a slight fading, and in contrast to the results of von Euler, found the acetate ion to have no effect on color development, but these two workers used different amines which very likely influenced the results they obtained. The authors have observed that salts in low concentrations affect the color adversely when aniline is used (*23*).

TABLE I. NICOTINIC ACID CONTENT OF BIOLOGICAL MATERIALS

| Sample | Waisman *et al.* | Melnick and Field | Bandier | Kringstad and Naess | Kodicek | Pearson | Swaminathan |
|---|---|---|---|---|---|---|---|
| | *Milligrams of nicotinic acid per 100 grams of fresh material* | | | | | | |
| Beef liver | 20.2 | ... | 12.2 | 20.0 | 17.0 | ... | 12.5 (sheep) |
| Pork liver | 28.2 | ... | 11.8 | ... | ... | ... | ... |
| Beef muscle | 4.8 | ... | 4.9 | 4.9 | 4.3 | ... | ... |
| Pork muscle | 5.3 | ... | 4.7 | 3.3 | ... | ... | ... |
| Beef spleen | 4.7 | ... | 4.4 | ... | 5.3 | ... | ... |
| Smoked ham | 8.3 | ... | ... | ... | ... | ... | ... |
| Brewer's yeast | ... | 43.0 | 53.0 | 44.5 | 57.5 | ... | 57.0 |
| Blood | ... | 0.72 (man) | ... | ... | 0.45 (sheep) | 0.80 (sheep) | 0.4 (man) |
| Cow's milk, 100 cc. | 0.82 | 0.45 | ... | ... | ... | ... | ... |
| Roe | ... | ... | 1.5 (cod) | 1.4 (frog) | 2.0 (herring) | ... | ... |
| Dried egg white | 5.2 | ... | ... | ... | ... | ... | ... |
| Polished rice | 10.5 (?) | ... | ... | ... | ... | ... | ... |
| Yellow corn | 10.7 (?) (acid hydrolysis) | ... | ... | ... | 4.5 (alkaline hydrolysis) | ... | ... |

EFFECT OF LIGHT ON COLOR. The effect of light on the stability of color is a consideration only when amines of high molecular weight are used. The color is developed more slowly and light seems to have a detrimental effect (*3*). With aniline the developed color is read within 5 to 7 minutes and this effect is of minor consideration. However, the color development is best carried out in diffuse light as a precaution against a possible detrimental effect of light.

QUANTITY OF AMINE. A number of workers have found that the amount of amine used is important, especially organic amines other than aniline. Bandier and Hald (*3*) found that the final intensity of the color depends on the metol concentration and not on the absolute amount of metol. The majority of the investigators have used an excess of the amine in the development of the color, but it appears (*10*) that for a given amount of cyanogen bromide a definite amount of amine should be used.

QUANTITY OF CYANOGEN BROMIDE. The amount of cyanogen bromide used by various investigators has varied from 0.35 to 6 ml. of the 4 per cent solution. Some workers have found that for a given amount of nicotinic acid a certain quantity of cyanogen bromide must be used. It appears from most of the available data that the exact amounts of cyanogen bromide used are not too important, but rather that if an excess is present no difficulties should be encountered in developing the maximum color.

EFFECT OF HEAT. Kodicek (*8*), Bandier and Hald (*3*), and Arnold and co-workers (*1*) heated the tubes in order to complete the reaction of cyanogen bromide and nicotinic acid. In early work in the authors' laboratory (*23*) they found that this procedure is especially advisable when higher molecular weight amines are used. Although heating the solution will likewise hasten the reaction when aniline is used, the added manipulations involved in the heating and cooling do not compensate by increasing the sensitiveness of the reaction.

## Some Difficulties Involved in the Determination

An analysis of biological material for nicotinic acid necessitates the liberation of the vitamin from combined forms such as coenzymes I and II. Many investigators have used either alkali or acid hydrolysis in order to obtain the free nicotinic acid or amide. Acid hydrolysis has the advantage that one obtains the free acid which is best suited for the color development. A number of workers have made water extracts of the material to be investigated followed by acid hydrolysis, thus preventing the interference by the yellow color due to the humin resulting from the hydrolysis of the proteins. Although no work has been published on enzymatic hydrolysis of naturally occurring materials, this procedure remains as a possibility for the liberation of nicotinic acid from complexes not easily broken down by short acid or alkaline hydrolysis.

The occurrence of interfering colors in natural materials such as bile pigments, riboflavin, carotenoids, and perhaps other unknown compounds has proved a definite handicap in reading the color developed by the reagents with the nicotinic acid present in the sample. Various workers thus found it necessary either to remove the nicotinic acid from the interfering pigments or to remove the pigments from the nicotinic acid. In some early work in their laboratory the authors found it desirable to eliminate the interfering yellow color that one obtains in liver extracts and in hydrolyzed biological materials. Attempts were made to remove interfering colors by adsorption on norite, by filtration methods, by extraction of the nicotinic acid into suitable solvents, and by differential solubility, but none of these methods was satisfactory. The use of preferential adsorption of interfering pigments on charcoal has been aided by the Melnick and Field (*12*) modification of the Shaw and McDonald (*19*) procedure of an acid alcohol adsorption with a special charcoal.

## Reagents Used

CYANOGEN BROMIDE. A cold saturated bromine water solution is carefully decolorized in the cold with 10 per cent sodium cyanide with the aid of a buret. Care is taken to prevent having any excess of the cyanide. Sodium is preferable to the potassium salt for making the reagent. This 4 per cent solution of the reagent is kept cold when not in use and is stable for long periods.

ALCOHOLIC ANILINE SOLUTION. A 4 per cent solution is prepared by dissolving freshly distilled aniline in absolute ethyl alcohol.

STANDARD NICOTINIC ACID SOLUTION. This contains 50 micrograms of nicotinic acid per cc. of absolute ethyl alcohol. A water solution containing 50 micrograms per cc. is also made.

BUFFER SOLUTION. This consists of 980 cc. of water, 15 cc. of 15 per cent sodium hydroxide, 5 cc. of 85 per cent phosphoric acid, and 167 cc. of absolute alcohol. This buffer solution corresponds to the pH of the test solution and the reagents.

## Procedure

The procedure used is essentially that of Melnick and Field (*12*), with slight modifications to simplify parts of the procedure. A sample containing 25 to 200 micrograms of nicotinic acid is weighed into each of two 15-cc. graduated centrifuge tubes. To one of the tubes are added 50 micrograms of nicotinic acid in water solution, to act as recovery. Five cubic centimeters of concentrated hydrochloric acid (sp. gr. 1.18) are added to both tubes together with 5 cc. of distilled water. The material is placed in a boiling water bath for 30 to 40 minutes with occasional stirring.

After hydrolysis the tubes are cooled, made up to the 15-cc. mark, and transferred to 125-cc. Erlenmeyer flasks. The centrifuge tubes are washed out carefully with exactly 10 cc. of absolute ethyl alcohol. To the flask are added 200 mg. of Darco (a charcoal obtained from the Coleman and Bell Co., Norwood, Ohio). The flasks are shaken, then filtered through quantitative filter paper into dry 50-cc. Erlenmeyer flasks. An 8.33-cc. aliquot of the filtrate is pipetted into a graduated centrifuge tube, 1 drop of phenolphthalein is added, and the solution is adjusted to pH 8.5 in the cold by the dropwise addition of saturated sodium hydroxide. The solution is now adjusted to pH 7.0 with the aid of an outside indicator, bromothymol blue, using dilute hydrochloric acid. Care must be taken in neutralizing so that the final volume is no more than 10 cc. The alcohol-water ratio at this final dilution is 1 to 2.

To carry out the color development so that readings can be made in the Evelyn photoelectric colorimeter, eight colorimeter tubes are used. To the first and second tubes are added 1 cc. of absolute ethyl alcohol and 2 cc. of water; to tubes 3, 4, and 5 are added 3-cc. portions of the solution of the sample; 3-cc. portions of the sample and recovery are added to tubes 6, 7, and 8; 7 cc. of the buffer solution are added to tubes 1, 3, and 6. Tubes 3 and 6 thus furnish the independent blank determination for the residual color of the sample remaining after decolorization with charcoal. Tube 2 acts as the blank on the reagents. To tubes 5 and 8, 5 micrograms of nicotinic acid are added in order to obtain the photometric density of this known amount of nicotinic acid.

To tubes 2, 4, 5, 7, and 8, 6 cc. of cyanogen bromide are added, followed as rapidly as possible by 1 cc. of alcoholic aniline. The tubes are shaken and the colorimetric readings made within 5 to 6 minutes after the addition of the aniline to the last tube. To read the color in the tubes, filter 400 or 420 millimicrons is used. Tube 1 is set so that the galvanometer reads 100. Tubes 3 and 6 are read at this setting. Tube 2 is now set at 100 and the readings for tubes 4, 5, 7, and 8 are made with this setting. In this way the double blank is obtained which checks both the reagents and the residual color in the test solutions.

### Calculations of Reading with Evelyn Photoelectric Colorimeter

$$K_1 = \frac{\text{5 micrograms of nicotinic acid}}{\text{log tube 5} - \text{log tube 4}}$$

$$X_1 = K_1 \times (\text{log tube 4} - \text{log tube 3})$$

$$K_2 = \frac{\text{5 micrograms of nicotinic acid}}{\text{log tube 8} - \text{log tube 7}}$$

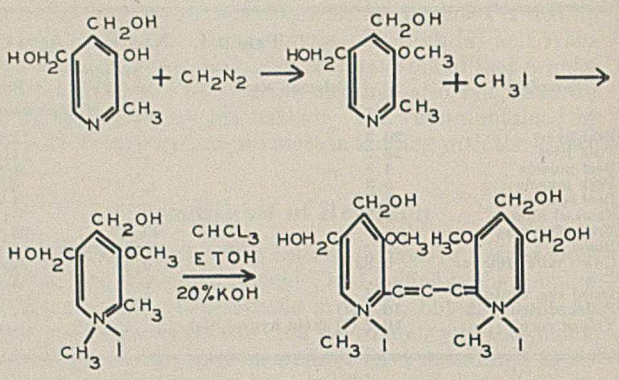
$X_2 = K_2 \times$ (log tube 7 − log tube 6), where $X_1$ is the nicotinic acid content of the aliquot of the sample

$X_2 - X_1$ should equal 5 micrograms, since the 3-cc. aliquot of the neutralized filtrate represents one tenth of the original sample, and the original recovery sample had 50 micrograms of nicotinic acid added. For more complete details on the method of calculation and procedure reference should be made to Melnick and Field (*12*) and to (*17*).

The methods now available to the investigator leave something to be desired. However, accepting the limitations of the cyanogen bromide method in general, it can be applied with a fair amount of success to the determination of nicotinic acid in animal tissues, urine, and blood. The analysis of plant materials must await further investigation before acceptable values are obtained. For practical purposes, however, the relative amounts of nicotinic acid in a variety of samples are important and the absolute values although desirable are not necessary. A few nicotinic acid values are given in Table I as determined in the authors' laboratory and by other investigators.

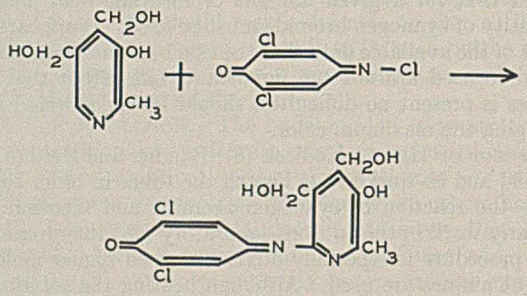
### Chemical Estimation of Vitamin $B_6$

The formula for pyridoxine (vitamin $B_6$) as now definitely established has the basic pyridine ring with two hydroxy methyl substituents, one alpha methyl group, and a phenolic hydroxyl in the beta position. Kühn and Löw (*11*) have applied the Folin Denis phenol reaction to the vitamin and found the color to be very sensitive. One difficulty which they observed was that of bleaching of the blue color by precipitates that are formed. Kühn and Löw (*11*) also attempted to determine this vitamin by forming carbopyridinium cyanin compounds. This reaction depends on the presence of a methyl group in the alpha position of the pyridine ring. In order to prepare the derivative, the beta-hydroxyl is methylated and the ether formed by diazomethane. Then by using methyl iodide and conversion into the quaternary pyridinium compound the product can be converted to a blue color by slight heating with chloroform and 20 per cent alkali in the presence of alcohol. The reactions are the following:




The carbopyridinium cyanin compound formed from the methyl ether methyl iodide is specific for vitamin $B_6$. Straight-line curves are obtained with crystalline vitamin, but the obvious difficulties are the quantitative conversion of the vitamin into its various derivatives. However, Kühn and Löw claim that the color developed either with the phenol reagent or with the quaternary pyridinium compound obeys Beer's law. These investigators have given no data on the application of their method to biological materials.

Scudi, Koones, and Keresztesy (*18*) have used a modification of the Gibbs phenol test (*5*), which depends on the formation of indophenols. These are products of the interaction of phenols and a dihalogen substituted quinone chloroimide. An intense blue solution results from the interaction of the phenolic hydroxyl of vitamin $B_6$ and the chloroimide.




It is believed that only those phenols react which have an open position para to the phenolic hydroxyl. The above-mentioned investigators used a veronal buffer to adjust the pH of the material, urine in this case, to pH 7.6 before the addition of the butanol solution of the chloroimide reagent. Extraneous substances are shaken out and the butanol layer is read after 40 minutes. No report has been made of applying this method to biological materials other than urine.

Swaminathan (*21*) has published a method for the chemical determination of vitamin $B_6$ which involves the use of diazotized sulfanilic acid. Contrary to the contention of Kühn and Löw that the diazotized sulfanilic acid reaction is unreliable, this investigator has obtained values which compare favorably with bioassays. The chemistry involved in the coupling reaction is simply that of forming an azo dye. The diazotized sulfanilic acid is treated with the vitamin solution or a concentrate of the vitamin and coupling takes place at the unsubstituted position para to the phenolic hydroxyl as indicated by the reactions:

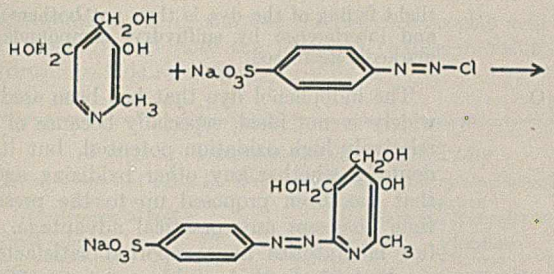


TABLE II. VITAMIN $B_6$ CONTENT OF FOODSTUFFS

| Foodstuff | Henderson, Waisman, and Elvehjem | Swaminathan |
|---|---|---|
| Brewer's yeast | 40 | 54 |
| Autoclaved brewer's yeast | 40 | 53 |
| Rice polishings | ... | 13.4 |
| Sheep liver | 12.6 | 13.4 |
| Sheep muscle | 11.7 | 4.5 |
| Cow's milk | ... | 1.7 |
| Yellow corn | 4.8 | 7.1 |
| Whole wheat | ... | 7.4 |
| Polished rice | ... | 3.0 |
| Soybean | ... | 8.0 |
| Cabbage | ... | 3.1 |
| Beet root | ... | 1.3 |
| Pork liver | 9.9 | ... |
| Pork ham | 24.0 | ... |
| Veal muscle | 16.3 | ... |
| Pork heart | 15.5 | ... |

The liberation of vitamin $B_6$ from naturally occurring materials depends on a pepsin digest followed by tungstate precipitation for removal of proteins and silver nitrate precipitation for the removal of purines, pyrimidines, and amidazole bases. The vitamin was absorbed, then eluted and further treated to remove interfering materials. The pH was adjusted to 7 and the color developed with the diazotized sulfanilic acid. This method is open to criticism since the reaction is not specific. However, the adsorption on an alkaline earth does minimize the carrying over of certain other cell constituents that are claimed to give the color by Kühn and Löw.

Swaminathan has also used the phenol reagent of Folin and Ciocalteau and by the use of either of these two methods, he has obtained the vitamin $B_6$ content of various foodstuffs. In Table II are listed his values as well as those obtained in the authors' laboratory using a new bioassay procedure for vitamin $B_6$.

### Literature Cited


(1) Arnold, A., Schreffler, C. B., and Lipsius, S. T., IND. ENG. CHEM., Anal. Ed., 13, 62 (1941).
(2) Bandier, E., *Biochem. J.*, 33, 1130 (1939).
(3) Bandier, E., and Hald, J., *Ibid.*, 33, 264 (1939).
(4) Euler, H. von, Schlenk, F., Heiwinkel, H., and Hogberg, B., *Z. physiol. Chem.*, 256, 208 (1938).
(5) Gibbs, H. D., *J. Biol. Chem.*, 72, 649 (1927).
(6) Harris, L. J., and Raymond, W. D., *Chemistry Industry*, 58, 652 (1939).
(7) Karrer, P., and Keller, H., *Helv. Chim. Acta*, 21, 463 (1938); 22, 1292 (1939).
(8) Kodicek, E., *Biochem. J.*, 34, 712, 722 (1940).
(9) König, W., *J. prakt. Chem.*, 69, 105 (1904) n. f.
(10) Kringstad, H., and Naess, T., *Z. physiol. Chem.*, 260, 108 (1939).
(11) Kühn, R., and Löw, I., *Ber.*, 72, 1453 (1940).
(12) Melnick, D., and Field, H., *J. Biol. Chem.*, 134, 1 (1940); 135, 53 (1940).
(13) Pearson, P., *Ibid.*, 129, 491 (1939).
(14) Perlzweig, W. A., Levy, E. D., and Sarett, H., *Ibid.*, 133, lxxiv (1940).
(15) Reitzenstein, F., *J. prakt. Chem.*, 68, 251 (1903).
(16) Ritsert, K., *Klin. Wochschr.*, 18, 934 (1939).
(17) Rubicon Co., Philadelphia, "Notes on Operation of Evelyn Photoelectric Colorimeter".
(18) Scudi, J. V., Koones, H. F., and Keresztesy, J. C., *Proc. Soc. Exptl. Biol. Med.*, 43, 118 (1940).
(19) Shaw, G. E., and McDonald, O. A., *Quart. J. Pharm. Pharmacol.*, 11, 380 (1938).
(20) Swaminathan, M., *Indian J. Med. Science*, 26, 427 (1938).
(21) Swaminathan, M., *Nature*, 145, 780 (1940).
(22) Vongerichten, E., *Ber.*, 32, 2571 (1899).
(23) Waisman, H. A., Henderson, L. M., and Elvehjem, C. A., unpublished data.


# Chemical Methods for Determination of Vitamin C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NEARLY all the methods that have been proposed for the estimation of vitamin C have been based upon the reversible oxidation of ascorbic acid to dehydroascorbic acid. Although several of the oxidation methods give reasonably satisfactory results, there are pitfalls to be avoided in all the procedures that have been published; hence it is of distinct value to have available an independent approach, such as that developed by Roe (*9*), in which ascorbic acid is decomposed to furfural for colorimetric estimation. The reaction with 2,6-dichlorophenolindophenol in metaphosphoric acid solution provides the most widely used, and in the author's experience the best, general basis for ascorbic acid analysis. The polarigraphic and oxidation-reduction titrimeter methods are of interest as specialized oxidation procedures, but neither has been developed sufficiently to merit confidence for general use.

Figure 1 will serve to outline the basic steps in the methods that have been proposed. In many types of investigation concerning vitamin C, both in foodstuffs and in physiological research, analyses for dehydroascorbic acid are nearly as important as tests for ascorbic acid itself. This situation arises primarily because dehydroascorbic acid is approximately as active antiscorbutically as ascorbic acid and theories concerning the function of vitamin C *in vivo* are based in part on the analyses for dehydroascorbic acid.

### Indophenol Method

For most investigations direct visual titration of ascorbic acid with the dye 2,6-dichlorophenolindophenol in a pH range of 1 to 3.5 gives a reasonably accurate and satisfactory measure of the vitamin C content of tissues. The extracts must be properly prepared and close attention must be given to details of the procedure (*1*, *2*, *7*). The two most crucial points to be watched, apparently, are avoidance of oxidative changes previous to titration, and awareness of the circumstances

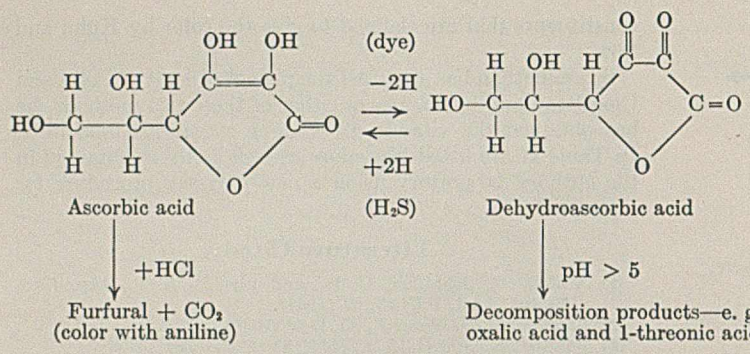


FIGURE 1

under which other reducing substances will or will not interfere with the titration.

In relation to the first point, aerobic oxidation through the agency of enzymes (chiefly copper- but to a lesser extent iron-containing specific proteins) or added copper (picked up from reagents and equipment) can generally be avoided by the use of 2 to 3 per cent metaphosphoric acid as an extractant, if sufficient care is given to other details. Insufficient grinding and extraction of the sample have also been common sources of error. The author has not observed evidence of the existence of significant amounts of combined, non-acid-extracted ascorbic acid as reported by Reedman and McHenry (*8*) and a few other investigators. Neither has he found sulfuric acid, as recommended by Tressler and associates (*5*), to be desirable as an extractant, even though it affords some economy. Although acetic and trichloroacetic acids were widely used in earlier work, and give good results with many products, they are not so satisfactory as metaphosphoric acid. The protective action of acids is not simply a pH phenomenon, because at the same pH, metaphosphoric acid is distinctly superior to sulfuric acid and the latter in turn is superior to hydrochloric acid.

In relation to the second point, sulfhydryl compounds and carbohydrate decomposition products are especially likely to interfere. The concentration of interfering substances in most natural products is not sufficient to cause significant errors, however. When making direct visual titrations it is very important to use an end point of about 5 seconds or less. With the photoelectric colorimeter method, two or more readings should be made at 15-second intervals, from which one can in part correct for the interference due to more slowly reacting substances. Thiosulfate, ferrous salts, and certain sulfhydryl compounds cause interference when present, as do a number of sugar decomposition products having a reductone type of structure.

In some natural products such as strawberries and beets the presence of coloring matter makes it nearly impossible to carry out direct titrations. In such cases the use of a photoelectric colorimeter is satisfactory, however, because one measures only the decrease in color caused by ascorbic acid, when the dye is added, and the natural pigment which masks the disappearance of the indophenol dye is not reduced during the test.

Bessey has described the details of procedure to be followed in using the instrument (*1*) and has cited many related publications. The papers by Mindlin and Butler (*6*) and Farmer and Abt (*3*) are of special interest in relation to blood analyses. It is now generally agreed that cyanide should not be used and that ascorbic acid is fairly stable in whole blood, plasma, and serum except when hemolysis has occurred. There may be a slight loss caused by oxidation arising from ferri forms of compounds and loosely bound oxygen carried by hemoglobin. Bessey has pointed out the value of using an extractant buffered at pH 3.5 for titrations with the instrument, since in that range the reaction with the dye is reasonably specific, the reading does not drift because of a pH effect on the dye, and conditions are about optimum for dehydroascorbic acid analyses. The same author has pointed out the need for a check reading after decolorizing all of the dye, in order to correct for light absorption by suspended matter and other material in the test preparation. In making direct visual titrations one can profitably use a lower pH (about 1) because the slight fading of the dye is then not bothersome and interference by sulfhydryl compounds is markedly decreased.

The indophenol dye that has been used so widely is not ideal, especially because of its relatively high oxidation potential, but it is doubtful whether any other oxidizing agent that has been proposed up to the present time possesses any practical advantage. A few laboratories have reported satisfactory results with methylene blue as an oxidant. The reaction in this case is slow, however, unless there is an accompanying exposure to light. For testing urine samples there have been encouraging reports based upon precipitation of the vitamin by 2,4-dinitrophenylhydrazine, followed by quantitative reduction of the nitro groups or by hydrolysis, reduction, and conversion to furfural. The method proposed by Roe (*9*), based upon furfural formation, has the advantage of completely avoiding interference by non-ascorbic acid reducing material such as sulfhydryl compounds and the 3- or 4-carbon sugar decomposition products, but the procedure is much more time-consuming and necessitates avoidance of possible interfering substances that yield furfural on decomposition, such as the uronic acids and pentoses.

In regard to the use of such special instruments as the oxidation titrimeter or the polarigraph, the author's experience has been very limited, but he has not found that they possess any significant advantage over the photoelectric colorimeter for general use. For meeting special difficulties these instruments have obvious advantages. The polarigraphic method holds promise for detecting and perhaps avoiding errors that arise from interfering reducing substances that exhibit slightly higher or lower reduction potentials, such as characterize the sulfhydryl compounds.

## Tests for Dehydroascorbic Acid

In studying ascorbic acid synthesis in tissue slices, Dr. Smythe in this laboratory observed that certain added substrates gave reactions with hydrogen sulfide that were comparable to the reaction generally used for estimating dehydroascorbic acid. The study was extended to establish the fact that many organic compounds give such a reaction. It is reasonably clear that many of the published data relative to the oxidized form of the vitamin have been misinterpreted, because of this interference by newly formed reducing material that results from hydrogen sulfide treatment. The risk of encountering such an error was pointed out in an earlier publication (*4*). The error that is introduced by hydrogen sulfide treatment is also inherent in the modified methods in which mercuric salts are added to remove sulfhydryl compounds from tissue extracts, because these methods include a final treatment with hydrogen sulfide.

Table I lists some of the compounds that were tested and found to interfere seriously with the dehydroascorbic acid determination, together with a number of compounds that did not cause serious interference under the conditions studied. In several cases the interference was almost mole-for-mole equivalent to the reversibly oxidized vitamin. During a considerable part of the titration, the reaction of the newly formed sulfhydryl compounds with the dye was apparently complete within 1 to 5 seconds. Toward the end of the titration there was generally a slower fading of the dye that resembled the typical glutathione reaction. Because of the rapidity of the reaction the interference could not be wholly avoided either by a correction curve with the photoelectric colorimeter or by visual titration at a pH of 1 or less. The best direct methods for detecting and avoiding such an error would seem to be the use of the furfural reaction, the use of a

polarigraph, or to decompose the dehydroascorbic acid at a pH above 7 and note the resultant change in value after hydrogen sulfide reduction.

TABLE I. DEHYDROASCORBIC ACID TESTS

($H_2S$ for 2 hours at pH 3.5; titration with 2,6-dichlorophenolindophenol)

| Positive (Interference > 20%) | Negative (Little or No Interference) | |
|---|---|---|
| Pyruvic acid | Acetone | Piperitone |
| Pyruvic aldehyde | Sorbose | Phorone |
| Glyceric aldehyde | Levulose | Oxalic acid |
| Dihydroxyacetone | Glucose | Kojic acid |
| Acetaldehyde | Xylose | β-Ketobutyric acid |
| Mannosaccharic acid | Cyclohexanone | β-Ketoglutaric acid |
| 5-Ketogluconic acid | Hexadienal | Glucuronic acid |
| 1,4-Benzoquinone | Butyraldehyde | |
| 1,4-Naphthoquinone | | |
| 2-Methylquinone (vitamin K) | | |


## Summary

When adequate precautions are taken, the reaction of ascorbic acid with 2,6-dichlorophenolindophenol can be used in direct titrations or with the photoelectric colorimeter to give relatively satisfactory quantitative analyses. Other methods of analysis may be preferable under special circumstances because of interference by other reducing materials.

Methods for the measurement of dehydroascorbic acid, however, are subject to great interference, because many aldehydes, ketones, and quinones give rise to an interfering reaction when reduced by hydrogen sulfide. Among the interfering compounds are pyruvic acid, pyruvic aldehyde, glyceric aldehyde, dihydroxyacetone, acetaldehyde, mannosaccharic acid, 5-ketogluconic acid, 1,4-benzoquinone, 1,4-naphthoquinone, and 2-methyl-1,4-naphthoquinone (thyloquinone). Three methods for detecting and avoiding such interference are pointed out.



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## Literature Cited


(1) Bessey, O. A., *J. Biol. Chem.*, 126, 771 (1938); *J. Am. Med. Assoc.*, 111, 1290 (1936).
(2) Bessey, O. A., and King, C. G., *J. Biol. Chem.*, 103, 687 (1933).
(3) Farmer, C. J., and Abt, A. F., *Proc. Soc. Exptl. Biol. Med.*, 34, 146 (1936).
(4) King, C. G., *Physiol. Rev.*, 16, 238 (1936).
(5) Mack, G. L. and Tressler, D. K., *J. Biol. Chem.*, 118, 735 (1937).
(6) Mindlin, R. L., and Butler, A. M., *Ibid.*, 122, 673 (1938).
(7) Musulin, R. R., and King, C. G., *Ibid.*, 116, 409 (1936).
(8) Reedman, E. J., and McHenry, E. W., *Biochem. J.*, 32, 85 (1938).
(9) Roe, J. H., *Science*, 80, 561 (1934); *J. Biol. Chem.*, 116, 609 (1936).


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# A Spectroscopic Method for the Quantitative Estimation of Vitamin D


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DURING the past 4 years three independent physicochemical methods (*1*, *5*, *6*, *15*, *16*) have been proposed for the estimation of vitamin D.

The method originally used by Brockmann and Chen has been more extensively studied and modified by several investigators (*3*, *8*, *12*, *13*, *14*). This method is based on the discovery that vitamin D forms with a saturated solution of antimony trichloride in chloroform a pink color which exhibits a prominent band in the region of 500 mμ. Figure 1 shows the antimony trichloride transmission spectra of vitamins $D_2$ and $D_3$ and that of the glucoside of vitamin $D_2$. Irrespective of the minor differences in structure, all transmit exactly in the same region with the head of the characteristic band located at 500 mμ. In the course of their work on the separation of vitamins A and D from fish liver oils (*10*, *11*), the authors followed the purification by measuring the extinction coefficients at 500 and at 620 mμ, the two maxima attributable to vitamins D and A, respectively. By the use of the appropriate correction factors, it was found possible to estimate vitamin D in the presence of considerable quantities of vitamin A with a reasonable degree of accuracy. This method has now been extended and modified to make possible the estimation of vitamin D in fish liver oils.

In preliminary attempts, the authors used as a standard a fish liver oil, the biological potency of which had been accurately determined a number of times by several laboratories and reported to be 50,000 U. S. P. vitamin D units per gram. This oil was kindly supplied by the Atlantic Coast Fisheries Corporation of New York. Using this standard, the potencies of some of the oils tested agreed fairly well with the reported biological potencies. However, a correction for the presence of vitamin A did not improve the spectroscopic values appreciably, since the presence in the oils of sterols and possibly carotenoids gives rise to colors which seem to have an additional effect on the 500 mμ maximum of vitamin D. This made necessary the use of an additional correction for the presence of sterols. Although such a procedure gave satisfactory results for oils containing more than 10,000 U. S. P. vitamin D units per gram, it was found cumbersome and not universally applicable, as it is necessary to have a method of measuring the visible spectrum accurately and rapidly.

In view of these objections the authors have adopted a modified procedure in which vitamin A, 7-dehydrocholesterol,

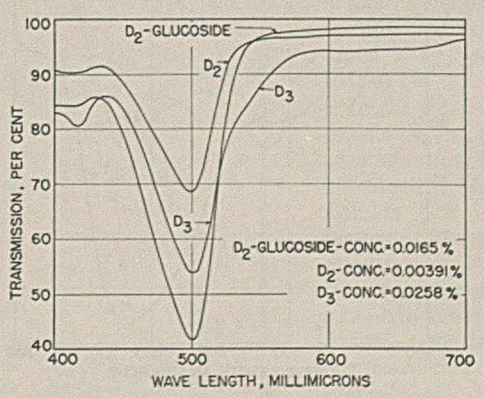


FIGURE 1. TRANSMISSION SPECTRA OF ANTIMONY TRICHLORIDE COLOR OF VITAMINS $D_2$ AND $D_3$ AND VITAMIN $D_2$–GLUCOSIDE

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Table I. Correction Due to Antimony Trichloride

| Maximum mμ | Correction |
|---|---|
| 430 | 0.006 |
| 455 | 0.006 |
| 480 | 0.005 |
| 500 | 0.004 |
| 580 | 0.002 |
| 620 | 0.000 |
| 690 | 0.000 |

and interfering carotenoids are destroyed by a preliminary treatment with maleic anhydride. By the application of this procedure, they obtained satisfactory estimates of vitamin D potency of oils containing in the neighborhood of 10,000 U. S. P. units per gram or higher. In all this latter work, they used as their standard purified crystalline calciferol which had been carefully assayed biologically by R. S. Harris of the Department of Biology. He reported a potency of 40,000,000 U. S. P. vitamin D units per gram. This calciferol was found to have an $E^{1\%}_{1\ cm.}$ value of 890 at 500 mμ (Figure 1, curve II). This modified procedure need not rely for its application upon the use of a rapid means of determining the visible spectrum such as the Hardy color analyzer. Filters suitable for the isolation of a band in the neighborhood of 500 mμ may be used in conjuction with an ordinary colorimeter for the estimation of color intensity. Furthermore, if the sterols are also removed by a preliminary digitonin treatment, it may be possible to estimate vitamin D potencies by measuring the absorption at 265 mμ, and thus avoid the use of antimony trichloride.

## General Procedure

To 0.2 cc. of a solution of vitamin D containing oil in chloroform in a 1-cm. special spectroscopic cell are added 3.8 cc. of a saturated solution of antimony trichloride in freshly purified chloroform. The transmission curve (or absorption curve) in the visible region of the spectrum is then determined in the Hardy color analyzer (7) in such a way that the maximum at 620 mμ is reached exactly 3 minutes after mixing. A second curve is then run, so that the maximum at about 500 to 520 mμ is reached 10 minutes after mixing. Finally a third curve is run 30 minutes after mixing.

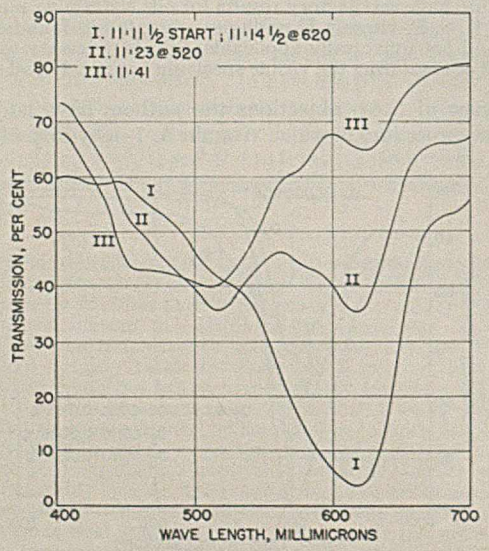


Figure 2. Effect of Time on Transmission Spectrum of Antimony Trichloride Color of a Fish Liver Oil

This gives a family of curves as shown in Figure 2. From the first curve the concentration of vitamin A is easily estimated by computing the extinction coefficient of a 1 per cent solution in a 1-cm. cell at 620 mμ, using the formula $E^{1\%}_{1\ cm.} = 1/c \times \log 1/T$, where $c$ is the per cent concentration and $T$ the per cent transmission as read from the curve. A small correction may be applied for the absorption due to antimony trichloride by adding to the per cent transmission the values given in Table I for the maxima of prominent bands which have been identified with the oils studied. The corrected $E^{1\%}_{1\ cm.} = E$ corrected/$c$.

The band at about 620 mμ appears most strongly in the first of each group of curves and fades very rapidly with time. It may be seen from Table II that the $E^{1\%}_{1\ cm.}$ values of five different oils at 620 mμ compare very favorably with the values of the same oils obtained at 328 mμ.

Table II. Comparison of $E^{1\%}_{1\ cm.}$ Values of Vitamin A at 328 and 620 mμ

| Oil Sample | $E^{1\%}_{1\ cm.}$ (328) | $E^{1\%}_{1\ cm.}$ (620) | $E^{1\%}_{1\ cm.}$ (328)/$E^{1\%}_{1\ cm.}$ (620) |
|---|---|---|---|
| 1616 | 51.1 | 96 | 0.5323 |
| 1617 | 32.8 | 57.5 | 0.5704 |
| 1618 | 39.7 | 75.5 | 0.5258 |
| 1619 | 32.9 | 60.7 | 0.5420 |
| 1620 | 43.6 | 81.5 | 0.5349 |

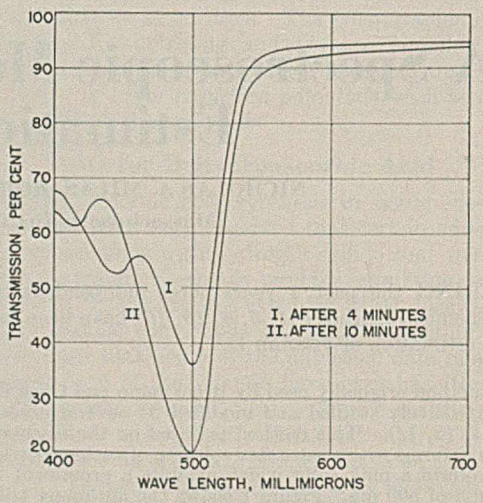


Figure 3. Effect of Time on Transmission Spectrum of Antimony Trichloride Color of Vitamin $D_2$

The band at 500 to 520 mμ attains a maximum value only after the lapse of about 10 minutes, irrespective of the material containing vitamin D. The effect of time on the development of this band is better illustrated by Figure 3.

A fairly complete spectroscopic study was then made of fifteen fish liver oils which had been assayed biologically, some of them by several laboratories. Six of these oils, marked $W_1$, $W_2$, etc., were supplied by C. L. Barthen of the White Laboratories, while the remaining nine were supplied by the Atlantic Coast Fisheries Corporation. All of the important bands in the visible region were accurately located and the extinction coefficient, $E^{1\%}_{1\ cm.}$ for each was estimated. These are recorded in Table III together with the biological potencies and the concentrations of solutions employed. At the bottom of Table III are appended for comparison the

TABLE III. SUMMARY OF SPECTROSCOPIC DATA OF FISH LIVER OILS

| Sample | Biological Potency U. S. P. units/g. | Concentration % | Time Min. | Band₃ | $E^{1\%}_{1\ cm.}$ | Band₂ | $E^{1\%}_{1\ cm.}$ | Band₃ | $E^{1\%}_{1\ cm.}$ | Band₄ | $E^{1\%}_{1\ cm.}$ | Band₃ | $E^{1\%}_{1\ cm.}$ | Band₂ | $E^{1\%}_{1\ cm.}$ | Band₁ | $E^{1\%}_{1\ cm.}$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ACF-CR 503 | 50,000 | 0.025 | 0 | 430 | 9.10 | | | 472 | 10.49 | 520 | 15.20 | | | | | 620 | 57.2 | 690 | 10.96 |
| | | | 11½ | | | 455 | 11.18 | | | 518 | 17.77 | 545 | 14.26 | 580 | 14.49 | 620 | 18.16 | 690 | 7.50 |
| | | | 30 | | | 455 | 14.38 | 480 | 14.70 | 512 | 15.83 | | | 580 | 8.54 | 627 | 7.49 | 690 | 3.92 |
| ACF-CR 726 | 18,000 | 0.05 | 0 | 430 | 2.72 | | | 480 | 3.76 | 520 | 5.12 | | | | | 616 | 14.10 | 690 | 4.60 |
| | | | 10 | | | ... | ... | 474 | 6.26 | 513 | 6.16 | ... | ... | 580 | 5.25 | 613 | 5.60 | 690 | 3.20 |
| | | | 30 | | | | | 474 | 5.90 | 500 | 5.52 | | | 580 | 3.38 | 620 | 2.92 | | |
| ACF 1614 | 50–100 | 0.4 | 0 | 430 | 0.286 | | | 474 | 0.42 | 525 | 0.70 | | | | | 604 | 1.49 | | |
| | | | 10 | | | ... | ... | 468 | 0.82 | 525 | 0.70 | ... | ... | ... | ... | 600 | 0.66 | ... | ... |
| | | | 30 | | | | | 468 | 0.86 | 520 | 0.78 | | | | | | | | |
| ACF 1615 | 50–100 | 0.2 | 0 | 430 | 0.835 | | | 472 | 1.02 | 520 | 1.52 | | | 580 | 1.41 | 612 | 4.27 | 690 | 0.61 |
| | | | 10 | | | ... | ... | 470 | 1.75 | 520 | 1.52 | ... | ... | 570 | 0.945 | 605 | 1.46 | | |
| | | | 30 | | | | | 468 | 1.96 | 520 | 1.46 | | | | | 612 | 0.79 | | |
| ACF 1616 | 10,000 | 0.02 | 0 | 430 | 13.65 | | | 476 | 16.67 | 520 | 26.3 | 545 | 28.5 | 580 | 27.95 | 620 | 96.0 | 693 | 15.35 |
| | | | 10 | | | ... | ... | 476 | 26.20 | 520 | 32.7 | | | 580 | 22.70 | 620 | 37.85 | 690 | 11.10 |
| | | | 16 | | | | | 476 | 27.50 | 520 | 32.1 | | | 580 | 22.70 | 622 | 29.25 | 690 | 10.00 |
| | | | 30 | | | | | 470 | 27.60 | 520 | 28.6 | | | 570 | 18.5 | 623 | 21.30 | | |
| ACF 1617 | 30,000 | 0.02 | 0 | 430 | 9.5 | | | 475 | 11.9 | 520 | 16.0 | | | | | 620 | 57.5 | 690 | 11.2 |
| | | | 10 | | | ... | ... | 475 | 15.2 | 518 | 18.3 | ... | ... | 584 | 14.95 | 620 | 18.85 | 690 | 7.1 |
| | | | 30 | | | 455–75 | | 455–75 | 14.35 | 510 | 14.45 | | | 570 | 8.00 | 620 | 9.1 | | |
| ACF 1618 | 42,500 | 0.01 | 0 | 430 | 8.6 | | | 455 | 15.2 | 518 | 17.7 | | | 580 | 15.7 | 618 | 75.5 | 690 | 13.6 |
| | | | 10 | | | ... | ... | 455 | 15.2 | 512 | 17.1 | ... | ... | 580 | 8.1 | 622 | 20.8 | 690 | 7.4 |
| | | | 30 | | | | | | | | | | | | | 624 | 9.9 | | |
| ACF 1619 | 50,000 | 0.01 | 0 | 430 | 5.3 | | | 455–80 | 12.6 | 518 | 13.4 | | | 580 | 10.7 | 619 | 60.7 | 684 | 9.8 |
| | | | 10 | | | ... | ... | 455–80 | 11.6 | 516 | 16.4 | ... | ... | 570 | 5.3 | 623 | 14.9 | | |
| | | | 30 | | | | | | | | 12.6 | | | | | 623 | 6.8 | | |
| ACF 1620 | 75,000 | 0.01 | 0 | 430 | 10.4 | 455 | 17.8 | .... | ... | 520 | 20.5 | ... | ... | 580 | 20.6 | 617 | 81.5 | 693 | 14.8 |
| | | | 10 | 420 | 11.8 | | | | | 518 | 28.4 | | | 580 | 12.4 | 617 | 26.1 | | |
| | | | 33 | 420 | 15.5 | 455 | 22.4 | | | 510 | 20.5 | | | 580 | | 617 | 11.2 | | |
| $W_1$ D-598 | 275 | 0.2 | 0 | 430 | 0.345 | | | 480 | 0.39 | 520 | 0.490 | | | | | 606 | 0.96 | | |
| | | | 10 | 430 | | ... | ... | 470 | 0.54 | 520 | 0.490 | ... | ... | ... | ... | 602 | 0.45 | ... | ... |
| | | | 30 | 430 | 0.62 | | | 460 | 0.65 | 520 | 0.605 | | | | | | | | |
| $W_2$ D-599 | 800 | 0.1 | 0 | | | | | 476 | 0.80 | 526 | 1.04 | | | | | 608 | 1.86 | | |
| | | | 10 | | | ... | ... | 476 | 1.41 | 526 | 1.36 | ... | ... | ... | ... | 608 | 1.50 | ... | ... |
| | | | 30 | 420 | 1.36 | | | 476 | 1.38 | 526 | 1.27 | | | | | | | | |
| $W_3$ D-600 | 1,500 | 0.6 | 0 | | | | | 472 | 1.52 | 520 | 1.40 | | | | | 620 | 4.2 | | |
| | | | 10 | | | ... | ... | 468 | 1.79 | 520 | 1.31 | 545 | 1.21 | 580 | 1.06 | 620 | 1.31 | ... | ... |
| | | | 30 | 430 | 0.81 | | | 468 | 1.95 | 526 | 1.11 | | | 572 | 0.66 | 620 | 0.77 | | |
| $W_4$ D-601 | 3,500 | 0.05 | 0 | | | | | 460 | 0.86 | 510 | 1.24 | | | | | 614 | 3.06 | 690 | 0.58 |
| | | | 11 | ... | .... | ... | ... | 470 | 1.76 | 516 | 1.76 | ... | ... | 580 | 1.32 | 610 | 1.32 | | |
| | | | 30 | | | | | 460 | 1.62 | 510 | 1.58 | | | | | | | | |
| $W_5$ D-602 | 8,000 | 0.05 | 0 | 430 | 2.42 | | | 475 | 3.0 | 520 | 4.1 | | | 580 | 8.26 | 616 | 11.1 | 690 | 1.92 |
| | | | 10 | | | ... | ... | 473 | 4.64 | 520 | 4.56 | ... | ... | 580 | 4.32 | 616 | 4.88 | | |
| | | | 30 | | | | | 471 | 4.42 | 508 | 3.94 | | | 570 | 2.64 | 620 | 2.44 | | |
| $W_6$ D-603 | 18,000 | 0.05 | 0 | 430 | 3.76 | | | | | 514 | 5.60 | | | | | 615 | 9.36 | 690 | 3.08 |
| | | | 10 | | | 460 | 5.16 | 476 | 5.34 | 514 | 5.88 | ... | ... | 580 | 4.76 | 615 | 5.40 | | |
| | | | 30 | | | | | 470 | 5.26 | 506 | 5.20 | | | 572 | 2.94 | 620 | 2.68 | | |
| Cholesterol acetate pure | .... | ... | 0 | 430 | | | | 484 | | | | | | | | 614 | | | |
| | | | 10 | 428 | .... | ... | ... | 484 | ... | ... | ... | ... | ... | ... | ... | 614 | ... | ... | ... |
| | | | 30 | 428 | | | | 484 | | | | | | | | 614 | | | |

bands for a very pure sample of cholesterol acetate free from 7-dehydrocholesterol. The band at 690 mμ is now attributed (*2*, *4*, *9*) to vitamin $A_2$, the maxima at 620 and 580 mμ to vitamin $A_1$, the one at 500 to 520 mμ to vitamin D, and those at 480 and 430 mμ, respectively, to cholesterol. The maxima at 545 and 455 mμ, respectively, are not yet well understood. Cholesterol shows an additional maximum (see Figure 4) at 614 mμ which may interfere with the 620 mμ vitamin $A_1$ maximum. However, this maximum is rather weak and does not appear prominently during the early stages of the measurements.

In Table IV the potencies are calculated by three different methods from the extinction coefficients, using the CR-503 sample as the standard of reference. In the first method no correction was made. In the second method, however, a correction was made for the presence of sterols by subtracting from $E^{1\%}_{1\ cm.}$ at 500–520 mμ, using the 10-minute curve, one half the value of $E^{1\%}_{1\ cm.}$ at 480 mμ, using the 30-minute curve. This choice is based on the fact that the maximum of the cholesterol band develops much more slowly than that of the vitamin D band, and in some preliminary studies this maximum was found to be about twice that at 500 mμ.

In the third method a correction for the presence of vitamin A was introduced. This correction is not a simple factor but increases more rapidly than the concentration of vitamin A. A graphic method was finally used to obtain the correction which is plotted against the $E^{1\%}_{1\ cm.}$ values of the vitamin A maximum at 620 mμ, us-

TABLE IV. COMPARISON OF BIOLOGICAL WITH SPECTROSCOPIC POTENCIES

| Oil Sample | Biological Potency | Method I | | Method II | | Method III | |
|---|---|---|---|---|---|---|---|
| | | $E^{1\%}_{1\ cm.}$ | Potency | $E^{1\%}_{1\ cm.}$ | Potency | $E^{1\%}_{1\ cm.}$ | Potency |
| | *U. S. P. units/g.* | | *U. S. P. units/g.* | | *U. S. P. units/g.* | | *U. S. P. units/g.* |
| ACF-CR 503 | 50,000 | 17.77 | 50,000 | 10.42 | 50,000 | 9.39 | 50,000 |
| ACF-CR 726 | 18,000 | 6.16 | 17,450 | 3.21 | 15,300 | 3.03 | 16,100 |
| ACF-CR 1614 | 50–100 | 0.7 | 1,975 | 0.27 | 1,290 | 0.24 | 1,270 |
| ACF-CR 1615 | 50–100 | 1.52 | 4,300 | 0.54 | 2,590 | 0.49 | 2,600 |
| ACF-CR 1616 | 10,000 | 32.7 | 92,300 | 18.9 | 90,000 | 1.5 | 8,000 |
| ACF-CR 1617 | 30,000 | 18.3 | 51,700 | 11.13 | 53,400 | 10.0 | 53,200 |
| ACF-CR 1618 | 42,500 | 22.5 | 63,500 | 14.9 | 71,500 | 12.0 | 63,800 |
| ACF-CR 1619 | 50,000 | 16.4 | 46,300 | 10.6 | 50,900 | 9.5 | 50,500 |
| ACF-CR 1620 | 75,000 | 28.4 | 80,200 | 17.2 | 82,500 | 15.2 | 70,300 |
| $W_1$-598 | 275 | 0.49 | 1,379 | 0.17 | 815 | 0.15 | 800 |
| $W_2$-599 | 800 | 1.36 | 3,826 | 0.67 | 3,200 | 0.62 | 3,300 |
| $W_3$-600 | 1,500 | 1.31 | 3,680 | 0.33 | 1,580 | 0.29 | 1,540 |
| $W_4$-601 | 3,500 | 1.76 | 4,950 | 0.95 | 4,560 | 0.86 | 4,580 |
| $W_5$-602 | 8,000 | 4.56 | 12,800 | 2.35 | 11,270 | 2.0 | 10,650 |
| $W_6$-603 | 18,000 | 5.88 | 16,580 | 3.25 | 15,500 | 3.15 | 16,800 |

ing the zero-minute curve. The resulting curve is shown in Figure 5. The correction so obtained is subtracted from the $E^{1\%}_{1\ cm.}$ values obtained by the second method. The corrected $E^{1\%}_{1\ cm.}$ values together with the potencies obtained from these values are shown in the last two columns of Table IV.

A number of measurements were made on the nonsaponifiable fractions of several fish liver oils, but the results were less satisfactory than those given in Table IV.

## Maleic Anhydride Procedure

Although the foregoing method seems to give somewhat satisfactory results, at least with high-potency oils, it is not universally applicable, as it is necessary to have spectroscopic equipment to measure the visible region of the spectrum accurately and rapidly. It also depends upon a number of corrections which make the method cumbersome. In view of these objections the authors developed the maleic anhydride procedure in which most of the substances that interfere with the vitamin D band are removed.

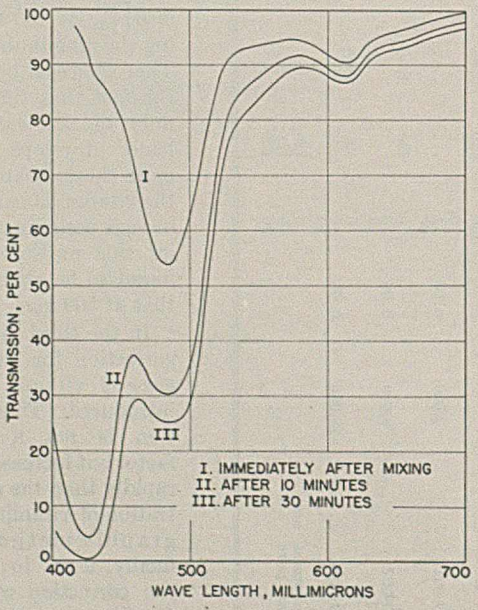


FIGURE 4. TRANSMISSION SPECTRUM OF ANTIMONY TRICHLORIDE COLOR OF PURE CHOLESTEROL ACETATE

A quantity of the fish liver oil in the neighborhood of 1 gram is accurately weighed and saponified in the usual manner. The nonsaponifiable matter is dried by distilling from it, under reduced pressure, an absolute alcohol–benzene mixture. To remove the greater portion of the sterols, the dried residue is then dissolved in about 5 cc. of pure methyl alcohol and the mixture is cooled to −10° to −15° C., then filtered at this temperature. The alcohol is completely removed from the filtrate, the residue is dissolved in about 5 cc. of freshly purified 1,4-dioxane (free from peroxides), and to it is added about 0.2 gram of maleic anhydride in about 2 cc. of dioxane. The maleic anhydride is more effective if it is freshly prepared by distilling in a moderate vacuum a 1 to 1.5 mixture of maleic acid and phosphorus pentoxide. The dioxane mixture is heated on the water bath for 1 hour, then cooled, 10 cc. of 0.5 *N* alcoholic potash are added, and it is allowed to stand at room temperature for 5 to 10 minutes. Fifteen cubic centimeters of water are then added and the mixture is extracted several times with pure ether; the ether extracts are washed twice with water, dried over anhydrous sodium sulfate, and filtered, and the ether is removed under reduced pressure.

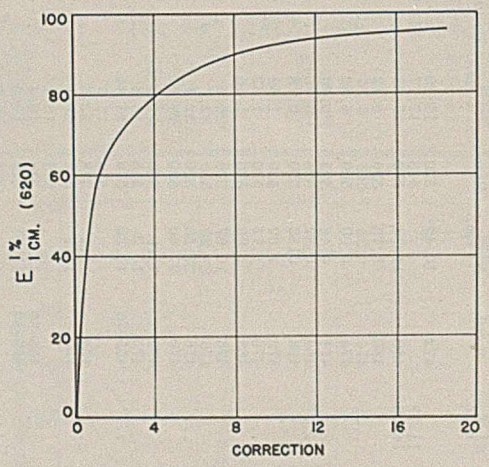


FIGURE 5. CORRECTION FOR PRESENCE OF VITAMIN A

Plotted against $E^{1\%}_{1cm.}$ at 620 mμ for 0-minute curve

Finally, the vitamin D in the residue is estimated spectrophotometrically, using the method described above.

The destruction of vitamin A by the maleic anhydride procedure is best illustrated by Figure 6. Three equal samples of fairly pure vitamin A were taken, two of which were heated on the water bath with maleic anhydride in dioxane in an atmosphere of nitrogen for 15 and 60 minutes, respectively. Transmission spectra were then taken of both the treated and untreated samples. The vitamin A absorption maximum was reached 3 and 10 minutes after mixing (lower and upper curves). It may be seen that 1-hour treatment with maleic anhydride destroys vitamin A almost completely, and the corresponding 10-minute curve, the standard time adopted for vitamin D measurements, shows almost zero vitamin A concentration. Figure 7 shows the 3- (I) and 10-minute (II) curves of one of the fish liver oils having moderate concentration of vitamin A after the treatment with maleic anhydride. Both curves show the complete absence of vitamin A.

Carotenoids having conjugated systems of double bonds and possibly 7-dehydrocholesterol are also removed by the maleic anhydride treatment. Although vitamin D contains a conjugated system of double bonds, it is known to react more sluggishly with maleic anhydride than its precursors (*17*).

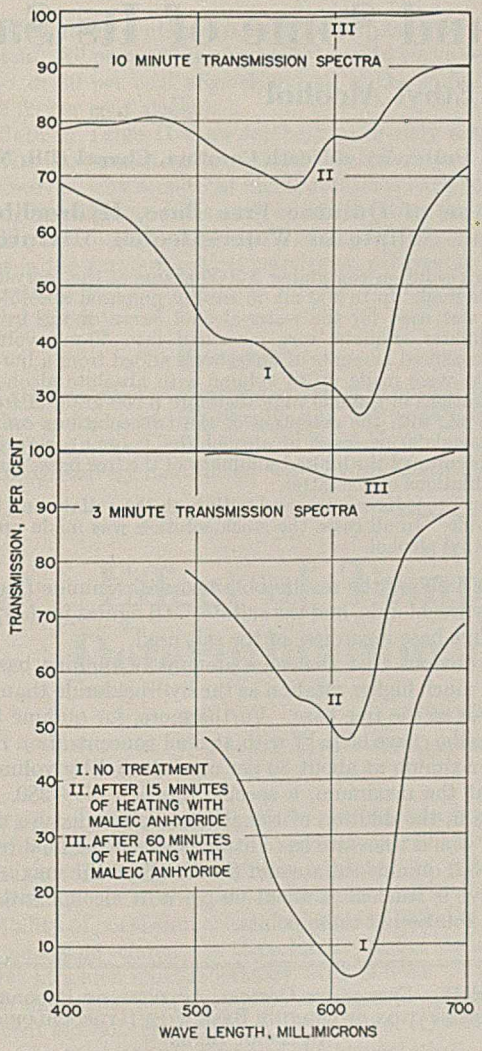


FIGURE 6. EFFECT OF MALEIC ANHYDRIDE ON PURE VITAMIN A

Table V shows the potencies and the $E^{1\%}_{1\,cm.}$ values at 500 mμ of six fish liver oils obtained by the maleic anhydride procedure. The potencies referred to calciferol are slightly higher than the biological potencies, while those referred to sample 1619 are in close agreement with the biological.

TABLE V. POTENCIES OBTAINED BY MALEIC ANHYDRIDE PROCEDURE

| Oil Sample | Biological Potency | $E^{1\%}_{1\,cm.}$ | Potency Referred to Calciferol | Potency Referred to 1619 |
|---|---|---|---|---|
| | *U. S. P. units/g.* | | *U. S. P. units/g.* | *U. S. P. units/g.* |
| 1616 | 10,000 | 0.235 | 10,700 | 9,550 |
| 1617 | 30,000 | 0.778 | 35,000 | 31,000 |
| 1618 | 42,500 | 1.067 | 48,000 | 44,000 |
| 1619 | 50,000 | 1.23 | 55,900 | 50,000 |
| 1620 | 75,000 | 1.775 | 80,000 | 72,100 |
| 1793 | 45,000 | 1.135 | 51,000 | 46,100 |

## Summary

Two independent physicochemical methods have been developed for the quantitative estimation of vitamin D in fish liver oils. One is based on the spectrophotometric estimation of $E^{1\%}_{1\,cm.}$ values at 500 to 520 mμ of the antimony trichloride color of various fish liver oils. Corrections have been made for the presence of sterols and vitamin A, both of which seem to interfere with the vitamin D absorption band.

The second method is based on a chemical treatment of the nonsaponifiable fraction of fish liver oils with maleic anhydride to destroy vitamin A, carotenoids, and possibly 7-dehydrocholesterol. The vitamin D in the treated nonsaponifiable portions is then estimated spectrophotometrically as in the first method. The results obtained seem to be in fair agreement with the biological.

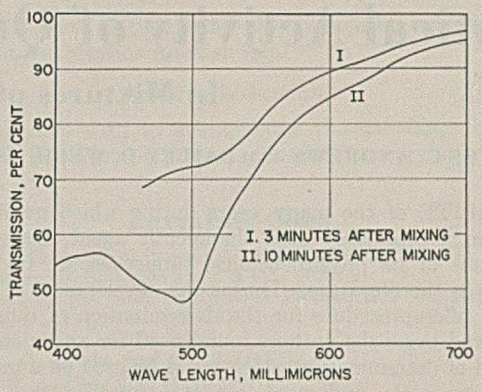


FIGURE 7. EFFECT OF MALEIC ANHYDRIDE ON VITAMIN A CONCENTRATION OF NONSAPONIFIABLE FRACTION OF A FISH LIVER OIL

## Literature Cited

(1) Brockmann and Chen, *Z. physiol. Chem.*, 241, 129 (1936).
(2) Edisbury, Morton, and Simpkins, *Nature*, 140, 234 (1937).
(3) Emmerie and van Eekelen, *Acta Brevia Neerland. Physiol. Pharmacol. Microbiol.*, 6, Nos. 9–10, 133 (1936).
(4) Gillam *et al.*, *Nature*, 140, 233 (1937).
(5) Halden, *Naturwissenschaften*, 24, 296 (1936).
(6) Halden and Tzoni, *Nature*, 137, 909 (1936).
(7) Hardy, *J. Optical Soc. Am.*, 25, 305 (1935).
(8) Heggie and Milas, paper presented before the American Chemical Society, Chapel Hill, N. C., April, 1937.
(9) Lederer and Rosanova, *Biokhimiya*, 2, 293 (1937).
(10) Milas, U. S. Patent 2,173,629 (Sept. 19, 1939).
(11) Milas and Heggie, paper presented before the American Chemical Society, Chapel Hill, N. C., April, 1937.
(12) Raoul and Meunier, *Compt. rend.*, 209, 546 (1939).
(13) Ritsert, *Merck's Jahresber.*, 52, 27 (1938).
(14) Russell, paper presented before Special Research Conferences of American Association for the Advancement of Science, Gibson Island, July, 1940.
(15) Solyanikova, *Biokhimiya*, 4, 482 (1939).
(16) Tzoni, *Biochem. Z.*, 287, 18 (1936).
(17) Windaus, Lüttringhaus, and Deppe, *Ann.*, 489, 262 (1931).

PRESENTED in part before the Special Research Conferences, Chemistry Section of the American Association for the Advancement of Science, Gibson Island, July, 1940. Contribution from the Research Laboratory of Organic Chemistry, Massachusetts Institute of Technology, No. 235.

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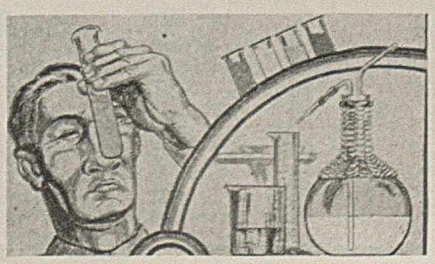

# Optical Activity of Quinine and Some of Its Salts

## In Mixtures of Water and Ethyl Alcohol

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IN SPITE of the many years during which quinine has been a commonly used therapeutic agent, exact determinations of its physicochemical properties are frequently lacking in the literature. During the development of an improved microprocedure for the determination of quinine in blood and urine the authors found need for an independent method of determination which could be used on a macro or semimicro scale and used for this purpose the optical activity of quinine or its salts. The use of such a procedure on a semimicro scale has necessitated a search for those conditions under which the highest optical activity could be attained.

The bibliographies provided by Allen (*1*), Schmidt and Grafe (*8*), and other books on alkaloids include many references to the optical activity of quinine and its salts but with the partial exception of the work of Oudemans (*7*), systematic studies of the effect of progressive neutralization of the free base, the effect of progressive change of solvent (ethyl alcohol, water), etc., under exact temperature control and with highly purified samples are largely lacking. Some data of this sort were reported by Hesse (*3*, *4*) and, more recently, by Schoorl (*9*), Dietzel and Soellner (*2*) and Lapp (*5*). The data in the present paper are not in any essential way in contradiction with those of the above authors but constitute an amplification and a partial confirmation of their work. Oudemans' determinations, while the most complete in the literature, frequently fail to cover the range desired for present purposes.

All determinations of optical activity were carried out at 25° ± 0.5° C. using a Schmidt and Haensch half-shadow polarimeter reading to ±0.01°, an electric sodium lamp providing practically monochromatic D light, and 4-dm. tubes.

Quinine as the free base was prepared by decomposition of either hydrochloride or sulfate with excess ammonia and crystallization at 0° C. from the water solution. Samples of the free base prepared from both salts gave identical optical activities.

Quinine dihydrochloride was prepared by recrystallization from absolute alcohol. The resulting addition compound with alcohol was then decomposed by drying at room temperature *in vacuo* and then briefly at 110° C. This product gave rotation values practically identical with those of the U. S. P. product dried *in vacuo* over phosphorus pentoxide at room temperature.

Quinine sulfate was prepared by several successive recrystallizations of the U. S. P. product from hot water and the product was dried to constant weight *in vacuo* over phosphorus pentoxide. Constancy of optical activity was used as the criterion of complete purification with both the free base and its salts. In all the following data, specific rotations are uniformly expressed in terms of the free base.

TABLE I. SPECIFIC ROTATION OF QUININE FREE BASE, QUININE DIHYDROCHLORIDE, AND QUININE SULFATE

(In varying percentages of water and alcohol)

| Alcohol (by Volume) % | Quinine Free Base $-[\alpha]_D^{25}$ | Quinine Sulfate $-[\alpha]_D^{25}$ | Quinine Dihydrochloride $-[\alpha]_D^{25}$ |
|---|---|---|---|
| 0 | ... | 176 | 270 |
| 6.16 | ... | ... | 272 |
| 8.60 | ... | 187 | ... |
| 10.0 | 120 | ... | ... |
| 18.5 | ... | 202 | ... |
| 20.0 | 138 | ... | 277 |
| 30.0 | 155 | 218 | 280 |
| 40.0 | 171 | 225 | 277 |
| 50.0 | 179 | 233 | 273 |
| 60.0 | 181 | 237 | 267 |
| 70.0 | 180 | 233 | 252 |
| 80.0 | 178 | 221 | 240 |
| 90.0 | 172 | 214 | 222 |
| 100.0 | 168 | 206 | 207 |

### Rotation of Quinine Free Base, Hydrochloride, and Sulfate in Water-Alcohol Mixtures

A stock solution containing 2.5000 grams of the recrystallized free base made up to 100 ml. in freshly prepared absolute ethyl alcohol was used for the water-alcohol curve of the free base. Five-milliliter aliquots were measured into 50-ml. volumetric flasks, measured amounts of water were added from a buret, and the flasks were made up to volume with absolute alcohol. All concentrations of quinine were therefore 0.250 gram of free base per 100 ml. with the exception of the two solutions containing only 10 and 20 per cent of alcohol (by volume), respectively. These, because of the limited solubility of the free base, contained higher dilutions of the latter.

Similar procedures were used with both the sulfate and the hydrochloride. In all cases the stock solution was made up in absolute ethyl alcohol.

Table I shows the results obtained with quinine free base, the dihydrochloride, and the sulfate. All figures for $[\alpha]_D^{25}$ refer to the free base regardless of the salt used.

It is obvious that the same amount of quinine base produces a much higher rotation as the hydrochloride than as the sulfate or as the free base. Furthermore, for quinine hydrochloride the curve of $[\alpha]_D^{25}$ with alcohol concentration reaches a flat maximum at about 30 per cent alcohol by volume and gives, at the maximum, a specific rotation of −280. Inaccuracies in the dilution of the alcohol-water mixture used as solvent would therefore have little effect on the final reading. With both quinine sulfate and the free base the maximum of the curve is reached at about 60 per cent alcohol with lower specific rotation at these points.

TABLE II. EFFECT ON OPTICAL ACTIVITY OF PROGRESSIVE NEUTRALIZATION OF QUININE BASE WITH HYDROCHLORIC AND SULFURIC ACIDS

| Hydrochloric Acid Molar ratio of HCl to quinine | $-[\alpha]_D^{25}$ | Sulfuric Acid Molar ratio of $H_2SO_4$ to quinine | $-[\alpha]_D^{25}$ |
|---|---|---|---|
| 0 | 153 | 0 | 177 |
| 0.258 | 175 | 0.028 | 184 |
| 0.516 | 198 | 0.141 | 200 |
| 0.774 | 216 | 0.283 | 217 |
| 1.032 | 236 | 0.424 | 234 |
| 1.290 | 252 | 0.565 | 246 |
| 1.548 | 270 | 0.706 | 252 |
| 1.806 | 278 | 0.848 | 256 |
| 2.064 | 283 | 0.989 | 257 |
| 2.322 | 286 | 1.130 | 258 |
| 2.580 | 289 | | |
| 2.838 | 290 | | |

### Neutralization Curves of Quinine Base with Sulfuric and Hydrochloric Acids

To determine the effect of progressive neutralization of the free base, fixed amounts were neutralized by addition of varying amounts of standard acid (hydrochloric or sulfuric). These solutions were so prepared as to contain that proportion of water and ethyl alcohol which was shown in Table I to give the maximum specific rotation of their respective salts (30 per cent alcohol for the hydrochloride and 60 per cent alcohol for the sulfate). The extent of neutralization is indicated by the molar ratio of acid to quinine. This ratio is obviously 0.5 for the sulfate and 2.0 for the hydrochloride in the case of the purified salts used in the measurements of Table I.

Table II shows the data obtained expressed as the specific rotation (as free base) for various molar ratios of acid to qui-

nine. All solutions contained 0.250 gram of quinine per 100 ml. of solution. All points of the hydrochloric acid curve were determined in 30 per cent alcohol and all of the sulfuric acid curve in 60 per cent alcohol.

The data in Table II show for both acids only a smooth curve with no breaks at any stoichiometric ratios. They show, however, why somewhat higher rotations have been obtained when excess acid has been added to either the sulfate or the dihydrochloride. The report of Liquier (*6*) concerning plateaus in the curve of optical activity *vs.* pH corresponding to the neutral and basic quinine sulfates has not been confirmed by the authors.

## Summary

Data are presented to show the variation in optical activity of quinine as free base, dihydrochloride, and sulfate in mixtures of water and ethyl alcohol, and the variation as the free base is progressively neutralized with hydrochloric and sulfuric acids, each in that water-alcohol solution which gives the maximum rotation for each salt.

## Acknowledgment

The authors wish to acknowledge the assistance of the Samuel S. Fels Fund in providing means for carrying out the work.

## Literature Cited

(1) Allen, "Commercial Organic Analysis", 5th ed., Vol. VII, Philadelphia, P. Blakiston's Son & Co., 1929.
(2) Dietzel, R., and Soellner, K., *Arch. Pharm.*, 268, 629 (1930).
(3) Hesse, O., *Ann.*, 176, 205 (1875).
(4) Hesse, O., *Ber.*, 4, 693 (1871).
(5) Lapp, C., *Compt. rend*, 201, 80 (1935).
(6) Liquier, J., *Ibid.*, 183, 195 (1926).
(7) Oudemans, A. C., *Ann.*, 182, 33 (1876).
(8) Schmidt, J., and Grafe, V., "Alkaloide", Berlin, Urban und Schwartzenberg, 1920.
(9) Schoorl, N., *Pharm. Weekblad*, 63, 469 (1926)

# Effect of Temperature of Alcohol in Determination of Potash in Fertilizers

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FOR a number of years the referee on potash in fertilizers of the Association of Official Agricultural Chemists has recommended investigation of the solubility of potassium chloroplatinate in alcohol and acid-alcohol. Pierrat gives the solubility of potassium chloroplatinate in alcohol at 14° C. (*3*) but makes no reference to the solubility at the higher temperatures at which most laboratory work is done. Allen reports on the greater solubility of potassium chloroplatinate in 80 per cent alcohol than in 95 per cent alcohol (*1*) but makes no reference to the temperature of the alcohol used. Archibald, Wilcox, and Buckley give the solubility of potassium chloroplatinate in alcohol-water mixtures of various alcohols at 20° C. (*2*) but do not mention the solubility in acid-alcohol. This work, while important in itself, has not been inclusive enough to encompass the conditions existing in the determination of potash by the official method, as, for example, the effect of temperature on the solubility of potassium chloroplatinate in alcohol and acid-alcohol.

During the summer of 1939 a large number of low-analysis potash fertilizers analyzed in the authors' laboratory were found to be running below guarantee. The daily temperatures during this period were unusually high but after cooling the alcohol to about 18° C. the results were from 0.1 to 0.3 per cent higher. Since a rise of about 8° C. was noted upon addition of concentrated hydrochloric acid to alcohol (when added at the rate of 0.6 ml. of hydrochloric acid to 6 ml. of alcohol) and the temperature remained above room temperature for the 15-minute extraction period, it was found advisable to mix the acid and alcohol and cool before adding it to the potassium chloroplatinate. Subsequently the effect of temperature on the solubility of potassium chloroplatinate in acid alcohol was studied. Known concentrations of pure potassium chloride were used for samples in place of commercial fertilizers in order to avoid other sources of error in the potash determination.

Table I. Loss of Potassium Chloroplatinate by Extraction with Acid-Alcohol

(0.1-gram portions of $K_2PtCl_6$ used)

| Treatment | $K_2O$ Lost 18° C. | 38° C. | 48° C. |
|---|---|---|---|
| | *Mg.* | *Mg.* | *Mg.* |
| Mixed acid-alcohol | 5.1 | 8.2 | 10.8 |
| Acid and alcohol separate | 7.6 | 9.7 | 11.6 |

## Procedure

The study is divided into two steps.

1. Extraction of weighed amounts of pure potassium chloroplatinate with definite volumes of acid and alcohol under controlled temperature conditions. Tenth-gram portions of pure potassium chloroplatinate were transferred to 250-ml. beakers. To these were added 137.5 ml. of acid alcohol at a definite temperature for the mixed acid-alcohol determinations and 125 ml. of alcohol and 12.5 ml. of concentrated hydrochloric acid for the determinations in which the acid and alcohol were added separately. The mixtures were stirred for 15 minutes at controlled temperatures. Finally the determinations were filtered into sintered-glass crucibles and washed with 137.5 ml. more of alcohol at corresponding temperatures. Table I gives the amounts of potassium chloroplatinate lost by extraction.

2. Determination of the effect of the temperature of acid-alcohol on the solubility of potassium chloroplatinate precipitated from two concentrations of pure potassium chloride. Two concentrations of potassium chloride were used. The first contained 6.25 grams of potassium chloride per liter, made to volume at 4° C. and kept at that temperature till used. The theoretical value of this solution is 0.3948 per cent $K_2O$. The second solution contained 12.5 grams of potassium chloride per liter and the theoretical $K_2O$ value for this solution is 0.7896 per cent.

Aliquots of the above concentrations (25 ml.) were measured into platinum dishes, measured amounts of chloroplatinic acid were added, and the solutions were evaporated to a thick paste on the steam bath. The dishes were then removed and to each dish were added 6 ml. of 83 per cent alcohol and 0.6 ml. of concentrated hydrochloric acid for the determinations in which the acid and alcohol were added separately or a mixture of 6 ml. of alcohol and 0.6 ml. of acid for the mixed acid-alcohol determinations. The samples were extracted for 15 minutes at 18° or at 38° C. The temperatures in each case were controlled by a constant-temperature bath. At the end of the 15-minute extraction, the samples were washed with 125 ml. more of 83 per cent alcohol adjusted to the corresponding temperature. It was estimated that 125 ml. of alcohol were normally used in the potash deter-

mination previous to the ammonium chloride washing, which was omitted in this case as no magnesium salts were present in the determinations.

All filtrations were made in the same set of sintered-glass crucibles (Jena B. G. 3). Actual potash results were determined by difference (the potassium chloroplatinate being dissolved out and the crucibles weighed back). Table II gives the solubility loss of potassium chloroplatinate at different temperatures.

TABLE II. SOLUBILITY LOSS OF POTASSIUM CHLOROPLATINATE

(Calculated as per cent $K_2O$ in acid-alcohol at 18° and 38° C.)

| Treatment | No. of Analyses Averaged | $K_2O$ Theoretical Value | At 18° C. $K_2O$ found | At 18° C. $K_2O$ lost | At 38° C. $K_2O$ found | At 38° C. $K_2O$ lost |
|---|---|---|---|---|---|---|
| | | % | % | % | % | % |
| Mixed acid-alcohol | 50 | 0.7896 | 0.7851 | 0.57 | 0.7760 | 1.72 |
| Acid and alcohol separate | 50 | 0.7896 | 0.7794 | 1.29 | 0.7640 | 3.24 |
| Mixed acid-alcohol | 50 | 0.3948 | 0.3907 | 1.04 | 0.3848 | 2.53 |
| Acid and alcohol separate | 50 | 0.3948 | 0.3852 | 2.43 | 0.3783 | 4.18 |

## Discussion of Results

Tables I and II indicate that a rise in temperature of the alcohol is accompanied by an increase in solubility of the potassium chloroplatinate. Although the differences in the amount of potassium chloroplatinate extracted when the acid and alcohol were added separately and when the mixed acid-alcohol was added are small, there is a significantly greater solubility with the former. This may be due to the increased temperature resulting from the mixing of the acid and alcohol.

When alcohol and acid are mixed in the proportions outlined in the official method for the determination of potash in fertilizers, there is a sharp rise in temperature of about 8° C. which does not entirely disappear at the end of the 15-minute extraction period. This indicates that it is advisable to mix the acid and alcohol and to control the temperature during the 15-minute extraction.

The percentage losses in Table II are greater at the lower concentration of $K_2O$. This may be due to the solvent action of the alcohol on the smaller crystals of potassium chloroplatinate which are formed in the lower concentration of $K_2O$. The small crystals present greater surface area than the larger crystals formed with the higher concentrations and therefore have a greater solubility.

## Conclusions

There is a definite increase in temperature when the acid is added to the alcohol, as outlined in the A. O. A. C. method for the determination of potash in fertilizers, which gives an error particularly under summer laboratory conditions. This can be prevented by mixing the acid and alcohol beforehand. In order to eliminate the effect of temperature it is advisable to cool the acid-alcohol mixture as well as the wash alcohol before using.

## Literature Cited

(1) Allen, H. R., *J. Assoc. Official Agr. Chem.*, 22, 162–7 (1939).
(2) Archibald, E. H., Wilcox, W. G., and Buckley, B. G., *J. Am. Chem. Soc.*, 30, 747–60 (1908).
(3) Pierrat, M., *Compt. rend.*, 172, 1041–3 (1921).

PRESENTED before the Division of Fertilizer Chemistry at the 100th Meeting of the American Chemical Society, Detroit, Mich.

# The Reducing Properties of *l*-Sorbose

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WITH the current commercial use of *l*-sorbose it seemed desirable to determine its reducing property by one of the standard methods and to compare this property with that of the other available ketohexose, fructose.

The *l*-sorbose was prepared by the method of Fulmer and others (*1*) and yielded eight-sided crystals in the orthorhombic system. The sugar was not fermented by *Saccharomyces cerevisiae* (a commercial baking strain and one isolated from cheese) nor by two strains of Torula. The product lost no weight upon heating for 2 hours in a vacuum oven at 70° C. Various samples melted with darkening from 162.4° to 164.2°, with a mean value of 163.5° (corrected), and the osazone melted at 163° (corrected). A 6 per cent solution showed $[\alpha]_D^{20} = -42.9°$ when corrected for temperature and concentration by the formula of Smith and Tollens (*4*). A recent value by Pigman and Isbell (*3*) records $[\alpha]_D^{20} = -43.4°$ for a 12 per cent solution.

TABLE I. CERIC SULFATE EQUIVALENTS AT VARYING CONCENTRATIONS OF SORBOSE

| Sorbose Concentrations $x$ | | 0.02 $N$ Ceric Sulfate, $y$ | Factor, $x/y$ |
|---|---|---|---|
| *Mg./5 ml.* | % | *Ml.* | |
| 0.05 | 0.001 | 0.20 | 0.250 |
| 0.10 | 0.002 | 0.19 | 0.526 |
| 0.50 | 0.01 | 1.21 | 0.413 |
| 1.00 | 0.02 | 2.46 | 0.407 |
| 5.00 | 0.10 | 11.40 | 0.439 |
| 10.00 | 0.20 | 20.29 | 0.493 |
| 15.00 | 0.30 | 28.47 | 0.527 |
| 20.00 | 0.40 | 36.46 | 0.549 |
| 25.00 | 0.50 | 43.94 | 0.569 |
| 30.00 | 0.60 | 51.18 | 0.586 |
| 35.00 | 0.70 | 58.42 | 0.599 |

TABLE II. CERIC SULFATE EQUIVALENTS AT VARYING CONCENTRATIONS OF FRUCTOSE

| Fructose Concentration $x$ | | 0.02 $N$ Ceric Sulfate, $y$ | Factor, $x/y$ | Fructose / Sorbose |
|---|---|---|---|---|
| *Mg./5 ml.* | % | *Ml.* | | |
| 1.00 | 0.02 | 2.77 | 0.361 | 1.13 |
| 5.00 | 0.10 | 12.24 | 0.408 | 1.07 |
| 10.00 | 0.20 | 21.66 | 0.462 | 1.08 |
| 15.00 | 0.30 | 30.88 | 0.486 | 1.08 |
| 20.00 | 0.40 | 40.01 | 0.500 | 1.10 |

The reducing power was determined by the method of Hildebrand and McClellan (*2*). In each case the desired quantity of the sugar was contained in 5 ml. of solution which was allowed to react with 10 ml. of the alkaline ferricyanide solution. The values are corrected for the blank which was determined daily and never exceeded 0.7 ml. of a 0.01773 $N$ ceric sulfate solution. All data are recorded as equivalents of 0.02 $N$ ceric sulfate and represent the mean of at least triplicate determinations.

In a similar way the equivalent reducing power of a sample of Pfanstiehl's C. P. special fructose was determined for comparative purposes. The data are recorded in Table II, where the last column indicates the relative reducing properties of the two ketosugars.

The data indicate that the direct titration with ceric sulfate of the formed ferrocyanide is satisfactory for sorbose in the concentration range of from 0.01 to 0.70 per cent. At concentrations below this range the accuracy is considerably reduced, and at higher concentrations the ferricyanide is completely reduced.

The results obtained in comparing the reducing properties of fructose and sorbose bear out the hypothesis of Sobotka and Reiner (*5*) that the configuration about the third and fourth carbon atoms of the hexose sugars is the important factor in determining the reducing properties, and that, if the configurations of the hydroxyl groups on carbon atoms 3 and 4 of two sugars are similar, these sugars will have approximately the same reducing properties. Fructose and sorbose have the same configuration on these carbon atoms and their reducing powers are of the same order of magnitude.

## Literature Cited

(1) Fulmer, E. I., Dunning, J. W., Guyman, J. F., and Underkofler, L. A., *J. Am. Chem. Soc.*, 58, 1012 (1936).
(2) Hildebrand, F. C., and McClellan, B. A., *Cereal Chem.*, 15, 107 (1938).
(3) Pigman, W. W., and Isbell, H. S., *J. Research Natl. Bur. Standards*, 19, 443 (1937).
(4) Smith, R. H., and Tollens, B., *Ber.*, 33, 1285 (1900).
(5) Sobotka, H., and Reiner, M., *Biochem. J.*, 24, 394 (1930).

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# Determining Copper and Nickel in Aluminum Alloys

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IN CONJUNCTION with the spectrographic analysis of aluminum alloys, this laboratory performs the routine chemical determination of copper in a large number and variety of aluminum alloys in which the copper concentration varies from 0.2 to about 10 per cent. The determination of nickel in aluminum alloys in amounts up to 2.5 per cent is also required.

The commonly used methods for copper (*2*, *4*, *5*) call for solution of the alloy in caustic solution and subsequent filtration. This leaves copper with other caustic-insoluble material on the filter, from which it is then removed with hot dilute nitric acid and determined by electrodeposition. This procedure serves only to separate the alloying elements (except zinc) from the bulk of the aluminum, and as commonly employed involves the danger of loss of material by spray in the sometimes violent reaction which occurs when the precipitate is washed from the filter with hot acid.

The present work was undertaken in an attempt to find suitable conditions for the direct electrodeposition of copper without preliminary separation of the bulk of the aluminum. Since aluminum alloys dissolve very slowly in acids other than hydrochloric and since hydrochloric acid is undesirable because it must be removed before electrolysis, the procedure used involved solution of the alloy in caustic solution, and, after dilution, addition of an excess of nitric acid. This method has found use in this laboratory in other cases where chlorides are objectionable—for instance, in the determination of manganese it is much to be preferred to the very slow method of dissolving in a mixture of nitric and sulfuric acids. This treatment gives complete solution of the alloy, except that with high-silicon alloys some silicon remains undissolved. It was found that this introduced no error in the determination of copper. If appreciable quantities of tin or other elements which interfere with the electrodeposition of copper are present, this method may not be used.

This method, since it cuts manipulation to a minimum, reduces the time required for a complete determination to less than one third of that required by the longer method. For a batch of twelve, the time elapsed between the completion of weighing the samples and the point at which they are ready to be electrolyzed is no more than 30 minutes, as compared with 1.5 to 2 hours required by the longer method. The sources of error due to incomplete solution of copper from the filter paper and the possibility of loss by spray are eliminated and the method has the further desirable feature of being carried out completely in a single vessel.

After deposition of the copper, the electrolyte can be used for the determination of nickel.

The method has been in continued use in this laboratory for some months and has proved satisfactory for routine analysis.

Table I. Determination of Copper and Nickel[a]

| Alloy No. | Alloying Elements | Copper Found | | Nickel Found | |
|---|---|---|---|---|---|
| | | Short method | Long method | Short method | Referee method |
| | % | % | % | % | % |
| 1 | 1.5 Zn, 1.5 Fe | 7.68 | 7.67 | .. | .. |
| 2 | 0.5 Mn, 0.5 Mg | 4.07 | 3.97 | .. | .. |
| 3 | 0.5 Mn, 1.5 Mg | 4.68 | 4.55 | .. | .. |
| 4 | 0.8 Mn, 0.8 Si | 4.58 | 4.54 | .. | .. |
| 5 | 0.2 Mg, 1.2 Fe | 9.84 | 9.78 | .. | .. |
| 6 | 0.8 Si, 0.6 Fe | 4.43 | 4.31 | .. | .. |
| 7 | 1.5 Si, 1.2 Fe | 7.18 | 7.16 | .. | .. |
| 8 | 12.0 Si, 0.8 Fe, 1.0 Mg | 0.73 | 0.76 | 2.38 | 2.43 |
| 9 | 1.5 Mg | 3.86 | 3.86 | 1.75 | 1.71 |
| 10 | 0.9 Si | 5.77 | 5.72 | 0.79 | 0.81 |
| 11 | 5.0 Si, 0.5 Mg | 1.34 | 1.29 | .. | .. |
| 12 | 2.5 Si | 4.45 | 4.42 | .. | .. |

[a] All values except referee nickel values are averages of two or more determinations.

## Procedures

Copper. A 1-gram sample is transferred to a 200-ml. electrolytic-type beaker and treated with 15 ml. of 20 per cent sodium hydroxide. If the reaction is too vigorous, water is added. After the reaction subsides, the beaker is heated on the steam bath until the reaction is completed, as indicated by the cessation of effervescence. The solution is then diluted to 100 ml. with hot water and stirred thoroughly, and 30 ml. of 1 to 1 nitric acid are added. This causes precipitation of gelatinous aluminum hydroxide, which redissolves on stirring and warming on the steam bath. Then 2 ml. of 1 to 1 sulfuric acid are added, the solution is stirred, and after cooling to room temperature is electrolyzed at 2 to 3 amperes, using a gentle air stream as a stirring device. A platinum gauze cathode 45 mm. in diameter and 50 mm. in height is used.

If the acid concentration is not sufficiently high, a white deposit of aluminum hydroxide will adhere to the copper deposit. This will be avoided if the quantities recommended above are used.

Nickel. The electrolyte from the copper determination is transferred to a 400-ml. beaker and 25 ml. of 25 per cent tartaric acid are added. The solution is then made ammoniacal and warmed on the steam bath. In order to facilitate the removal of silica, 5 ml. of a 5 per cent zinc chloride solution are added and the solution is stirred and warmed further. It is then filtered into a 600-ml. beaker and the residue is washed with hot water. The filtrate is made slightly acid with dilute hydrochloric acid and 20 ml. of 1 per cent alcoholic dimethylglyoxime solution are added (for samples containing over 2 per cent of nickel 5 ml. more are used for each additional 1 per cent). Dilute ammonia is then

added while stirring until a slight excess is present. The mixture is warmed on the steam bath for an hour, filtered onto a weighed Gooch or sintered-glass crucible, washed with hot water and once with methanol, and dried at 110°.

## Results

To check the method twelve different types of aluminum alloys were analyzed for copper by the procedure herein described and by the longer method involving separation from aluminum (*2*). Three were nickel-bearing and were analyzed for nickel both by the procedure here described and by a referee method (*3*). The results are shown in Table I.

The first alloy listed (No. 1) is National Bureau of Standards light aluminum alloy standard sample No. 86, the copper value of which is reported as 7.66 per cent.

## Discussion

The values for copper obtained by the shorter method are in most cases slightly higher than those obtained by the long method. Hence, to check on the accuracy of the shorter method, in ten determinations the deposited copper was dissolved from the electrodes and redeposited. In all cases the weights of copper changed by no more than 0.5 mg. and the average change was 0.3 mg. As this change is of the same order of magnitude as the experimental error, it is believed that the difference in the values obtained by the two methods is due to the slight solubility of copper in the alkali used in making the aluminum separation in the longer method (*1*).

To demonstrate this, the filtrates from the treatment of 1-gram samples of alloys 5 and 11 (Table I) by the longer procedure (*2*) were concentrated to 35 ml. and were then analyzed spectrographically by comparison with standards. This examination showed that the filtrate from alloy 5 contained between 0.5 and 1.0 mg. of copper, and the filtrate from alloy 11 contained about 1.0 mg. of copper. A blank treated in the same way showed no appreciable amount of copper. Since alloy 5 contains more than seven times as much copper as alloy 11, it is indicated that the amount of copper passing into the alkali filtrate is not a function of the copper content of the alloy but may depend on other factors, such as the amounts of other alloying elements present, the physical subdivision of the sample, and the length of time of treatment prior to filtration.

## Literature Cited

(1) Aluminum Co. of America, New Kensington, Penna., "Chemical Analysis of Aluminum", p. 20, 1941.
(2) *Ibid.*, p. 36.
(3) *Ibid.*, p. 59.
(4) Aluminum Research Institute, Chicago, Ill., "Methods of Aluminum Research Institute", p. 9, 1939.
(5) Am. Soc. Testing Materials, Philadephia, Penna., "A. S. T. M. Methods of Chemical Analysis of Metals", p. 93, 1939.

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# Simplification of the Petering-Wolman-Hibbard Method for Determination of Chlorophyll and Carotene

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IN VIEW of the interest shown in the method for the determination of chlorophyll and carotene in green plant tissue recently published by Petering, Wolman, and Hibbard (*3*) it seems advisable to make generally available a subsequent simplification of the barium hydroxide technique for removing chlorophyll from the acetone extracts of plant tissue.

Instead of adding the solid barium hydroxide octahydrate reagent to the aqueous-acetone extract, a saturated solution of barium hydroxide is added to the pure acetone extracts in suitable amount to remove all of the chlorophyll, and this mixture is then treated as directed in the original procedure. This technique eliminates the necessity of preparing an active solid reagent from anhydrous barium hydroxide, of having to handle finely divided barium hydroxide octahydrate, or of keeping the solid reagent free from carbonate. During the preparation of the solution the carbonate is removed because of its insolubility, and the addition of dissolved barium hydroxide to the acetone extract produces a very finely divided precipitate of barium hydroxide octahydrate which is extremely active.

This technique has been extensively used by Petering, Morgal, and Miller (*2*) in their work on the isolation of carotene from green plant tissue and its possible use in the analytical procedure has been suggested by Benne, Wolman, Hibbard, and Miller (*1*). Peterson (*4*) recently has pointed out that this general technique is satisfactory. However, no specific directions have been given which could be easily followed in an analytical method.

The following procedure is therefore suggested: Five grams of fresh plant tissue (or 1 gram of dried tissue) are ground and extracted with pure acetone and the extract is brought to a volume of 200 ml. A 100-ml. aliquot is taken and to it are added 15 ml. of saturated solution of barium hydroxide. This mixture is then refluxed for 30 minutes as in the original procedure, which results in the complete removal of chlorophyll from the extract.

Fifteen milliliters of a solution of barium hydroxide saturated at room temperature contain about 0.60 gram of barium hydroxide, which according to the data of Petering, Morgal, and Miller is sufficient to remove about 100 mg. of chlorophyll from an 85 per cent aqueous-acetone extract. Since 5 grams of fresh, highly pigmented plant tissue contain about 20 mg. of chlorophyll, it is obvious that the amount of barium hydroxide in the volume of solution recommended is adequate for its complete removal from the extract. This procedure, furthermore, automatically takes care of the water-acetone ratio which has been found very important to the efficiency of the barium hydroxide technique (*2*).

## Literature Cited

(1) Benne, E. J., Wolman, W., Hibbard, R. P., and Miller, E. J., *J. Assoc. Official Agr. Chem.*, 23, 709 (1940).
(2) Petering, H. G., Morgal, P. W., and Miller, E. J., IND. ENG. CHEM., 32, 1407 (1940).
(3) Petering, H. G., Wolman, W., and Hibbard, R. P., *Ibid.*, Anal. Ed., 12, 148 (1940).
(4) Peterson, W. J., Abstracts of the Biological Division, American Chemical Society, 100th Meeting, Detroit, Mich., September, 1940.

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# Removal of Thiocyanate in the Detection of the Halides

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MANY methods have been proposed for the removal of thiocyanate, which is the most serious interference in the detection of the halides.

Sneed and Duschak (*8*) destroy the thiocyanate by igniting the silver halide precipitate and then fusing it with a mixture of sodium carbonate and potassium carbonate. Curtman and Schneiderman (*1*) also destroy the thiocyanate by igniting the silver precipitate. Both Curtman and Wikoff (*2*) and Dobbins and Ljung (*3*) remove thiocyanate by precipitating it as the cuprous salt. Noyes (*7*) oxidizes it with potassium permanganate, while MacAlpine and Soule (*6*) oxidize it by means of persulfate. Heisig and Heisig (*5*) present a modified procedure of Prescott and Johnson. Swift (*9*) oxidizes the thiocyanate by means of nitrate ion in a solution wherein the acidity is adjusted (using a sulfate-hydrosulfate buffer). In a previous paper (*4*) the authors proposed the use of concentrated sulfuric acid to destroy the thiocyanate.

Procedures for the detection of the halides involving the above methods for removing thiocyanates were found to be very time-consuming. Therefore, the desire for a rapid method of detecting thiocyanate and the halides when as little as 1 per cent of the total concentration is present, stimulated the authors to develop a simple, rapid, and efficient procedure for the removal of thiocyanate. In this new procedure a large part of the thiocyanate is removed with lead nitrate and the remainder destroyed by boiling with sodium nitrite in dilute nitric acid solution.

After the silver halide precipitate has been brought into solution by boiling with ammonia sulfide, the solution to be tested for halides is acidified and then rendered just alkaline. Lead nitrate is added to remove a large portion of the iodide and thiocyanate, since large amounts of iodide interfere with the test for thiocyanate (*4*). This provides a fixed concentration of anions in the form of a saturated solution of their lead salts except when the anions are present in small concentrations.

The precipitate is removed by centrifuging and a portion of the centrifugate is taken for the test for thiocyanate and iodide. Ferric nitrate and carbon tetrachloride in a dilute nitric acid solution are used to detect these anions. The remainder of the centrifugate is acidified with nitric acid, sodium nitrite is added, and the solution is boiled. In this manner iodide and thiocyanate are removed simultaneously. Potassium permanganate and carbon tetrachloride are then used to detect the bromide in a portion of this solution.

Using the procedure described below, only 1 ml. of 0.02 *M* permanganate was required to detect the bromide in all cases except where a large amount of bromide was present. In this instance the addition of potassium permanganate was discontinued after 3 ml. had been added, since sufficient bromide had already been oxidized.

The remainder of the solution (after boiling with nitrite) is used for detecting the chloride with silver nitrate. If bromide is present it is removed by boiling with concentrated nitric acid.

## Procedure

Dilute the solution to be tested (the silver sulfide has been filtered off and the sulfide and ammonia removed by boiling) to 22 ml. in a 100-ml. beaker and add 3 *M* nitric acid dropwise and with constant stirring until the solution is just acid. Add 1.5 *M* sodium carbonate dropwise and with constant stirring until the solution is distinctly blue to nitrazine paper. Then add 12 ml. of *M* lead nitrate dropwise and with constant stirring. Stir for 2 minutes, transfer to a large centrifuge tube, and centrifuge.

A. DETECTION OF THIOCYANATE. To one quarter of the centrifugate add 3 ml. of 3 *M* nitric acid and 5 ml. of 2 *M* ferric nitrate. A blood-red solution shows the presence of thiocyanate.

B. DETECTION OF IODIDE. Transfer the solution of a 50-ml. glass-stoppered Erlenmeyer flask which contains 2 ml. of carbon tetrachloride. Let it stand for 3 minutes, shaking at 1-minute intervals. A violet-colored carbon tetrachloride layer shows the presence of iodide.

C. DETECTION OF BROMIDE. (1) *Thiocyanate and Iodide Present.* Under a hood transfer the remaining three quarters of the centrifugate to a beaker. Add 5 ml. of 3 *M* nitric acid and then 1.5 ml. of 6 *M* sodium nitrite dropwise and with constant stirring. Heat to boiling with constant stirring, boil for 2 minutes, cool, and filter off any precipitate.

(2) *Thiocyanate and Iodide Absent.* Add 5 ml. of 3 *M* nitric acid to the remaining three quarters of the centrifugate. To two thirds of the solution resulting from either (1) or (2) add 1 ml. of 15 *M* nitric acid and then 0.02 *M* potassium permanganate in 1-ml. portions until the solution remains pink or until a maximum of 3 ml. of permanganate has been added. Transfer to a 50-ml. glass-stoppered Erlenmeyer flask containing 1 ml. of carbon tetrachloride and shake. A yellow or brown carbon tetrachloride layer shows the presence of bromide.

D. DETECTION OF CHLORIDE. (1) *Bromide Present.* Dilute the remaining one third solution from C to 20 ml. and add with stirring 10 ml. of 15 *M* nitric acid. Heat to boiling with stirring and boil gently for at least 4 minutes and until all the bromine has been given off. Filter off any precipitate.

(2) *Bromide Absent.* To the remaining one-third solution from C add one tenth of its volume of 15 *M* nitric acid.

To the solution from (1) or (2) add 3 ml. of 0.5 *M* silver nitrate. Let stand for at least 5 minutes. A white precipitate shows the presence of chloride. Compare with a 1-mg. chloride control which has undergone the same procedure.

TABLE I. TEST ANALYSES

| | | | | Results | | | |
|---|---|---|---|---|---|---|---|
| $CNS^-$ | $I^-$ | $Br^-$ | $Cl^-$ | $CNS^-$ | $I^-$ | $Br^-$ | $Cl^-$ |
| *Mg. per 3 ml.* | | | | | | | |
| 1 | 1 | 1 | 1 | + | + | + | + |
| 100 | 1 | 1 | 1 | + | + | + | + |
| 1 | 100 | 1 | 1 | + | + | + | + |
| 1 | 1 | 100 | 1 | + | + | + | + |
| 1 | 1 | 1 | 100 | + | + | + | + |
| 100 | 0 | 0 | 0 | + | − | − | − |
| 0 | 100 | 0 | 0 | − | + | − | − |
| 0 | 0 | 100 | 0 | − | − | + | − |
| 0 | 0 | 0 | 100 | − | − | − | + |

## Test Analyses

The test analyses of Table I were carried out on 12 ml. of solution, using the above procedure.

## Literature Cited

(1) Curtman and Schneiderman, *Rec. trav. chim.*, 54, 158 (1935).
(2) Curtman and Wikoff, *J. Am. Chem. Soc.*, 37, 298 (1915).
(3) Dobbins and Ljung, *J. Chem. Education*, 12, 586 (1935).
(4) Hart and Meyrowitz, IND. ENG. CHEM., Anal. Ed., 12, 318 (1940).
(5) Heisig and Heisig, *Ibid.*, 7, 249 (1935).
(6) MacAlpine and Soule (Prescott and Johnson), "Fundamentals of Qualitative Chemical Analysis", pp. 466, 572, New York, D. Van Nostrand Co., 1933.
(7) Noyes, A. A., "Qualitative Chemical Analysis", p. 151, New York, Macmillan Co., 1920.
(8) Sneed and Duschak, *J. Chem. Education*, 8, 1388 (1931).
(9) Swift, E. H., "System of Chemical Analysis", pp. 453–4, New York, Prentice Hall, 1939.

# Determination of Formaldehyde with 5,5-Dimethylcyclohexanedione-1,3

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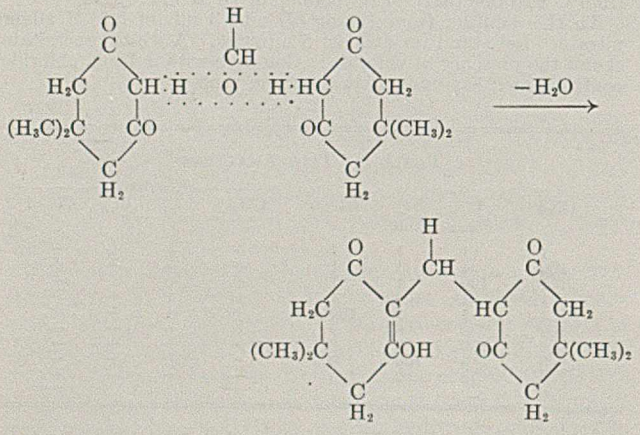
VORLÄNDER (*5*) and Ionescu and Bodea (*2*, *6*) found that for quantitative results the precipitation of formaldehyde with 5,5-dimethylcyclohexanedione-1,3, commonly called "dimedon", must be carried out in a neutral or very weakly acid solution. They did not, however, determine the influence of the hydrogen-ion concentration on the precipitation. The object of this investigation, therefore, was to find the effect of changing pH, to determine the optimum pH for the precipitation, and to study the determination of formaldehyde with dimethylcyclohexanedione in buffered solutions. This work was extended to finding the optimum pH for the precipitation of acetaldehyde and of propionaldehyde, and a comparison was made between the properties of these precipitates and that from formaldehyde.

## Standard Formaldehyde Solution

A solution containing approximately 1 gram per liter of formaldehyde was prepared by adding 2.7 ml. of a 37 per cent formaldehyde solution (reagent quality) to distilled water and diluting to 1 liter. This solution was standardized by the method of Orchard (*4*), a 10-ml. sample being taken for the standardization. Assuming that 10 ml. of the solution contained 10 mg. of formaldehyde, the sample would require 0.107 gram of dimedon for a 15 per cent excess—two molecules of dimedon condense with one molecule of formaldehyde through loss of a molecule of water. Two 10-ml. samples of the formaldehyde solution were




withdrawn and each was added to 0.107 gram of dimedon dissolved in 50 ml. of water. The mixtures were shaken, allowed to stand overnight in stoppered flasks, and filtered through previously weighed sintered-glass crucibles. The precipitates and crucibles were dried in vacuum over phosphorus pentoxide to constant weight, and multiplication of weight of precipitate by 0.1027 (ratio of molecular weights) gave the amount of formaldehyde in the 10-ml. samples. The pH of the filtrate from a dimedon-formaldehyde precipitation made exactly as above was found to be 4.3.

Table I shows a comparison of the results with Orchard's method and with dimedon. From the mean the strength of the formaldehyde solution was taken to be 1.086 grams of formaldehyde per liter.

## Determination of Optimum pH

A saturated solution of dimedon was prepared and filtered; 1 ml. of this solution contains approximately 0.004 gram of dimedon at room temperature. A formaldehyde solution of 0.1 gram per liter was prepared by diluting the standard in a volumetric flask. As buffers, Walpole's sodium acetate–hydrochloric acid mixtures (*1*) for pH 1.0 to 5.2, and Snell's monobasic phosphate–sodium hydroxide mixtures (*3*) for pH 5.8 to 7.0 were used. The method in general was to add 6.42 ml. (10 per cent excess) of the saturated dimedon solution to 250 ml. of the buffer in a 300-ml. Erlenmeyer flask and to this solution, by means of a precision pipet, add 25 ml. of the 0.1 gram per liter formaldehyde solution. The flask was stoppered and allowed to stand approximately 24 hours with occasional shaking. The solution was then filtered through a previously weighed sintered-glass crucible, and the precipitate and crucible were dried to constant weight at 55° to 60° C. In the process of filtering, a sample of the filtrate was caught in a test tube placed in the filter flask, and was set aside for several days. Further precipitation, if any, was noted, and then its pH was determined by means of a Youden hydrogen-ion apparatus using quinhydrone electrodes. As in the low pH range (2.9 to 4.2) precipitation was incomplete after 24 hours, several days were allowed for these precipitations.

The results of this experiment are plotted in Figure 1. Theoretically, 25 ml. of the 0.1086 gram per liter formaldehyde solution should have yielded 0.0264 gram of precipitate. The greatest amount of precipitate was obtained at pH 4.55, and this amount is less than the theoretical by 0.5 mg. This is equivalent to 0.05 mg. of formaldehyde which failed to precipitate in approximately 300 ml. of solution containing a 10 per cent excess of dimedon, and corresponds to an error of only 2 per cent in the precipitation of as little as 3 mg. of formaldehyde.

A similar method was used to determine the optimum pH for the precipitation of acetaldehyde.

Ten-milliliter samples of a 1 gram per liter acetaldehyde solution were added to 250-ml. buffer mixtures containing 17.5 ml. of saturated dimedon solution (10 per cent excess), and the solutions were filtered after 24 hours. Samples of the filtrates were caught as before, but examination after several days' standing

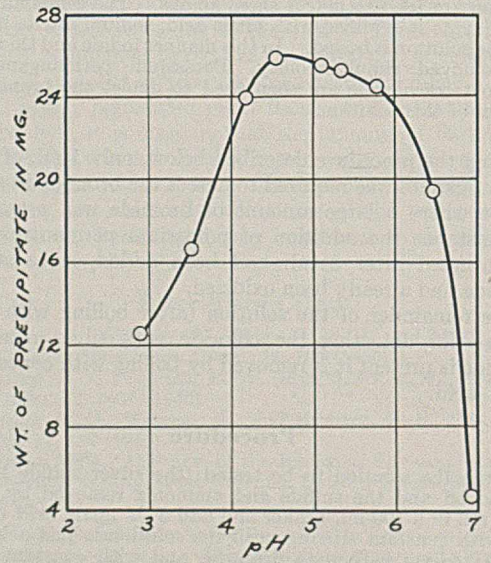



FIGURE 1. INFLUENCE OF pH ON FORMALDEHYDE-DIMEDON PRECIPITATE

TABLE I. DETERMINATION OF FORMALDEHYDE

| Method | Formaldehyde Found G./10 ml. |
|---|---|
| Reduced silver | 0.01075 |
| Excess silver | 0.01096 |
| Dimedon, sample 1 | 0.01090 |
| Sample 2 | 0.01084 |

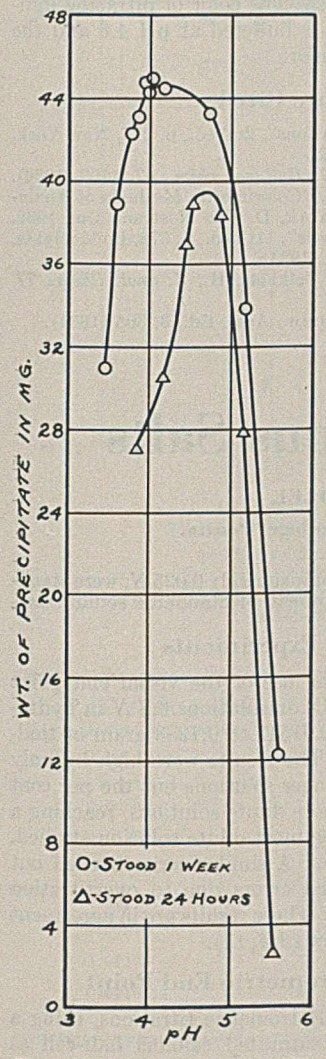



FIGURE 2. INFLUENCE OF pH ON ACETALDEHYDE-DIMEDON PRECIPITATE

showed that precipitation had been incomplete at the time of filtering. It was concluded that the rate of precipitation of acetaldehyde is slower than that of formaldehyde, and this was proved by qualitative tests. A new series of buffers was prepared and acetaldehyde was precipitated as before, except that the solutions were shaken several times a day and allowed to stand a week before being filtered. The precipitation was found to be complete in these solutions. Figure 2 shows the results of these investigations with acetaldehyde.

The solution from which 10-ml. samples were taken for these determinations was standardized by the method of Ripper (*4*) and found to contain 0.9588 gram of acetaldehyde per liter. Each 10-ml. sample should theoretically have yielded 0.0667 gram of precipitate. The greatest precipitation, at pH 4.02, was less than the theoretical by 0.0221 gram, corresponding to 3.2 mg. of acetaldehyde which failed to precipitate in approximately 300 ml. of solution containing a 10 per cent excess of dimedon.

In the determination of the optimum pH for precipitation of propionaldehyde, the method was the same as for formaldehyde except that 13.3 ml. of saturated dimedon solution were used for a 10 per cent excess over the amount required for reaction with 10 ml. of a propionaldehyde solution of 1 gram per liter, and 5 days were allowed for complete precipitation. Qualitative tests showed that the rate of precipitation of propionaldehyde is slower than that of formaldehyde but greater than that of acetaldehyde. The precipitation was incomplete after standing 24 hours, but complete after 5 days. The results are plotted in Figure 3.

The solution from which the 10-ml. samples were taken for these determinations was also standardized by Ripper's method (*4*) and found to contain 0.8480 gram of propionaldehyde per liter. The theoretical yield from a 10-ml. sample is 0.0468 gram of precipitate. The greatest precipitation, at pH 4.72, was less than the theoretical by 0.0095 gram, corresponding to 1.7 mg. of propionaldehyde which failed to precipitate in approximately 300 ml. of solution containing a 10 per cent excess of dimedon.

The results in the determination of the optimum pH for precipitating acetaldehyde and propionaldehyde indicated that small amounts of these aldehydes would not introduce an error in the quantitative precipitation of formaldehyde under these conditions. The determination of formaldehyde was unaffected by the presence of 3.2 mg. of acetaldehyde in 300 ml. of solution, and the presence of 1.7 mg. of propionaldehyde gave results in error by only 0.05 mg.

## Solubility

To determine directly the solubilities of the precipitates in the buffer, a solution was prepared at pH 4.6 and filtered, and 400 ml. were added to weighed amounts of each of the condensation products in 500-ml. Erlenmeyer flasks. The flasks were then stoppered and placed in a constant-temperature water bath at 25° ± 0.1° C. The solutions were allowed to remain in the bath for 4 days with occasional shaking, and were then filtered. The undissolved residues were washed with a little water and dried to constant weight at 60° C.

To find the effect of excess dimedon on the solubility of the precipitates, the above procedure was repeated, except that 10 ml. of saturated dimedon solution were added to each solution before placing them in the bath.

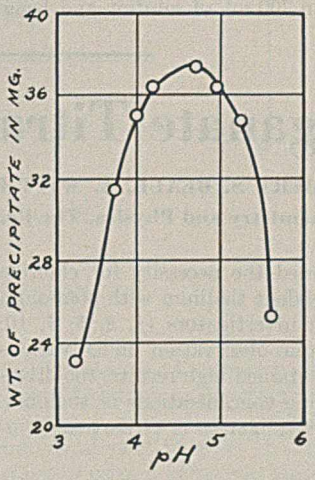



FIGURE 3. INFLUENCE OF pH ON PROPIONALDEHYDE-DIMEDON PRECIPITATE

The results are shown in Table II. Melting points of the undissolved residues showed that the compounds had undergone no change. It is clearly seen that a 10-mg. excess of dimedon per 100 ml. of buffer greatly decreases the solubility of the precipitates.

It was found that acid or alkaline solutions can be analyzed accurately for formaldehyde if first neutralized to the purple

TABLE II. SOLUBILITY DETERMINATIONS

| Dimedon Precipitate | Solvent | Weight of Sample Gram | Weight of Undissolved Residue Gram | Solubility Mg./100 ml |
|---|---|---|---|---|
| HCHO | 400 ml. of buffer | 0.0813 | 0.0808 | 0.1 |
| $H_3C.CHO$ | 400 ml. of buffer | 0.0820 | 0.0508 | 7.8 |
| $H_3C.CH_2.CHO$ | 400 ml. of buffer | 0.0956 | 0.0822 | 3.4 |
| HCHO | 400 ml. of buffer and 10 ml. of saturated dimedon solution | 0.0788 | 0.0786 | 0.05 |
| $H_3C.CHO$ | 400 ml. of buffer and 10 ml. of saturated dimedon solution | 0.0362 | 0.0234 | 3.2 |
| $H_3C.CH_2.CHO$ | 400 ml. of buffer and 10 ml. of saturated dimedon solution | 0.0648 | 0.0624 | 0.6 |

color of bromophenol blue or to the yellow color of nitrazine indicator. The buffer (50 ml. of 1 *N* sodium acetate and 25 ml. of 1 *N* hydrochloric acid) to keep the solution at pH 4.6, and a 10 per cent excess of dimedon are then added and the solution is allowed to stand about 18 hours with occasional shaking before being filtered.

### Stability to Heat

Results obtained in the determination of the stability of the formaldehyde-dimedon precipitate to heat showed that a 0.02-gram precipitate is at constant weight after heating 1 to 2 hours at 60° C., while heating for only 1 hour at 100° C. leads to a loss of approximately 0.5 mg. through sublimation.

### Summary

Very accurate results can be obtained in the determination of formaldehyde with dimethylcyclohexanedione if the precipitation is carried out in a sodium acetate–hydrochloric acid buffer solution at pH 4.6. In such a solution containing a 10 per cent excess of reagent, only 0.05 mg. of formaldehyde remains unprecipitated in 300 ml. of solution and 1 mg. each of acetaldehyde and propionaldehyde do not precipitate in this same solution.

Twelve hours or longer should be allowed for the precipitation to take place, during which time the solution should be shaken occasionally. After being filtered through a sintered-glass crucible and washed with distilled water, the precipitate may be dried to constant weight in several hours at 60° C.

If an acid or alkaline solution of formaldehyde is to be analyzed, the sample is first neutralized to the purple color of bromophenol blue or to the yellow color of nitrazine indicator. The resulting solution is buffered at pH 4.6 and the precipitation carried out as before.

### Literature Cited

(1) Britton, H. T. S., "Hydrogen Ions", 2nd ed., p. 217, New York, D. Van Nostrand Co., 1932.
(2) Ionescu, M. V., and Bodea, C., *Bull. soc. chim.*, 47, 1408 (1930).
(3) Snell, F. D., and Snell, C. T., "Colorimetric Methods of Analysis", Vol. I, p. 265, New York, D. Van Nostrand Co., 1936.
(4) Sutton, F., "Volumetric Analysis", 11th ed., p. 372, Philadelphia, P. Blakiston's Son and Co., 1924.
(5) Vorländer, D., Ible, C., and Volkholz, H., *Z. anal. Chem.*, 77, 321 (1929).
(6) Weinberger, W., IND. ENG. CHEM., Anal. Ed., 3, 365 (1931).

# Permanganate Titration of Thallous Salts

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SINCE Willm (*15*) first noted the necessity for chloride ion in titrations of monovalent thallium with potassium permanganate solutions, other investigators (*1*, *2*, *3*, *5*, *10*, *11*, *12*, *14*) have made the same observation under various conditions. They, however, obtained high results, the divergence increasing with decreasing concentrations of thallium. The work of three of these investigators is of particular interest.

Hawley (*5*), reporting his results in terms of a factor, gram of thallium per ml. of potassium permanganate, found that hot solutions, containing in a total volume of 60 ml. 4 ml. of hydrochloric acid (specific gravity 1.2) and about 0.1 gram of thallium gave nearly constant factors but that solutions containing smaller amounts of thallium gave lower factors. Similar results were obtained in cold solutions. Berry (*2*), following the method of Hawley, made a few titrations using an electrometric apparatus to determine the end point and obtained results 3 per cent high for a solution containing 0.0563 gram of thallium in 60 ml. He reported satisfactory results for a solution containing 0.253 gram of thallium in this volume. Swift and Garner (*14*) varied the concentration of hydrochloric acid from 0.5 to 1 *N* and the temperature from 55° to 90° C. but the end point was uncertain and the results were from 0.6 to 0.7 per cent high. They also observed little or no improvement upon addition of phosphoric acid alone or with manganous sulfate, addition of potassium chloride, or use of the iodine monochloride end point.

It seems to be generally believed that the high results obtained in the permanganate titration to a visual end point are due to oxidation of chloride ion. Since very little investigation of the electrometric end point has been reported, it seemed worth while to determine whether satisfactory results could be obtained by this method. In addition, titrations to a visual end point of solutions containing fluoride ion have been investigated.

### Substances and Solutions

All reagents were of C. P. or equivalent grade. Stock solutions of thallous nitrate were standardized with concordant results by the chloroplatinate method (*4*) and by titration with standard potassium bromate (*7*, *16*). In some experiments, solutions of thallous sulfate similarly standardized were used. Aged potassium permanganate solutions, approximately 0.025 *N*, were standardized by means of National Bureau of Standards sodium oxalate.

### Preliminary Experiments

A series of titrations making use of the visual end point was carried out at 65° to 70° C. on solutions 0.8 *N* in hydrochloric acid containing from 0.01541 to 0.1233 gram of thallium in a volume of 60 ml. The results were high by only 0.2 to 0.3 per cent in the stronger solutions but the per cent error became greater in the more dilute solutions, reaching a value of over 4 per cent in the most dilute solution studied. The end points faded rapidly. A similar series carried out at room temperature exhibited errors due to overtitration ranging from 2 to 10 per cent. These results are in agreement with those previously mentioned (*2*, *5*, *14*).

### Titration to Electrometric End Point

The results of a series of electrometric titrations, using a bright platinum wire and a saturated calomel half-cell as

TABLE I. ELECTROMETRIC TITRATION IN HOT 0.8 *N* HYDROCHLORIC ACID SOLUTION WITH NITROGEN ATMOSPHERE

| Thallium Taken | Thallium Found | Error |
|---|---|---|
| *Gram* | *Gram* | % |
| 0.00783 | 0.00792 | 1.0 |
| | 0.00787 | 0.5 |
| 0.01565 | 0.01579 | 0.9 |
| | 0.01573 | 0.7 |
| 0.02348 | 0.02362 | 0.6 |
| | 0.02357 | 0.4 |
| 0.03131 | 0.03134 | 0.1 |
| | 0.03132 | 0.0 |
| 0.04696 | 0.04706 | 0.2 |
| | 0.04717 | 0.4 |
| 0.06262 | 0.06238 | −0.4 |
| | 0.06235 | −0.4 |
| 0.07827 | 0.07805 | −0.3 |
| | 0.07811 | −0.2 |
| 0.09392 | 0.09375 | −0.2 |
| | 0.09367 | −0.3 |
| 0.1096 | 0.1096 | 0.0 |
| | 0.1095 | −0.1 |
| 0.1252 | 0.1251 | −0.1 |
| | 0.1250 | −0.2 |

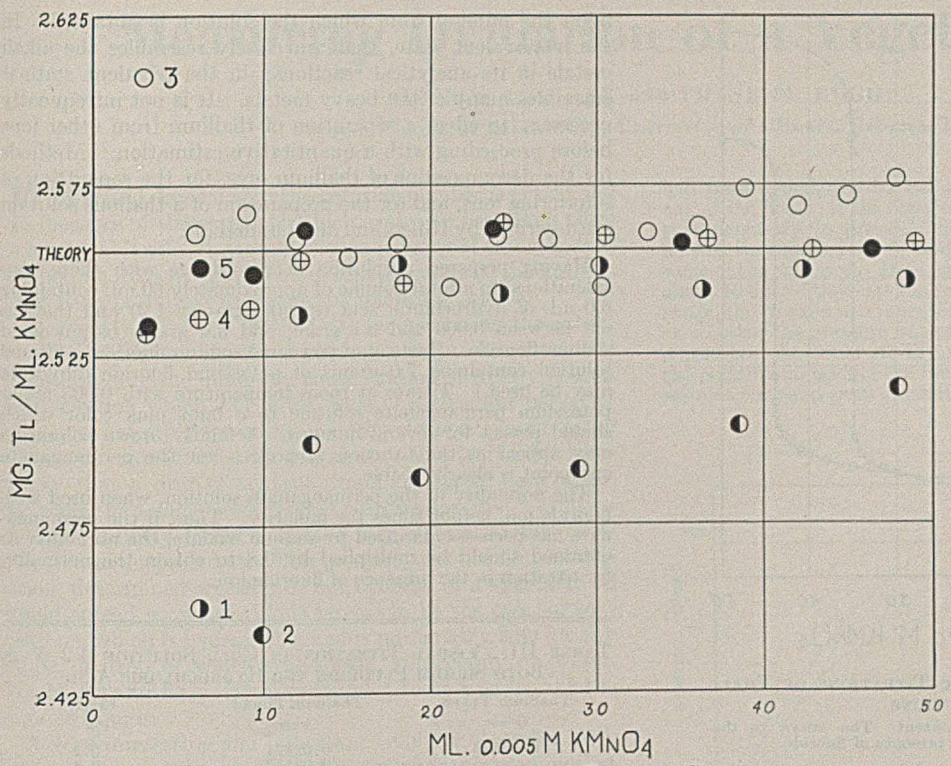


FIGURE 1. PERMANGANATE TITRATIONS OF THALLOUS SOLUTIONS

1. Hot solution with visual end point
2. Cold solution with visual end point
3. Hot solution with electrometric end point
4. Hot solution in nitrogen atmosphere with electrometric end point
5. Cold solution in presence of fluoride with visual end point

TABLE II. VISUAL TITRATION IN COLD SOLUTION 0.8 *N* IN BOTH SODIUM FLUORIDE AND HYDROCHLORIC ACID

| Thallium Taken | Thallium Found | Error |
|---|---|---|
| *Gram* | *Gram* | % |
| 0.00641 | 0.00648 | 1.1 |
| | 0.00644 | 0.5 |
| 0.01282 | 0.01287 | 0.4 |
| | 0.01280 | −0.2 |
| 0.01922 | 0.01928 | 0.3 |
| | 0.01924 | 0.1 |
| 0.02563 | 0.02558 | −0.2 |
| | 0.02554 | −0.4 |
| 0.04870 | 0.04856 | −0.3 |
| | 0.04856 | −0.3 |
| 0.07177 | 0.07165 | −0.2 |
| | 0.07173 | −0.1 |
| 0.09483 | 0.09481 | 0.0 |
| | 0.09471 | −0.1 |

electrodes, at 65° to 70° C. in 0.8 *N* hydrochloric acid solution indicated that detectable amounts of thallium were oxidized by atmospheric oxygen in the time required for these titrations. As a check on this point the loss of thallous thallium in warm hydrochloric acid solutions in beakers open to the atmosphere was shown, by the chloroplatinate method, to be as much as 4 per cent in 1 hour. With the elimination of this factor by constantly blowing a stream of nitrogen over the surface of the solution while it was being heated and during the titration, very satisfactory results were obtained. The solutions were all 0.8 *N* in hydrochloric acid and contained from 0.00783 to 0.1252 gram of thallium in a volume of 60 ml. Titrations to a visual end point with a nitrogen atmosphere showed no improvement over those conducted in air. The results of titrations to an electrometric end point are given in Table I.

The serious overtitration in the dilute solution range found with the visual end point is nearly eliminated. Further experiments indicated a very slow rate of attainment of equilibrium at room temperature and that 0.8 *N* was nearly the optimum concentration of hydrochloric acid for the warm solutions.

### Titration in Presence of Fluoride Ion

Solutions 0.8 *N* in hydrochloric acid and containing an equivalent amount of sodium fluoride were titrated to the visual end point at room temperature. A faintly brown coloration appeared as titration progressed but the permanganate end point was clearly visible and the results were satisfactory, as shown in Table II. In the calculation of these results, account has been taken of the fact that the equivalent strength of permanganate in these titrations is 0.8 its normality as standardized, since permanganate ion is reduced only to the trivalent state in the presence of fluoride ion. The substitution of an equivalent amount of potassium fluoride dihydrate for the sodium fluoride in another series of titrations yielded similar results.

Further experiments were performed with solutions containing about 0.04 gram of thallium in order to ascertain the effect of certain variations from this procedure on the results. It was found that replacing a portion of the hydrochloric acid with an equivalent amount of sulfuric acid slowed the rate of reduction of permanganate. The results, however, were not seriously affected until approximately equivalent amounts of the two acids were present, when the end point became very uncertain. With the acidity maintained at 0.8 *N*, reduction of the sodium fluoride content to about 0.2 *N* was possible. Below this, the results were low and dropped off rapidly with further reduction of the sodium fluoride strength. In another series, the hydrochloric acid content was increased from 0.8 to 2.8 *N* in steps of 0.4 *N* and for each acidity the sodium fluoride strength was varied from 0.8 to 2.0 *N* in steps of 0.2 *N*. Solutions of high acidity and low sodium fluoride content gave low results. Those in which the acidity was low and the sodium fluoride high tended to be turbid but were otherwise satisfactory.

Consideration of these effects suggested the use of solutions 1.2 *N* in hydrochloric acid and 1.2 *N* in sodium fluoride for the titrations. The results of a series containing 0.02569 to 0.07707 gram of thallium in 60 ml. of this solution are listed in Table III and are seen to be satisfactory.

The data of Tables I and II together with the results of some of the preliminary experiments have been represented graphically in Figure 1, which permits a ready comparison of these results with the theoretical value as well as with each other.

The nature of the potentiometric titrations for thallium solutions may be seen in Figure 2. Both solutions were 1.2 *N* in hydrochloric acid, the one containing in addition an

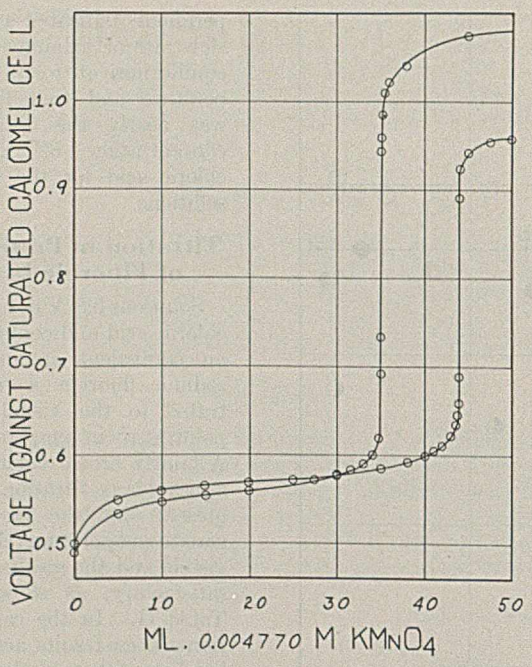


FIGURE 2. POTENTIOMETRIC TITRATIONS OF THALLOUS SOLUTIONS

Solutions had same thallium content. The curve to the right was obtained in the presence of fluoride.

equivalent amount of sodium fluoride. The titrations were carried out slowly at room temperature (27.5° C.).

It is interesting to compare the values of the potentials for these solutions when half oxidized, with the standard oxidation potential, −1.25 volts (*8*), for the system $Tl^{+}$–$Tl^{+++}$ based on studies made in other mineral acid solutions. The value, referred to the normal hydrogen electrode, of about −0.799 volt obtained in the case of the solution free from fluoride ion indicates clearly the strong tendency of trivalent thallium to form complex chloride ions. This phenomenon causes also a very real dependence of the oxidation potential on the chloride-ion concentration of the solution, as was shown some time ago by Spencer and Abegg (*13*). These authors reported a value of −0.859 volt for a solution 0.105 *N* in hydrochloric acid and −0.828 volt for one 0.190 *N* in hydrochloric acid.

By analogy to ferric ion, the existence of a stable fluorothalliate complex ion might be expected. Although such an ion may be formed, the value of −0.815 volt obtained when thallium is half oxidized in the presence of fluoride ion indicates that its stability, if greater, is evidently not much more so than the corresponding chlorothalliate ion. It is not immediately evident why the potentials obtained in the presence of fluoride ion are larger than those in its absence. In view of the profound influence of the chloride-ion concentration on the $Tl^{+}$–$Tl^{+++}$ potential, it is perhaps worth noting that the mean ion activity coefficient of hydrochloric acid is greater than that of sodium chloride in moderately strong solutions (*9*).

## Recommended Procedure

Of the two satisfactory methods evolved in this work for the permanganate titration of thallous salts—namely, the electrometric titration in nitrogen atmosphere in hot 0.8 *N* hydrochloric acid solution and the visual titration in fluoride-ion solution at room temperature—the latter obviously is to be preferred because of its greater convenience.

The nature of the treatment of the solution prior to titration with potassium permanganate will, of course, depend upon the mineral from which the solution is obtained. In the monovalent state, thallium closely resembles the alkali metals in its analytical reactions; in the trivalent state it resembles many of the heavy metals. It is not infrequently necessary to effect a separation of thallium from other ions before proceeding with a quantitative estimation. Methods for the decomposition of thallium ores, for the separation of interfering ions, and for the preparation of a thallous solution are described by Hillebrand and Lundell (*6*).

Having prepared a solution in accordance with these considerations, to a total volume of approximately 60 ml. containing 6.0 ml. of hydrochloric acid (specific gravity 1.2) and thallous ion between 0.006 and 0.1 gram, add 3.0 grams of powdered sodium fluoride. (Instead of powdered sodium fluoride, a filtered solution containing 7.0 grams of potassium fluoride dihydrate may be used.) Titrate at room temperature with 0.005 molar potassium permanganate solution to a faint pink color which should persist for several minutes. A faintly brown coloration may appear as the titration progresses but the permanganate end point is clearly visible.

The normality of the permanganate solution, when used with fluoride ion, is four times the molarity. Thus, if the permanganate has been standardized by sodium oxalate, the normality so obtained should be multiplied by 0.8 to obtain the normality for titration in the presence of fluoride ion.

TABLE III. VISUAL TITRATION IN COLD SOLUTION 1.2 *N* IN BOTH SODIUM FLUORIDE AND HYDROCHLORIC ACID

| Thallium Taken | Thallium Found | Error |
|---|---|---|
| *Gram* | *Gram* | % |
| 0.02569 | 0.02574 | 0.2 |
| | 0.02572 | 0.1 |
| 0.03853 | 0.03857 | 0.1 |
| | 0.03855 | 0.1 |
| 0.05138 | 0.05141 | 0.1 |
| | 0.05139 | 0.0 |
| 0.06422 | 0.06409 | −0.2 |
| | 0.06419 | 0.0 |
| 0.07707 | 0.07705 | 0.0 |
| | 0.07711 | 0.1 |

## Summary

The work of previous investigators has been confirmed.

Electrometric titrations have been studied and found to be satisfactory in hot 0.8 *N* hydrochloric acid solutions under a nitrogen atmosphere.

A new method has been developed for the titration of thallous salts with permanganate in cold solutions by the use of fluoride ion.

## Acknowledgments

Grateful acknowledgment is made to H. H. Geist and J. R. Hayes of this laboratory for making titrations to check the recommended procedure.

## Literature Cited

(1) Benrath, A., and Espenschied, H., *Z. anorg. allgem. Chem.*, **121** 361 (1922).
(2) Berry, A., *J. Chem. Soc.*, **121**, 394 (1922).
(3) Bodnar, J., and Terenyi, A., *Z. anal. Chem.*, **69**, 33 (1926).
(4) Cushman, A., *Am. Chem. J.*, **24**, 222, (1900).
(5) Hawley, L., *J. Am. Chem. Soc.*, **29**, 300 (1907).
(6) Hillebrand, W. F., and Lundell, G. E. F., "Applied Inorganic Analysis", pp. 373–6, New York, John Wiley & Sons, 1929.
(7) Kolthoff, I. M., *Rec. trav. chim.*, **41**, 172 (1922).
(8) Latimer, W., "Oxidation Potentials", p. 153, New York, Prentice-Hall, 1938.
(9) Lewis, G. N., and Randall, M., "Thermodynamics", p. 362, New York, McGraw-Hill Book Co., 1923.
(10) Marshall, H., *J. Soc. Chem. Ind.*, **19**, 994 (1900).
(11) Noyes, A., *Z. phys. Chem.*, **9**, 608 (1892).
(12) Proszt, J., *Z. anal. Chem.*, **73**, 401 (1928).
(13) Spencer, J., and Abegg, R., *Z. anorg. allgem. Chem.*, **44**, 379 (1905).
(14) Swift, E., and Garner, C., *J. Am. Chem. Soc.*, **58**, 113 (1936).
(15) Willm, Ed., *Bull. soc. chim.*, **5**, 352 (1863).
(16) Zintl, E., and Rienäcker, G., *Z. anorg. allgem. Chem.*, **153**, 276 (1926).

# Measuring Oxidation of a Vegetable Oil

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IT IS WELL known that the peroxide number is not a valid measure of the extent to which oxidation has taken place in a vegetable oil. In view of the inadequacy of this standard method and the need for a more reliable method, the authors have been prompted to report the following studies.

A recently proposed method (*1*) for measuring the extent to which oxidation has taken place in a vegetable oil consists in determining the minimum concentration of freshly poured soap solution which will, after lapse of a constant period of time, prevent rapid spreading of a drop of the oil placed on the surface of the solution. Having already observed in this laboratory that a drop of benzene would not spread on a monomolecular film under considerable pressure and that a solution of Dreft quickly forms a surface film of considerable pressure, it was suspected that film pressure, and not film composition, determined whether or not benzene or a vegetable oil would spread on water. This proved to be the case for soybean oil, which is hydrophilic, and is presumably true for any liquid, a drop of which will spread appreciably on water. Washburn and Keim (*2*) have found it to be true for volatile organic liquids.

A very interesting and important behavior of soybean oil, when added to a water surface in an amount far exceeding that required to form a monomolecular film, is that some sections will be covered by a monomolecular film and other regions by a thick, plainly visible film, with very distinct and abrupt boundaries between these sections. The film pressure just at the point where drops will not spread when added to a surface covered by thin film, or just below which the drop will begin to spread, is readily determined by observation of the boundaries of the drops (or between areas with thin and thick layers), and thus constitutes a measure of the hydrophilic nature, or degree of oxidation, of an oil. The results are more easily reproducible if the drops are added to a region covered by a thin film.

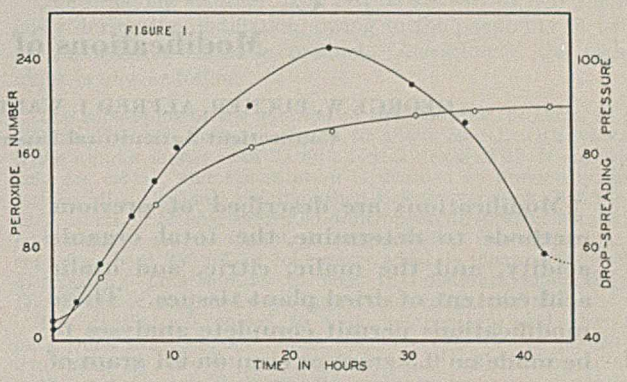
## Experimental

All readings were taken on the Cenco Hydrophil balance, using distilled water as the substrate. The soybean oil was added to the water surface by means of a medicine dropper. After 2 drops had been added, the barrier was advanced to within about 10 cm. of the float. If the observed pressure was much greater than that necessary to prevent the third drop from spreading, the barrier was moved back and lifted for a moment to release some of the excess oil. If on again advancing the barrier to within about 10 cm. of the float the pressure was not within a reasonable range, the process was repeated or more oil was added, depending on whether the pressure was too great or too small. With a little experience one could readily approximate from the behavior of the pressure on adding the first 2 drops the pressure necessary to prevent the drop from spreading.

Table I. Oxidation of Soybean Oil

| Time of Blowing of Oil | Peroxide No. | Drop-Spreading Pressure[a] |
|---|---|---|
| *Hours* | | |
| 00.00 | 9.44 | 44.3<br>44.6 |
| 4.00 | 65.5 | 53.3<br>53.7 |
| 8.50 | 135 | 65.8<br>65.9 |
| 16.75 | 197 | 81.0<br>81.1 |
| 23.75 | 249 | 83.9<br>84.3 |
| 30.25 | 218 | 87.5<br>87.2 |
| 35.50 | 185 | 87.7<br>87.9 |
| 42.75 | 72.7 | 88.5<br>88.6 |
| 48.00 | 48.4 | 89.0<br>88.9 |

[a] In scale units: to convert to dynes per cm. multiply by 0.2696.




With the pressure adjusted to a reasonable value, the third drop was added to a thin-film section. If the film pressure was sufficiently great, the drop would not spread and would remain indefinitely as a very thick lens (drop). The barrier was then moved back at a rate of about 0.5 cm. per second until the drop spread out and apparently lost its identity, as judged by the very distinct shadow which was cast on the bottom of the trough by the outer edge of the drop (lens). The pressure was always taken when this shadow became indistinct. This pressure is referred to as drop-spreading pressure.

It is significant that the thickness of the drop after it has lost its identity is about the same as that of the thick film and that, on moving the barrier still farther back, the pressure changes only very slowly, making it easy to obtain reproducible values. To test the applicability of this method air was blown through an edible soybean oil at a temperature of 100° C., samples being removed at intervals. Peroxide values in milliequivalents of peroxide per kilogram of oil and drop-spreading pressure values were obtained for each sample. Then the results were compared, as shown by the curves in Figure 1 and the numerical data in Table I.

It is at once apparent that the peroxide number rises to a maximum value in about 30 hours and then decreases to form a nearly symmetrical curve with two possible time values for each peroxide number; whereas the drop-spreading pressure rises continuously with time, though tapering off after about 15 hours.

## Advantages

No weighing and no volumetric measurements are involved. The water surface does not have to be swept clean; the composition of the film apparently does not alter results; the barrier position before and after expansion of drop is of no appreciable significance; and after spreading of the drop the

pressure decreases very slowly as the film is expanded still farther, thus providing reproducible results. A medicine dropper gives a drop of convenient size; a drop may be added directly from the oil container; small variations in rate of expanding the film introduce no observable error; and the values do not rise to a maximum and then decrease (as peroxide numbers do). Presumably the method has wide applications, especially to the evaluation of lubricating oil addition agents which contain hydrophilic groups. It appears at present that the drop-spreading pressures are directly proportional to collapse pressures of monomolecular films of corresponding substances.


## Summary

The measurement of the spreading pressure of a drop of liquid placed on a monomolecular film on the hydrophilic balance is a far more accurate evaluation of oxidation in a vegetable oil such as soybean oil or the presence of hydrophilic groups in any liquid than the familiar peroxide number. The evaluation of lubricating addition agents is an especially valuable application.


## Literature Cited


(1) Marzolf, Jack, private communication.
(2) Washburn and Keim, *J. Am. Chem. Soc.*, 62, 1747 (1940).


# Organic Acids in Plant Tissues

## Modifications of Analytical Methods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**Modifications are described of previous methods to determine the total organic acidity, and the malic, citric, and oxalic acid content of dried plant tissues. These modifications permit complete analyses to be made on 0.5 gram or even on 0.1 gram of material, without sacrifice of accuracy and with greater convenience.**


PLANT tissues, especially leaves and fleshy fruits, as a rule contain substantial proportions of relatively simple organic acids, chiefly polybasic hydroxy acids of the malic and citric acid type. Although much study has been given to the chemistry and physiology of these substances, surprisingly little of their exact functions in the metabolism of the plant is yet known. This is a field in which speculation still plays a dominating role, a consequence largely of the fact that satisfactory analytical methods for the more important of these substances have been developed only in recent years.

Methods for determining the total acidity of plant tissues due to organic acids (*6*), and malic (*7*), citric (*3*, *4*) and oxalic acid (*6*), together with nitric acid (*8*), have been described in previous papers from this laboratory. These methods depend on the observation that nitric acid and the organic acids can be quantitatively extracted by means of ether from the acidified dried tissue provided that an efficient extraction device is employed. Several years' experience in routine analyses of a variety of tissues has led to the introduction of a number of minor modifications that, taken together, materially increase the convenience and broaden the applicability of these methods. In the following description of these modifications, familiarity with the details of the earlier procedures will be assumed in order to save unnecessary repetition. The changes have chiefly to do with a reduction of scale so that complete analyses can be carried out upon 0.5 gram of dry tissue, or even on 0.1 gram instead of 2.0 grams without sacrifice of accuracy, and with an important simplification of the ether-extraction technique which eliminates the use of paper extraction thimbles.

### Preparation of "Organic Acid Fraction"

The fresh tissue is prepared for analysis by being dried at 80° C. in a ventilated oven and is ground to a fine powder; of this 0.500 gram is thoroughly mixed in a small beaker with 1.0 ml. of 4 *N* sulfuric acid; 1.0 gram of asbestos is added and the whole is mixed with a rod until a homogeneous mass is obtained. The glass siphon cup of the extraction apparatus (Eimer & Amend catalog No. 30,754) is charged with angular quartz pebbles (about 5-mm. size) to a depth of about 1.5 cm., and over these is placed a folded pad consisting of several thicknesses of cotton gauze (cheesecloth) previously extracted with ether. The sample is then added, being packed evenly, and a second pad of gauze is used to wipe the last traces of tissue from beaker and glass rod and is gently pressed down above the sample in such a way as to cover it completely. The quantities of pebbles, gauze, and sample are such that the sample occupies approximately the middle third of the depth of the glass cup and sufficient gauze is used to prevent escape of the particles of tissue.

Extraction with specially purified ether is then conducted for about 16 hours (usually overnight) as previously described (*6*, *8*). The siphoning rate should be between 40 and 60 cycles per hour. (The completeness of the extraction depends on the number of times of siphoning. If extraction devices are used which siphon less frequently, the total extraction time must be correspondingly increased.) The ether in the extraction flask is then treated with 7 ml. of water and 5 ml. of 1 *N* sodium hydroxide (free from carbon dioxide) and the ether is evaporated in a water bath at 50° to 60° C. The aqueous solution is transferred to a 25-ml. flask and made to volume and is referred to hereafter as the "organic acid fraction".

### Determination of Total Organic Acids

The changes introduced into the technique previously described for the titration of the total organic acids (*6*) are the substitution of a glass electrode for the quinhydrone electrode, the complete removal of carbon dioxide immediately before the titration, and the use of a more dilute nitric acid reagent. The advantages of the glass electrode on grounds of convenience do not need emphasis. All difficulties with platinum wire electrodes are eliminated and greater sensitivity is obtained.

No prescription with respect to the details of the apparatus is necessary; in this laboratory a Beckman glass electrode is used in conjunction with a Leeds & Northrup thermionic amplifier and student potentiometer with accessory equipment. The electrode is mounted, for convenience, on a discarded microscope stand, the rack and pinion of which provide for easily controlled introduction of the electrode into the solution. An agar bridge serves to make the connection with the standard calomel half-cell and has been found entirely satisfactory. The instru-

ment is calibrated against buffer solutions of known hydrogen-ion activity.

A 5-ml. aliquot of the organic acid fraction is transferred to a 30-ml. beaker with calibration marks at 20 and 25 ml. To this, 2 ml. of water, 3 drops of 0.04 per cent solution of bromocresol purple, and 1 ml. of 1 *N* nitric acid are added, the beaker is covered, and the contents are boiled gently for about a minute to expel carbon dioxide. The solution is cooled and neutralized with 1 *N* sodium hydroxide free from carbon dioxide, added from a fine-tipped buret, until a pale blue color is obtained. At this point, the glass electrode is introduced and the connections with the potentiometer system and half-cell are established. The potentiometer is set to the voltage that corresponds to a reaction of pH 8.0 as determined from the calibration. The solution should be slightly more acid than pH 8.0; if it is not, the reaction is adjusted with 0.1 *N* nitric acid, and 0.1 *N* sodium hydroxide is then added until the galvanometer deflects in the opposite direction. The galvanometer is finally brought to zero deflection by the dropwise addition of 0.05 *N* nitric acid. The potentiometer is next adjusted to the voltage that corresponds to pH 2.60, the acid buret is read, and acid is rapidly added until no deflection of the galvanometer is obtained. The solution is then diluted to the 20-ml. mark, and the titration is carefully completed. If more than 0.5 ml. of acid is required, it is necessary to dilute to the 25-ml. mark and again complete the titration by dropwise additions of the reagent.

A titration of water, completed at 20- and at 25-ml. total volume, is also made with the potentiometer set at pH 2.60, the result being subtracted from the titration value of the organic acid fraction. This blank value needs to be determined only occasionally; in this laboratory values of from 0.96 to 1.00 ml. were obtained during several months of routine titrations.

Two corrections are required before the titration data can be calculated in terms of total organic acidity. Oxalic acid must be separately determined, since this acid is titrated only to the extent of 50 per cent by the technique described, and sulfuric acid must also be determined, since the amount extracted by the ether under the present conditions, while small, is not entirely negligible if exact information is required.

To this end, the solution, after titration, is evaporated on a steam bath to about 12 ml., and is cooled and acidified with 0.5 ml. of 2 *N* hydrochloric acid; 50 to 100 mg. of asbestos are added and the solution is filtered with gentle suction through a Gooch crucible that contains a thin asbestos mat, into a 25-ml. wide-mouthed test tube. An apparatus constructed from a desiccator or a small bell jar with side tubulature provides for this filtration. The asbestos is washed with water until a volume of not more than 20 ml. of filtrate is obtained. To this, 6 *N* ammonium hydroxide is added until the indicator already present turns a faint blue color, and then 2 *N* acetic acid is added until a yellow color is produced, followed by 0.5 ml. of 5 per cent calcium acetate solution. After being thoroughly mixed, the solution is allowed to stand for at least 2 hours.

Filtration is more satisfactory than centrifugation (*2*) and is accomplished on a 1.5-ml. Gooch microcrucible that contains a disk of filter paper covered with a pad of fine asbestos specially prepared according to the directions of Kirk and Moberg (*1*). The test tube and the precipitate are washed with a saturated solution of calcium oxalate prepared according to Stanford and Wheatley (*9*) and the filtrate is reserved (see below). The test tube used for the precipitation is placed in the filtration apparatus under the crucible, and the calcium oxalate is dissolved by the careful addition of about 2 ml. of boiling 2 *N* sulfuric acid. This is stirred in the crucible with a fine rod and is then drawn through with gentle suction (see *2*). The treatment with hot sulfuric acid is repeated three or four times; the filtrate is then heated in a boiling water bath and titrated with 0.02 *N* potassium permanganate to an end point that is permanent for 10 to 15 seconds. One milliliter of the permanganate is equivalent to 0.09 mg. or to 0.02 milliequivalent of oxalic acid.

The sulfuric acid correction is ascertained from a sulfate determination in the filtrate from the calcium oxalate precipitate, and is calculated on the assumption that 3.6 per cent of the sulfuric acid extracted by the ether is titrated between the limits chosen for the organic acid titration. The filtrate is acidified to Congo red by the addition of 2 *N* hydrochloric acid and 5 ml. of 1 per cent barium chloride are added. The weight of the barium sulfate, obtained according to the usual technique, multiplied by 0.0063 gives the number of milliliters of 0.05 *N* nitric acid equivalent to the sulfuric acid titrated within the pH limits of the organic acid titration; this quantity is added to the water blank before calculating the total organic acids according to the formula given in the original paper (*6*). The magnitude of the sulfuric acid correction varies between 0.02 and 0.10 ml. of 0.05 *N* nitric acid reagent for a 5.0-ml. aliquot of the organic acid fraction.

## Determination of Malic Acid

The modifications of the technique for the determination of malic acid have entirely to do with the details of the distillation of the oxidation product and with the filtration of the dinitrophenylhydrazine precipitate.

A suitable aliquot of the organic acid fraction, usually 5 ml., is prepared and oxidized as previously described (*7*). After removal of the pentabromoacetone with petroleum ether, the subaliquot (usually 25 ml.) is distilled directly, the addition of permanganate and of sodium sulfite being omitted. The original procedure occasionally gave low recoveries of malic acid since the addition of the sulfite reduced the acidity to a point where slight decomposition of the oxidation product might occur.

The receiving solution must not be allowed to boil vigorously during the distillation, owing to the possibility of loss of the volatile oxidation product. Accordingly, the technique is now as follows:

The subaliquot of the solution of the oxidation product, in a 300-ml. Kjeldahl flask, is diluted to about 50 ml. with water; if the aliquot is less than 25 ml., 1 or 2 drops of 18 *N* sulfuric acid are added, and the solution is distilled as previously described into a 250-ml. wide-mouthed Erlenmeyer flask that contains 10 ml. of filtered saturated dinitrophenylhydrazine solution and 20 ml. of water. The flask is supported in a small pan that contains about 250 ml. of cold water which is renewed for each distillation. This suffices to cool the distillate adequately. Distillation is continued until the residual volume is 5 ml.

The distillate is cooled to room temperature and filtered on a small sintered-glass funnel (Büchner type of fine porosity equivalent to Jena G4, treated if necessary with boiling alkali until a satisfactory filtration rate is obtained), and is dissolved in pyridine and treated as previously described. The calibration curve, from which the malic acid equivalent of the readings of the Pulfrich spectrophotometer is obtained, has been repeatedly checked within 5 per cent of the data given in the earlier paper. A Pulfrich spectrophotometer is not essential for the determination of the intensity of the color. There are several photoelectric devices available for which calibration curves can be established as described in the original paper (*7*) or, if necessary, a colorimeter can be used. In this case a standard solution of malic acid must be carried through the procedure in parallel with the unknown and a suitable quantity of the pyridine solution, found by trial and error, used for the final comparison.

## Determination of Citric Acid

Only a slight change in the technique of the titration method for the halogen content of the pentabromoacetone obtained in the course of the method for citric acid (*4*) has been found necessary. It is essential to acidify the sodium sulfide solution that contains the decomposition products of the pentabromoacetone and to boil it to eliminate the hydrogen sulfide immediately. The solution may then be titrated for the halogen content when convenient.

A more satisfactory hydrogen peroxide reagent than that previously described may be prepared by pouring 16 to 18 ml. of 18 *N* sulfuric acid into 100 ml. of water, cooling the mixture to 5° C. in an ice bath, and adding about 10 grams of fresh sodium peroxide slowly so that the temperature does not rise above 30° C. The final solution should still be acid to Congo red paper. It is cooled to 5° C. and the fluid is decanted or centrifuged from the sodium sulfate. Prepared in this way the reagent contains between 2.5 and 3.5 per cent of hydrogen peroxide.

## Analysis of 0.1-Gram Quantities of Tissue

Although the use of a 0.5-gram sample of dried tissue for the preparation of the organic acid fraction is more convenient and is recommended for routine use, occasions may arise when this quantity cannot be spared for the analysis. With attention to details of technique, it is possible to secure

TABLE I. DETERMINATIONS OF ORGANIC ACIDS IN TOBACCO LEAF TISSUES

(Data are given in milliequivalents per 100 grams of dry tissue)

| Amount of Tissue Used for Preparation of Organic Acid Fraction | Total Organic Acids | Oxalic Acid | Malic Acid | Citric Acid |
|---|---|---|---|---|
| *Grams* | *M. e./100 grams* | *M. e./100 grams* | *M. e./100 grams* | *M. e./100 grams* |
| 2.0 | 213 ± 9 | 40 ± 2 | 122 ± 6 | 30 ± 0.5 |
| 0.500 | 211 ± 6 | 39 ± 0.75 | 122 ± 3 | 30.3 ± 0.5 |
| 0.100 | 215 ± 3 | 39 ± 1 | 119 ± 2 | 30.3 ± 0.03 |

accurate analyses when only 0.100 gram of dried tissue is available.

The accurately weighed sample, in a watch glass, is treated with 0.3 ml. of 4 *N* sulfuric acid, placed in a depression in the heap of powder. Mixing is accomplished with a fine glass rod, and 0.2 gram of fine asbestos is then incorporated. The siphon cup of the extraction apparatus is filled with sufficient quartz pebbles so that the sample, on its pad of gauze, will occupy the middle of the height of the cup. Extraction is accomplished as already described. The ether is treated with 3 ml. of water and 1 ml. of 1 *N* sodium hydroxide solution, and is then evaporated. The aqueous solution is drained into the calibrated titration beaker for half a minute, and the residual traces are washed in with water in two successive 3-ml. portions, each being allowed to drain as before. The titration of the total organic acids is carried out as described, and the entire solution is used for the determination of the oxalic acid. The filtrate from the calcium oxalate is acidified with hydrochloric acid, and the sulfuric acid is determined as before. The filtrate from the barium sulfate is acidified with 3 ml. of 50 per cent sulfuric acid, is evaporated to 15 to 20 ml., and is used for the determination of the citric and malic acids.

### Nitrate Nitrogen

The organic acid fraction contains the whole of the nitric acid present in the sample of plant tissue. The method of determining the nitric acid content, as originally described (*8*), involved the use of a colorimeter, and this modification has been studied by a group of collaborators and recommended to the Association of Official Agricultural Chemists as a tentative official method (*10*). The original description required the use of an aliquot of the organic acid fraction that contained the equivalent of 0.200 gram of dry tissue. Equally satisfactory results have been secured with the use of an aliquot that represents 0.100 gram of tissue (5 ml. of the organic acid fraction obtained as described above), and by reading the color of the nesslerized solution in a Pulfrich spectrophotometer, as described by Pucher, Vickery, and Leavenworth (*5*). Attention is merely called to this slight change in the interests of convenience to those laboratories equipped with this instrument. A photoelectric apparatus may doubtless be used equally well if calibrated correctly or, alternatively, the ammonia may be titrated with 0.02 *N* acid under appropriately standardized conditions.

### Experimental

Many control analyses of pure acids and of previously analyzed tissues have been carried out to check the details of the modified techniques. A summary of the data secured is given in Table I, which shows mean values with the standard deviation of a single determination. In each case, four or more duplicate determinations are represented. It is clear that there is no choice between the use of 2 grams and 0.5 gram of tissue on the grounds of accuracy or precision; the smaller quantity is, however, somewhat more convenient to manage and is recommended for routine work. The use of 0.1 gram of tissue likewise gives satisfactory results but is recommended only when the supply of tissue is inadequate.

### Literature Cited

(1) Kirk, P. L., and Moberg, E. G., IND. ENG. CHEM., Anal. Ed., 5, 95 (1933).
(2) Kirk, P. L., and Schmidt, C. L. A., *J. Biol. Chem.*, 83, 311 (1929).
(3) Pucher, G. W., Sherman, C. C., and Vickery, H. B., *Ibid.*, 113, 235 (1936).
(4) Pucher, G. W., Vickery, H. B., and Leavenworth, C. S., IND. ENG. CHEM., Anal. Ed., 6, 190 (1934).
(5) *Ibid.*, 7, 152 (1935).
(6) Pucher, G. W., Vickery, H. B., and Wakeman, A. J., *Ibid.*, 6, 140 (1934).
(7) *Ibid.*, 6, 288 (1934).
(8) Pucher, G. W., Vickery, H. B., and Wakeman, A. J., *J. Biol. Chem.*, 97, 605 (1932).
(9) Stanford, R. V., and Wheatley, A. H. M., *Biochem. J.*, 19, 710 (1925).
(10) Vickery, H. B., *J. Assoc. Official Agr. Chem.*, 17, 273 (1934).

# Rapid Determination of Starch (Root) with Sodium Hypochlorite

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THE usual methods employed for the determination of starch are long and cumbersome and are unsuited for the control of manufacturing processes, especially in the starch industry. Rapid methods of analysis have been proposed from time to time and have found limited applicability. These methods depend upon complete dispersion of the starch into a colloidal solution which can be separated from extraneous matter (plant tissues) and then upon measuring it by precipitation and weighing, by combining with iodine which may be quantitatively determined, or by measuring the optical rotation of the starch solution with a polariscope or saccharimeter.

The principal difficulty encountered with the rapid methods is obtaining complete dispersion of the starch without undue hydrolysis or without solubilizing plant constituents other than starch. A very large list of chemical agents has been proposed for solubilizing starch for analytical work, but only a very few have found much favor. On certain materials hydrochloric acid (*6*) is satisfactory, but it requires carefully controlled conditions as to temperature and time of reaction which cannot always be adhered to in analyzing certain other plant materials because they filter with such extreme difficulty. Magnesium chloride (*2*) and calcium chloride (*5*) have been found fairly satisfactory in certain cases, but here solutions of high salt concentration are required and complete dispersion of the starch under the conditions specified, particularly in the analysis of sweet potato starch factory products, was not always obtained. Although dispersion with enzymes such as amylases might be considered ideal from a theoretical viewpoint, their application has also been found limited, at least under the conditions specified (*3*). The principal faults were that the solutions frequently filtered with difficulty and were often somewhat too turbid to be polarized with accuracy. Complete dispersion of the starch in the products being analyzed was not always obtained, especially in those products which had been treated with lime water (used in the factory process).

Since a rapid method for the determination of starch in

Table I. Effect of Varying Quantity of Sodium Hypochlorite on Polarization

(Solution contains 2.5 grams of starch)

| 5.25% NaOCl | Polarization | Clearness of Solution | Remarks |
|---|---|---|---|
| *Ml.* | ° *V.* | | |
| 1 | 24.00 | Good | No excess NaOCl in any sample after 5 minutes' boiling |
| 2 | 24.05 | Good | |
| 3 | 24.00 | Better | |
| 4 | 24.00 | Better | |
| 5 | 23.85 | Best | |

sweet potato starch factory products was so desirable, a study was made of various agents which might be employed in solubilizing starch for its determination with a saccharimeter. Sodium hypochlorite, a strong oxidizing and bleaching agent known to have also a high power of converting raw starch even at room temperatures, was found to give excellent results. This agent is easily available and can be purchased under various trade names as a 5 to 5.25 per cent (by weight) solution in most grocery stores at relatively low cost; only small quantities of the active agent (2 to 15 ml. of commercial solution) are required per determination; the final solution filters easily, as a rule, yielding clear solutions that usually can be read in a polariscope without difficulty; and, with the simple procedure developed, the starch is completely dispersed (judging by the fact that the residue can be washed entirely free from starch with water). The principal disadvantage of the method is its apparent limitation to root starches. With the procedure outlined below, grain starches are apparently from 93 to 94 per cent converted; the unconverted fraction remains insoluble and is filtered off, yielding a solution whose polarization is, naturally, too low.

This observation suggests a possible rapid method for separating the so-called amylohemicellulose (*4*, *7*) from the amylopectin and amylose fractions of starch. It is likely that the portion remaining insoluble after the action of hypochlorite is amylohemicellulose because of its obvious presence in grain, but not in root, starches. This fraction, whatever it may prove to be, is, of course, convertible with malt diastase or hydrochloric acid, so that it is included in the determination of starch by the usual methods.

## Preliminary Experiments in Developing Procedure

After it was determined that sodium hypochlorite would disperse starch satisfactorily in the presence of plant tissues—that is, without converting the starch to an objectionable degree—and yield a solution of starch which was clear and, hence, could be read in a polariscope, the details of a workable procedure had to be developed.

Although most starches are gelatinized at a temperature close to 70° C., it was found desirable actually to boil the samples of vegetable plant materials during the gelatinization period and during the reaction with hypochlorite; otherwise complete liberation of the starch from plant tissues might not be obtained. This procedure also ensured complete usage of the hypochlorite. Attempts were made to conduct the gelatinization and conversion operations in a 100-ml. volumetric flask in order to eliminate transferring, but because of erratic results and the tendency of some samples to foam when boiled, it seemed preferable to use a 400-ml. tall-form beaker. After treatment, the samples can be quantitatively transferred to a 100-ml. volumetric flask. However, gelatinization and hypochlorite conversion operations can be performed in a hot-water bath in cases where the starch is easily freed from the plant tissue and made soluble.

The amount of hypochlorite required to effect a desired degree of conversion of starch in the sample depends upon the material being analyzed. With pure or commercial starch, a suitable proportion of hypochlorite to starch is 1 ml. of 5.25 per cent solution per gram of starch in the sample. The tolerance is considerable, as may be noted from the tables, so that no undue precautions need to be observed in measuring the hypochlorite solution precisely.

In Table I are given the saccharimeter readings for 2.5 grams of sweet potato starch treated as follows:

Five 2.5-gram portions of starch were separately suspended in 50-ml. portions of water and treated with 1, 2, 3, 4, and 5 ml. of 5.25 per cent hypochlorite solution. These were heated to boiling and boiled for exactly 5 minutes; they were then transferred to 100-ml. volumetric flasks, cooled, made to the mark, filtered, and polarized in a 2-dm. tube with a saccharimeter.

Table II. Polarization of Starch Solutions

(Employing 3 ml. of NaOCl as a dispersing agent)

| 5.25% NaOCl | Starch in Sample | Polarization |
|---|---|---|
| *Ml.* | *Grams* | ° *V.* |
| 3 | 1 | 9.60 |
| 3 | 2 | 19.75 |
| 3 | 3 | 29.75 |
| 3 | 4 | 40.10 |

Table III. Polarization of Starch Solutions

(Prepared with a constant proportion of 1 ml. of 5.25% NaOCl per gram of starch)

| 5.25% NaOCl | Starch | Polarization | Remarks |
|---|---|---|---|
| *Ml.* | *Grams* | ° *V.* | |
| 1 | 1 | 9.95 | All samples filtered clear, those of higher concentration increasingly slower. |
| 2 | 2 | 19.80 | |
| 3 | 3 | 29.90 | |
| 4 | 4 | 39.95 | |

In the ratio of 5 ml. of the hypochlorite to 2.5 grams of starch, it is obvious that the reading is lowered. This effect is also apparent in Table II, which gives the readings obtained when employing 3 ml. of sodium hypochlorite solution with 1, 2, 3, and 4 grams of starch per 100 ml. following a similar procedure.

Under these conditions the polarization is not quite a linear function of the concentration. However, a linear relationship is shown by results, believed to be within experimental error, if a constant proportion of sodium hypochlorite to starch is employed, as may be observed from the data in Table III.

When, following the same technique as with commercial starch, 1 ml. of sodium hypochlorite solution was added to 5 grams of sweet potatoes containing approximately 1 gram of starch and boiled 5 minutes, etc., the starch was not completely dispersed. By varying the time of preboiling for gelatinizing the starch and softening the tissues, and by increasing the proportion of sodium hypochlorite to starch, conditions were established under which a limpid solution of starch was obtained with the tissues showing only traces of undigested starch, if any at all, when tested with iodine solution. The color reaction of the starch solution with iodine was purplish blue. These color reactions of starch solutions and of extracted tissue with iodine were the criteria used to establish the quantities of sodium hypochlorite solution to be used on different products. From the experiments it was evident that the tissues comprising the extraneous matter of the sample, in addition to the starch, were acted upon by the hypochlorite and that increasing quantities of hypochlorite must be used in analyzing products of a starch factory in which the ratio of starch to fiber is decreasing.

As with all other indirect methods of analysis, it was necessary to establish a factor for converting saccharimeter readings into starch. This is not a simple procedure because of uncertainties in the existing methods of starch analyses and one may rightfully question the accuracy of the factor to be suggested, because it is based upon the starch value obtained with malt diastase followed by acid hydrolysis. And again there arises the old question as to whether one should employ

TABLE IV. RELATIONSHIP BETWEEN POLARIZATION AND STARCH VALUE OF SWEET POTATO PRODUCTS DETERMINED BY OFFICAL MALT-DIASTASE METHOD

| Determination No. | Sweet Potatoes (5-Gram Samples) Starch by malt | Polarization[a] | Factor | Residual Pulp (5-Gram Samples) Starch by malt | Polarization[a] | Factor | Commercial Starch (2-Gram Samples) Starch by malt | Polarization[a] | Factor |
|---|---|---|---|---|---|---|---|---|---|
| | *Grams* | ° *V.* | | *Grams* | ° *V.* | | *Grams* | ° *V.* | |
| 1 | 1.2405 | 14.15 | 0.0877 | 1.3600 | 15.65 | 0.0873 | 1.2724[b] | 14.20[b] | 0.0899 |
| 2 | 1.3805 | 15.70 | 0.0879 | | | | | | |
| 3 | 1.2175 | 13.85 | 0.0879 | 1.0745 | 12.85 | 0.0836 | 1.6628 | 18.90 | 0.0880 |
| 4 | 1.2395 | 14.10 | 0.0879 | | | | | | |
| 5 | 1.1845 | 13.50 | 0.0877 | 1.0705 | 12.10 | 0.0885 | 1.6966 | 19.10 | 0.0888 |
| 6 | 1.1700 | 13.60 | 0.0860 | | | | | | |
| 7 | 1.0810 | 12.30 | 0.0879 | 1.2070 | 13.70 | 0.0881 | 1.6684 | 18.90 | 0.0883 |
| 8 | 0.9915 | 11.40 | 0.0870 | | | | | | |
| 9 | 1.0840 | 12.10 | 0.0896 | 1.1305 | 13.00 | 0.0870 | 1.6684 | 18.90 | 0.0883 |
| 10 | 0.9715 | 10.90 | 0.0891 | | | | | | |
| 11 | 1.0355 | 11.60 | 0.0901 | 1.1075 | 12.40 | 0.0892 | 1.6616 | 19.00 | 0.0875 |
| 12 | 1.0240 | 11.60 | 0.0883 | | | | | | |
| 13 | 1.0030 | 11.25 | 0.0891 | 0.8470 | 9.40 | 0.0901 | 1.6740 | 18.80 | 0.0890 |
| 14 | 1.1085 | 12.25 | 0.0905 | | | | | | |
| 15 | 0.9890 | 11.10 | 0.0891 | 0.9340 | 10.35 | 0.0901 | 1.6628 | 18.85 | 0.0882 |
| 16 | 0.9495 | 10.75 | 0.0883 | | | | | | |
| 17 | 1.0500 | 12.00 | 0.0875 | .... | ... | .... | 1.6188 | 18.65 | 0.0868 |
| 18 | 0.9530 | 10.75 | 0.0887 | | | | | | |
| 19 | 0.9610 | 10.90 | 0.0882 | .... | ... | .... | .... | ... | .... |
| 20 | 0.9050 | 10.15 | 0.0891 | | | | | | |
| Av. | | | 0.0880 | | | 0.0880 | | | 0.0882 |

Grand average, 0.0880 gram per ° V.

[a] 2-dm. tube.

[b] 1.5 grams of commercial starch in this sample.

the usual equation of 0.9 × glucose = starch in this method to obtain the starch value or use a larger factor to compensate for the discrepancy between the determined value and the actual weight of starch corrected for known impurities. In this paper the writer used this equation and assumed the malt-diastase method would yield correct results, although in another paper (*1*) it is shown that serious errors may arise in its application to plant materials. The saccharimeter readings on solutions prepared from duplicate samples in which the starch was dispersed with sodium hypochlorite led to the conversion factor of 1° V. = 0.0880 gram of starch, which corresponds to a specific rotation of +193° for the starch in solution. This average factor held for the analysis of sweet potatoes and residual pulp, and was very close to that for commercial sweet potato starch. The results are summarized in Table IV.

## Description of Method

It is, of course, essential that the sample employed for starch analysis be finely disintegrated. Sweet potatoes are most accurately and conveniently sampled by making a V-shaped cut in each of 10 or more of them with a sugar-beet sampling rasp and compositing. Residual pulp from the starch plant in the wet state is sufficiently fine to require no further disintegration. It is generally necessary, however, to grind dry pulp in order to reduce to a fine state the lumps and balls formed during drying operations. Commercial starch requires no further grinding unless it contains lumps in sufficient proportion to cause sampling errors. If grinding is necessary, care should be exercised to avoid breaking down the starch granules.

Weigh out a portion of the sample containing from 1 to 2 grams of starch and transfer to a 400-ml. tall-form beaker with about 100 ml. of water or of about 25 per cent alcohol solution in case of dried pulp containing gelatinized starch. After allowing mixture to digest at room temperature for about half an hour, add 10 ml. of a 10 per cent suspension of diatomaceous earth and transfer to a Gooch crucible fitted with an asbestos mat prepared in some standard manner. Wash the sample with about 100 ml. of water or dilute alcohol. Transfer the washed sample with asbestos mat quantitatively to the same beaker with from 50 to 75 ml. of water. Add a small quantity of sodium hypochlorite solution (consult Table V) and heat on a hot plate; boil the mixture 15 minutes, giving the beaker a rotating motion, or stirring occasionally to keep the insolubles suspended in the water the greater part of this period. Add the main portion of hypochlorite solution at the end of the gelatinization period and continue boiling for exactly 5 minutes, again agitating at times to rinse down the material which may collect on the sides of the beaker. Set aside for a few minutes and transfer while still warm to a 100-ml. volumetric flask. Cool quickly to room temperature; add about 5 drops of concentrated acetic acid (as an aid to clarification and filtration), make up to the mark with water; and add an extra quantity of water equal to the volume of the insoluble matter (chiefly asbestos and diatomaceous earth). In the tests reported, 1 ml. was taken as the correction volume, but each analyst should determine this correction for each type of material or change of procedure which might influence the value. Filter through fluted paper and read the rotation of the clear solution in a 2-dm. tube in a saccharimeter (or polarimeter). Calculate the starch from the reading in degrees Ventzke in accordance with the equation:

$$\% \text{ starch} = \frac{(^\circ \text{V.} \times 0.0880)\ 100}{\text{weight of sample taken}}$$

If the determination is carried out properly the starch solution, after the hypochlorite treatment, usually filters readily and is clear. However, a slight turbidity sometimes apparent seems to cause no appreciable difficulty in reading the solution because of its high dispersion. The starch solution should produce a purple to blue coloration with iodine; its reaction should be between 7 and 8 pH; and it should show no excess of hypochlorite when tested by adding a few drops of potassium iodide solution. If iodine is not liberated, giving a blue coloration in the presence of starch, hypochlorite is absent.

In Table V are given the pertinent conditions under which starch determinations are conducted, as applied in the analysis of sweet potato starch factory products. Although there has been no opportunity to analyze many other types of starchy plant materials, it is believed that the method is generally applicable to roots or tubers but not to grains or grain products.

TABLE V. RECOMMENDED CONDITIONS FOR ANALYSIS OF SWEET POTATO STARCH FACTORY PRODUCTS

| Product | Weight of Sample | 5.25% NaOCl Required Gelatinization | 5.25% NaOCl Required Conversion | Time of Boiling Gelatinization | Time of Boiling Conversion |
|---|---|---|---|---|---|
| | *Grams* | *Ml.* | *Ml.* | *Min.* | *Min.* |
| Fresh potatoes | 5.0 | 1 | 4 | 15 | 5 |
| Potato flour | 1.5 | 1 | 4 | 15 | 5 |
| Wet pulp residue | 5.0 | 1 | 14 | 15 | 5 |
| Dry pulp residue | 1.5 | 1 | 14 | 15 | 5 |
| Starch (dry) | 2.0 | 0.2 | 1.8 | 15 | 5 |

## Acknowledgment

The writer wishes to express his appreciation to J. K. Phillips, Barberton, Ohio, formerly of this division, for assisting with the analyses reported in Table IV.

## Literature Cited

(1) Balch and Phillips, IND. ENG. CHEM., Anal. Ed. (in press).
(2) Jirak, L. W., *Z. Spiritusind.*, 58, 81 (1935).
(3) Kingsbury, unpublished work.
(4) Ling and Dinshaw, *J. Chem. Soc.* (London), 127, 652 (1925).
(5) Mannich and Lenz, *Z. Untersuch. Nahr. u. Genussm.*, 40, 1 (1920).
(6) Rask, Official and Tentative Methods of Analysis, Assoc. Official Agr. Chem., 5th ed., pp. 221–2, 1940.
(7) Schryner and Thomas, *Biochem. J.*, 17, 497 (1923).

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# Rapid Determination of Reducing Sugars

## Extension of Forsee's Photocolorimetric Ferricyanide Method

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IN CONNECTION with a genetic study on the distribution of sugars in different varieties of watermelons it was necessary to devise a method whereby a large number of samples could be analyzed daily for both reducing and total sugars. Of the many sugar methods described in the literature, the clarification procedure and ferricyanide oxidation of Hassid (*7, 8*), as modified by Forsee (*4*) for rapid photocolorimetric determinations, seemed best adapted to the problem.

The principle of the Forsee technique is that the yellow color of alkaline ferricyanide, when heated with reducing sugars, diminishes in proportion to the quantity of sugar present, owing to the production of colorless ferrocyanide. This color decrease is then measured quantitatively with a photoelectric colorimeter, and a standard curve relating sugar concentrations to the colorimeter readings (or photometric densities) is prepared for routine use.

In the volumetric determination of reducing sugars by ferricyanide, a titration is conducted on either the residual reagent, as in the clinical iodometric methods of Hagedorn and Jensen (*5*) and its macromodification of Hanes (*6*), or the ferrocyanide formed, as in the ceric sulfate oxidation of Hassid (*7, 8*). A colorimetric modification for clinical micro work was introduced by Folin (*3*) who measured the ferrocyanide formed by conversion to Prussian blue. A more rapid technique based on the ferricyanide color itself was first used by Hawkins and Van Slyke (*9*), who measured the time required for the complete decolorization of the reagent. This procedure, however, requires continuous observation of each sample during an approximate 5-minute heating period. Hoffman (*10*) then described a photocolorimetric measurement of the decrease in yellow color and utilized this procedure for blood sugar determinations. It was applied to plant materials by Forsee (*4*).

The range of the present colorimetric ferricyanide methods was inconveniently narrow for this investigation: up to 0.2 mg. for the clinical Prussian blue and Hoffman methods, and 0.4 mg. for that of Forsee. The present modification makes possible the rapid estimation of up to 1.2 mg. of reducing sugar per aliquot oxidized; with two assistants it was possible to analyze daily about one hundred samples. It has been standardized for both glucose and invert sugar. No essential changes from Forsee's method have been made except as regards the volumes and concentrations of the reagents employed and an increase in the period of heating. A comparison of the ferricyanide oxidation to the standard copper methods has already been made by several investigators (*4, 7, 8*) and found very satisfactory for plant materials. Although the volumetric procedures permit far greater range—i. e., up to 10 mg. in Hassid's original procedure (*7*)—they are not so well adapted to the large number of determinations which can be rapidly and conveniently handled by the technique described below.

### Apparatus and Reagents

The photoelectric colorimeter described by Evelyn (*2*) was used, equipped with a blue filter No. 420 (transmission limits 380 to 469 millimicrons). Six dozen 17.5 × 2.2 cm. (7 × 0.875 inch) absorption test tubes were selected which agreed to within 0.25 galvanometer unit. Complete analyses, including mixture of sugar aliquot with reagent, heating, and colorimeter reading, were conducted in the test tubes. In this manner rapid serial determinations were made without transfer of samples. Since the blue filter transmitted a weak light, the colorimeter lamp was always set to the bright position for taking readings.

A fresh solution of alkaline ferricyanide, exactly 0.05 per cent in potassium ferricyanide and approximately 1 per cent in sodium carbonate concentration, was prepared daily by pipetting 50 ml. of each of stock solutions A and B to a 2-liter volumetric flask and diluting to volume: Stock A, 2 per cent potassium ferricyanide–sodium carbonate prepared by dissolving 40.00 grams of reagent grade potassium ferricyanide and 40 grams of sodium carbonate monohydrate in water and diluting to 2 liters; stock B, 40 per cent sodium carbonate monohydrate. The reagents used for clarification by means of Hassid's (*7*) procedure were molar neutral lead acetate and 20 per cent disodium phosphate. For preparing the standard sugar curve, Bureau of Standards' samples of dextrose and sucrose were used.

The selection of 0.05 per cent ferricyanide concentration as the oxidizing reagent was determined by the necessity of obtaining a good spread in galvanometer readings, from approximately 10 to 90, as the sugar concentrations progressively increased. When the galvanometer was set at 100 with a tube of distilled water, a constant "center setting" (no absorption tube in the slot) of 74.75 was maintained. This ferricyanide concentration resulted in a consistent blank reading of 10.0. The galvanometer was read to the nearest quarter of a division.

### Procedure for Calibrating Standard Sugar Curves

GLUCOSE. A solution containing 1 mg. per ml. was prepared by dissolving 2.000 grams of dextrose in 2000 ml. In order to obtain a series of a dozen 2-ml. aliquots containing from 0.1 to 1.2 mg., 5, 10, 15, etc., to 60 ml. were diluted to 100 ml. The 2 ml. aliquots were pipetted to the dry colorimeter test tubes, and 25.0 ml. of the reagent alkaline ferricyanide, 0.05 per cent potassium ferricyanide–1 per cent sodium carbonate, added from a 250-ml. dispensing buret. Blanks were included using 2 ml. of distilled water. The tubes were placed in a wire basket and set into a gently boiling steam-heated water bath in such manner that the contents were immersed to approximately two thirds of their depth. A piece of flat sheet metal was kept between the steam coils and the basket to prevent any uneven heating of tubes placed directly above the coils. Heating was maintained for exactly 10 minutes and the basket then quickly immersed in a beaker of water. A stream of tap water was continuously run into the beaker for approximately 10 minutes.

If care is observed to prevent splashing, especially during the heating period, the volume within the tubes remains sufficiently constant for the determination. The condensed water formed

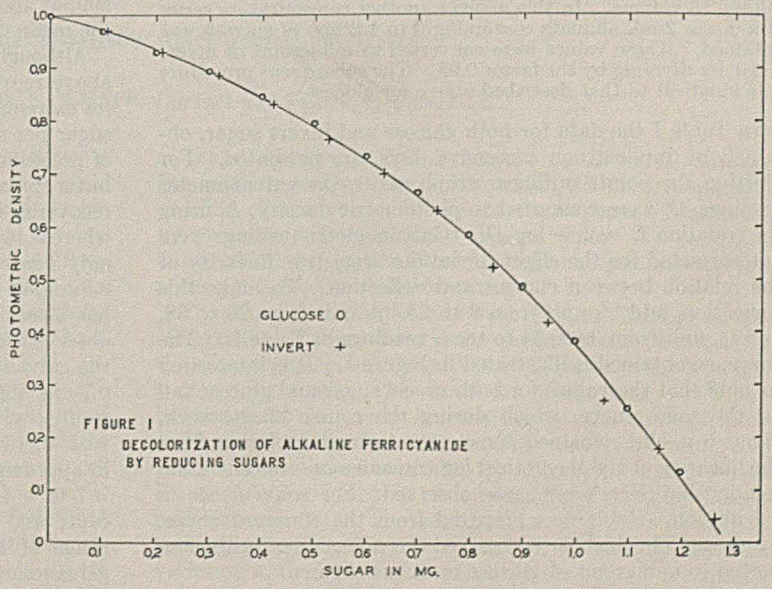


FIGURE 1
DECOLORIZATION OF ALKALINE FERRICYANIDE BY REDUCING SUGARS

TABLE I. DECOLORIZATION OF ALKALINE FERRICYANIDE BY REDUCING SUGARS[a]

| Glucose | | | | Invert Sugar[b] | | | |
|---|---|---|---|---|---|---|---|
| Mg. per 2-cc. aliquot | Galvanometer Readings[c] Sept. 6 | Sept. 7 | Optical density (av.)[d] | Mg. per 2-cc. aliquot[e] | Galvanometer Readings[c] Sept. 6 | Sept. 7 | Optical density (av.)[d] |
| 0.0 | 10.00 | 10.00 | 1.000 | 0.00 | 10.00 | 10.00 | 1.000 |
| | 10.00 | 10.00 | | | 10.00 | 10.00 | |
| 0.1 | 10.75 | 10.75 | 0.969 | 0.105 | 10.75 | 10.75 | 0.969 |
| | 10.75 | 10.75 | | | 10.50 | 10.75 | |
| 0.2 | 11.75 | 11.75 | 0.930 | 0.211 | 11.75 | 11.75 | 0.930 |
| | 11.75 | 11.75 | | | 11.75 | 11.75 | |
| 0.3 | 12.75 | 13.00 | 0.894 | 0.316 | 13.00 | 13.00 | 0.886 |
| | 13.00 | 12.50 | | | 13.00 | 13.00 | |
| 0.4 | 14.25 | 14.25 | 0.846 | 0.421 | 15.00 | 15.00 | 0.831 |
| | 14.25 | 14.00 | | | 14.75 | 14.50 | |
| 0.5 | 15.75 | 16.00 | 0.796 | 0.526 | 17.00 | 17.75 | 0.763 |
| | 16.25 | 16.00 | | | 17.00 | 17.25 | |
| 0.6 | 18.50 | 18.50 | 0.733 | 0.632 | 20.00 | 20.75 | 0.699 |
| | 19.00 | 18.25 | | | 19.50 | 20.25 | |
| 0.7 | 22.25 | 21.75 | 0.663 | 0.737 | 24.25 | 23.25 | 0.629 |
| | 22.25 | 21.00 | | | 23.00 | 23.50 | |
| 0.8 | 27.00 | 26.00 | 0.581 | 0.842 | 30.00 | 31.00 | 0.520 |
| | 27.00 | 25.00 | | | 30.25 | 30.00 | |
| 0.9 | 33.00 | 32.75 | 0.485 | 0.947 | 37.25 | 39.00 | 0.417 |
| | 33.00 | 32.25 | | | 38.50 | 38.00 | |
| 1.0 | 42.25 | 42.00 | 0.382 | 1.053 | 56.25 | 54.75 | 0.270 |
| | 43.25 | 38.00 | | | 55.00 | 53.75 | |
| 1.1 | 55.75 | 56.75 | 0.255 | 1.158 | 67.50 | 66.00 | 0.179 |
| | 58.25 | 52.25 | | | 65.25 | 66.00 | |
| 1.2 | 73.00 | 73.25 | 0.135 | 1.263 | 86.25 | 92.25 | 0.046 |
| | 74.50 | 72.25 | | | 90.50 | 91.25 | |

[a] 25 cc. of 0.05% $K_3Fe(CN)_6$–1% $Na_2CO_3$ heated with 2-cc. aliquots containing 0 to 1.2 mg. of sugar.
[b] On Sept. 7 inversion was conducted by official A. O. A. C. method (*1*); on Sept. 6 by immersion in boiling water for 10 minutes.
[c] Observed to nearest quarter division.
[d] Optical density = 2 − $\log_{10}$ galvanometer readings.
[e] Mg. of sucrose divided by 0.95 for conversion to invert sugar.

in the necks of the tubes was mixed with the contents by gentle shaking. The tubes were wiped dry with clean cheesecloth and read in the colorimeter. The instrument was set at 100 with distilled water and a constant center setting of 74.75 maintained. After cooling, the colors remained extremely stable. No change was detected up to 2 hours, and even when left overnight a maximum increase equivalent to only 1 per cent of the sugar present was observed. The tubes were therefore read whenever convenient within 2 hours.

INVERT SUGAR. A solution containing 10 mg. of sucrose per ml. was prepared by dissolving 2.000 grams of sucrose in 200 ml. Aliquots of 0.5, 1.0, 1.5, etc., to 6.0 ml. were transferred to 100-ml. Pyrex volumetric flasks and water was added to make a total volume of 5 to 6 ml. Ten milliliters of *N* hydrochloric acid were added and inversion was conducted in a bath at 70° C. according to the standard A. O. A. C. method (*10*) or by immersion in the boiling water bath for 10 minutes. The methods gave identical results (see Table I). The flasks were cooled in running water, 10 ml. of *N* sodium hydroxide were added, and the solution was diluted to volume. In this manner another concentration series of a dozen 2-ml. aliquots containing 0 to 1.2 mg. of sucrose was obtained. These values were converted to milligrams of invert sugar by dividing by the factor 0.95. The subsequent procedure was identical to that described above for glucose.

In Table I the data for both glucose and invert sugar, obtained in duplicate on successive days, are presented. For plotting the points on linear graph paper, the galvanometer readings, *G*, were converted to photometric density, *L*, using the equation $L = 2 - \log_{10}G$. (Galvanometer readings were not corrected for the slight deviations from true linearity of the relation between current and deflection. To make this correction, add $^1/_4$ unit from 9 to 25, $^1/_2$ unit from 26 to 38, and $^1/_4$ unit from 39 to 54 to the *G* readings in Table I.) The curve, so obtained is illustrated in Figure 1. It is interesting to note that the values for both invert sugar and glucose fall on the same curve, which during the course of the work, about 6 weeks, remained constant. A small deviation from the linearity of the theoretical log transmission–concentration relationship (Beer's law) was observed. For convenience in calculation, a table was prepared from the standard curve, by means of which any observed *G* reading was readily converted to milligrams of glucose or invert sugar.

## Procedure for Plant Materials

The preparation of the samples for analysis as described by Hassid (*7, 8*) and Forsee (*4*) has proved entirely satisfactory. In the case of watermelon juice, a direct clarification with neutral lead acetate and disodium phosphate was conducted. Approximately 300-ml. samples of juice were prepared from the hearts of melons by reaming out with a spoon and gently pressing through two layers of cheesecloth. A clear juice was readily obtained by centrifuging and 10-ml. portions were transferred to 100-ml. volumetric flasks. Two milliliters of molar neutral lead acetate were added and the contents mixed. Five milliliters of 20 per cent disodium phosphate were added, mixed, and diluted to volume. The solutions were filtered through Whatman No. 12 fluted papers into dry screw-cap jars. Whenever it was necessary to preserve samples for subsequent analysis, several drops of a saturated solution of benzoic acid in toluene were added and the jars stored at 4° C.

For the determination of reducing sugars, 5-ml. portions of the filtrates were diluted to 100 ml. and 2-ml. aliquots were analyzed as described above for calibrating the glucose curve. For the determination of total sugars, 5-ml. portions were inverted and analyzed as described above for calibrating the invert sugar curve. By reference to the table the galvanometer readings were converted to milligrams of glucose and a factor of 10 directly converted the values to per cent sugar in the original juice (grams per 100 ml.).

As a test of the accuracy of the method, the recovery of various sugars added to watermelon juice samples was studied.

Three melons were selected at random and the juice was extracted and centrifuged as described. For each melon 10-ml. samples were analyzed in duplicate. A mixed sugar solution was prepared containing a final sugar concentration exactly 2.5 per cent in glucose, 2.5 per cent in fructose, and 5.0 per cent in sucrose. To the first two melon samples 5 ml. were added, or a supplement of 250 mg. of invert sugar and 250 mg. of sucrose (producing 263 mg. of invert sugar after hydrolysis) the samples from the third melon were supplemented with 10 ml., equivalent to 500 mg. of invert and 500 mg. of sucrose (526 mg. after inversion), respectively.

In Table II the results of these experiments are summarized. The recoveries were treated statistically as six pairs by subtracting the differences before and after supplementing with sugar. In the case of melon 3 the differences were divided by two, so that they could be combined with the results of the first two melons. The average recovery for 250 mg. of invert sugar added was 245, or about 98 per cent, with a standard error of 7 mg. for the mean of two determinations. The average recovery for 513 mg. of total sugar added was 509, or about 99 per cent, with a standard error of 10 mg. for the mean of two determinations.

Although the concentration range of the method described above represents a threefold extension of that of Forsee (*4*), for extremely dilute solutions, up to approximately 0.3 mg. of sugar per sample, the latter method affords a greater degree of precision. The calibration data were not given by Forsee, but a spread of 13 colorimeter scale divisions was reported for recoveries involving 0.1 to 0.3 mg. of glucose [(*4*), Table I]; whereas in the method described above (Table I) a spread of only 3 galvanometer divisions was observed in the range up to 0.3 mg. of glucose. (In a private communication, the author has been informed that a spread of 24.7 scale divisions was observed in the range 0 to 0.4 mg. of glucose.) If one reads the galvanometer to the nearest quarter division, then at the 0.2-mg. sugar level an error of approximately ±10 per cent results, which progressively increases with greater dilution; with increasing concentration, however, this error decreases in approximately the following manner: from 0.3 to 0.4 mg., ±7 to ±4 per cent; from 0.5 to 0.7 mg., ±2 to ±1 per cent; and from 0.8 to 1.2 mg., less than 1 per cent. In the range of higher concentration a variation of even several galvanometer divisions results in only a relatively small

TABLE II. RECOVERY OF SUGARS ADDED TO WATERMELON JUICE

| Melon No. | Reducing Sugars in 10 Cc. of Juice — Before Inversion: Galvanometer readings | Mg. per 2-cc. aliquot | Mg. per 10-cc. juice | After Inversion: Galvanometer readings | Mg. per 2-cc. aliquot | Mg. per 10-cc. juice | Sugar Added to 10 Cc. of Juice: Invert Mg. | Sucrose as invert[b] Mg. | Reducing Sugars in 10 Cc. of Supplemental Juice — Before Inversion: Galvanometer readings | Mg. per 2-cc. aliquot | Mg. per 10-cc. sample | After Inversion: Galvanometer readings | Mg. per 1-cc. aliquot[e] | Mg. per 10-cc. sample | Recovery of[a] Invert sugar[c] Mg. | % | Total sugar[d] Mg. | % |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 17.00 | 0.528 | 528 | 20.75 | 0.661 | 661 | 250 | 263 | 24.75 | 0.763 | 763 | 18.75 | 0.594 | 1188 | 238 | 95 | 518 | 101 |
| | 17.00 | 0.528 | 528 | 20.75 | 0.661 | 661 | | | 25.00 | 0.768 | 768 | 18.50 | 0.585 | 1170 | ±3 | ±1 | ±9 | ±2 |
| 2 | 23.75 | 0.740 | 370 | 21.25 | 0.675 | 675 | 250 | 263 | 19.50 | 0.621 | 621 | 18.75 | 0.594 | 1188 | 248 | 99 | 511 | 100 |
| | 22.75 | 0.717[f] | 359 | 20.75 | 0.661 | 661 | | | 19.00 | 0.603 | 603 | 18.50 | 0.585 | 1170 | ±14 | ±6 | ±16 | ±3 |
| 3 | 13.75 | 0.364 | 364 | 21.25 | 0.675 | 675 | 500 | 526 | 30.00 | 0.857 | 857 | 28.00 | 0.825 | 1650 | 500 | 100 | 996 | 97 |
| | 14.00 | 0.380 | 380 | 21.50 | 0.682 | 682 | | | 32.25 | 0.887 | 887 | 29.50 | 0.849 | 1698 | ±15 | ±5 | ±28 | ±3 |

[a] ± values represent maximum deviations from average recoveries.
[b] Mg. of sucrose added divided by 0.95 to convert to invert sugar formed after hydrolysis.
[c] Obtained by subtracting mg. of reducing sugars per 10 cc. of juice before inversion (column 4) from that observed after supplementing with known quantity of invert sugar (column 12).
[d] Obtained by subtracting mg. of reducing sugars per 10 cc. of juice after inversion (column 7) from that observed after supplementing with known quantity of invert sugar plus sucrose (column 15).
[e] Owing to high total sugar content, half of usual aliquot supplemented with 1 cc. water was oxidized.
[f] 10 cc. of clarified juice, rather than 5 cc. as usually taken, were diluted to 100 cc. before oxidation of 2-cc. aliquot.

error, due to the logarithmic relation between concentration and galvanometer deflection; at the 1.1-mg. glucose level, for example, the observed variation of 52.25 to 58.25 (the greatest galvanometer variation observed in Table I) results in an error of only ±2 per cent. In the routine analysis of large numbers of plant samples, usually involving great variation in sugar concentration, the sacrifice of a certain degree of precision in the extremely dilute range is well repaid by the saving of time and material consumed in reanalyzing samples requiring greater dilution.

## Summary

A rapid photocolorimetric method for determining reducing sugars in aliquots containing up to 1.2 mg. has been described. The method is an extension of the procedure developed by Forsee for plant materials and represents a threefold increase in concentration range. The decrease in yellow color produced by heating reducing sugars with alkaline ferricyanide was measured with an Evelyn photoelectric colorimeter. Test-tube absorption cells were employed, permitting complete serial analyses without transfer. A reference curve for glucose and invert sugar has been prepared. The method was developed for rapidly analyzing large numbers of watermelons during a short harvesting period. Good recoveries were obtained when various sugars were added to watermelon juice.

## Literature Cited

(1) Assoc. Official Agr. Chem., Official and Tentative Methods of Analysis, 4th ed., p. 473, 1935.
(2) Evelyn, K. A., *J. Biol. Chem.*, 115, 63 (1936).
(3) Folin, O., *Ibid.*, 77, 421 (1928).
(4) Forsee, W. T., IND. ENG. CHEM., Anal. Ed., 10, 411 (1938).
(5) Hagedorn, H. C., and Jensen, B. N., *Biochem. Z.*, 135, 46 (1923).
(6) Hanes, C. S., *Biochem. J.*, 23, 99 (1929).
(7) Hassid, W. Z., IND. ENG. CHEM., Anal. Ed., 8, 138 (1936).
(8) *Ibid.*, 9, 228 (1937).
(9) Hawkins, J. A., and Van Slyke, D. D., *J. Biol. Chem.*, 81, 459 (1929).
(10) Hoffman, W. S., *Ibid.*, 120, 51 (1937).

CONTRIBUTION No. 7 from U. S. Regional Vegetable Breeding Laboratory, Charleston, S. C.

# Decomposition Temperatures of Some Analytical Precipitates

## Calcium Carbonate and Lead Sulfate

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ONE of the essential steps in many gravimetric analyses is the ignition of a precipitate either to remove a volatile impurity or to change the composition to a more desirable weighing form. The higher the temperature used, the less time will be required to fulfill the purposes of the ignition. However, since all compounds will decompose if heated to a high enough temperature, it is necessary to set a maximum temperature at which it is safe to ignite a precipitate without danger of decomposition.

In analytical chemistry we are concerned mainly with the decompositions which result in a loss of weight or, in other words, in which at least one of the decomposition products is a gas or a volatile substance. Only for those substances whose gaseous decomposition products are normal constituents of the atmosphere will it be possible to establish definite decomposition temperatures. For the class of substances whose gaseous decomposition products are not constituents of the atmosphere, it is not possible to state with exactness the ignition temperature below which no decomposition is possible. The statement of the maximum permissible ignition temperature will necessarily depend upon the conditions under which the ignition is carried out and upon the degree of accuracy desired in the determination.

The aim of these studies is to work out a satisfactory method of determining these dissociation pressures and of correlating the dissociation pressures with the maximum allowable ignition temperatures. The classes of precipitates which have been selected for study are the carbonates, sulfates, phosphates, and chromates. In deciding which individual compounds in each class to study, the authors have selected the list of weighing forms of various elements given by Lundell and Hoffman (*24*). The need for studying these decomposition temperatures becomes evident if we consider the great variation in the ignition temperatures for various precipitates

which are recommended by different investigators and in the standard texts on quantitative analysis.

In reviewing the various investigations pertaining to this subject, two types of results are found. The first are merely qualitative statements, such as "ignition at red heat produces partial decomposition" or "no decomposition results on heating with a blast lamp". Many of these statements were published before the advent of the thermocouple which made possible exact determination of high temperatures. The second type is more of a quantitative nature, either measurements of the dissociation pressures or loss in weight experiments.

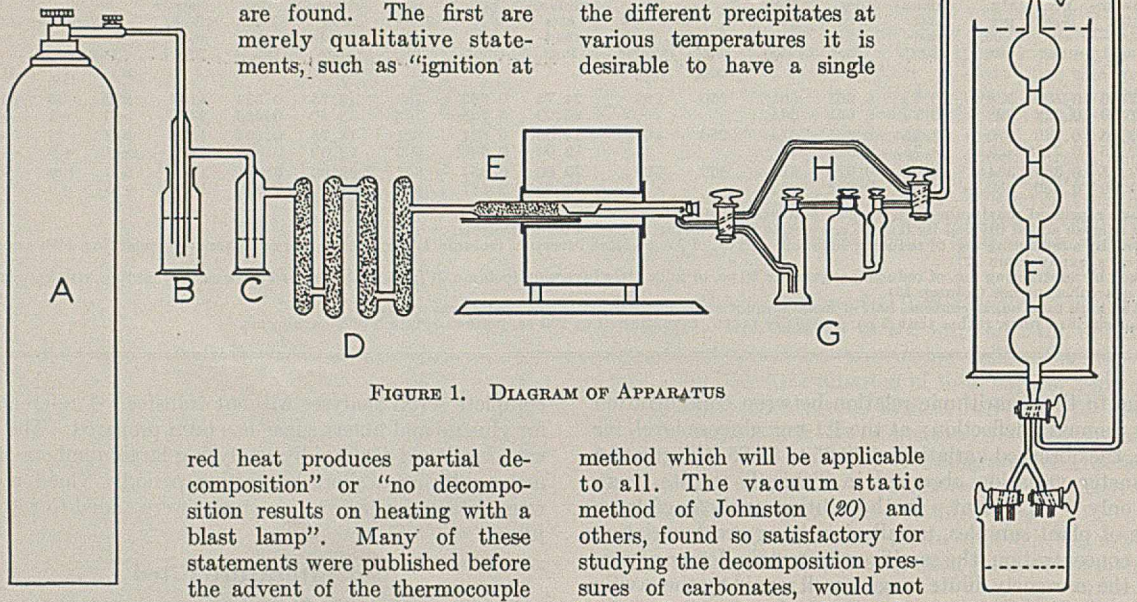


FIGURE 1. DIAGRAM OF APPARATUS

One of the compounds which have been the most extensively and probably the most accurately studied is calcium carbonate, formed by the ignition of calcium oxalate at the proper temperature.

Willard and Boldyreff (*40*) state that it is advisable to carry out this ignition above 450° C. and that decomposition results above 550° C. The methods which have been used for this study include the determination of dissociation pressure (*8*, *16*, *20*, *23*, *26*, *30*, *34-36*, *38*, *41*), loss in weight (*6*, *15*), rate of gas evolution (*1*), gas saturation (*10*, *22*), "differential" method (*7*), emanation (*31*, *42*), and the use of a high-temperature vacuum balance (*4*, *32*, *37*). Probably the most accurate results are those of Johnston (*20*) who used a static method. His values are slightly lower than those of Pott or Zavriev (*41*) and he explained this discrepancy by the fact that in using a very small sample he was able to obtain a uniform temperature throughout the charge which cannot be done with a larger charge. This is a necessary precaution, since the pressure developed will be that in which the carbon dioxide is in equilibrium with the calcium carbonate at the lowest temperature.

The precipitate selected for this first investigation was lead sulfate.

Its decomposition was first mentioned by Berthier (*3*) who in 1822 stated that it decomposes slightly at white heat. Erdman (*11*) in 1854 found that this substance lost weight when heated in an open or loosely covered crucible. According to Boussingault (*5*) a loss of weight occurs on ignition at white heat. Percy (according to Mellor, *25*) in 1870 stated that lead sulfate was permanent at a low red heat. It was found by Bailey (*2*) that no decomposition results on igniting at 500° C. Friedrich and Blickle (*13*) gave the decomposition temperature as 850° C., while Hofman and Wanjukow (*17*) gave 637° C. as the temperature of the beginning of decomposition and 705° C. as the temperature at which dissociation becomes marked. Both these investigations were made by the thermal method. Mostovich (*27*) found that up to 800° C. lead sulfate does not change when heated in a current of dry air but at this temperature sulfur trioxide begins to be liberated. In 1906 Doeltz and Grauman (*9*) found no loss in weight between 700° and 800° C. and only a very slight loss at 900° C. but a big loss at 1000° C. Schenck and Rassbach (*33*) in 1908, Grahmann (*14*) in 1913, and Jaeger and Germs (*19*) in 1921 confirmed the decomposition at between 900° C. and 1000° C.

## Method and Apparatus

For the determination of the dissociation pressures of the different precipitates at various temperatures it is desirable to have a single method which will be applicable to all. The vacuum static method of Johnston (*20*) and others, found so satisfactory for studying the decomposition pressures of carbonates, would not be equally satisfactory for studying sulfates and other substances whose gaseous products are not permanent gases at room temperature and would condense in the cooler part of the apparatus. Therefore the dynamic or gas saturation method was adopted. This method was first used for the determination of vapor pressures of solids by von Wartenberg (*39*) although Regnault (*29*) and others had used it earlier to determine the vapor pressure of liquids. This method consists of passing a measured volume of an inert gas over the heated substance, determining the amount of vapor in the gas, then from Dalton's law calculating the dissociation pressure. The method has also been used by Keppeler and d'Ans (*21*), Dutoit (*10*), Isambert (*18*), and Finkelstein (*12*) for determining the vapor pressures of various substances. Since these authors give insufficient details of their work and their results are not in agreement with those obtained by other methods, this method was first tried on calcium carbonate, as the dissociation pressures of this substance are probably the best known of any materials.

The apparatus used is shown in Figure 1. The nitrogen, which was used as the inert gas, passes from the cylinder, *A*, to a pressure regulator, *B*, through a bubble counter, *C*, and then through a series of tubes, *D*, filled with Ascarite and Dehydrite to remove all carbon dioxide and water. From these tubes it passes to the reaction tube, *E*, a microcombustion tube with a side arm, made of Pyrex or transparent quartz, depending upon the temperature being used. The front part of the tube is filled with small pieces of quartz which serve as baffles, making certain that the nitrogen is heated to the temperature of the sample. Following the quartz granules is the platinum boat containing the sample. The boat is 5 cm. long with the other dimensions such that it will just fit inside the combustion tube.

The combustion tube is heated by an electric furnace, *E*. From the combustion tube the nitrogen passes to the gas buret, *F*, going through either the absorption train, *G*, or the by-pass, *H*. The absorption train used with calcium carbonate consisted of a Stetser-Norton absorption bulb filled with Dehydrite for removing any water and a Wesson absorption bulb filled with Ascarite and Dehydrite. In several experiments Pregl micro absorption tubes were used. In all cases the microprocedure of wiping with moist flannel and chamois was used and a similar tube was used as a tare in weighing. The by-pass was used to permit flushing after introduction of the sample and to make it unnecessary to stop the flow of gas between the flushing and the beginning of the experiment.

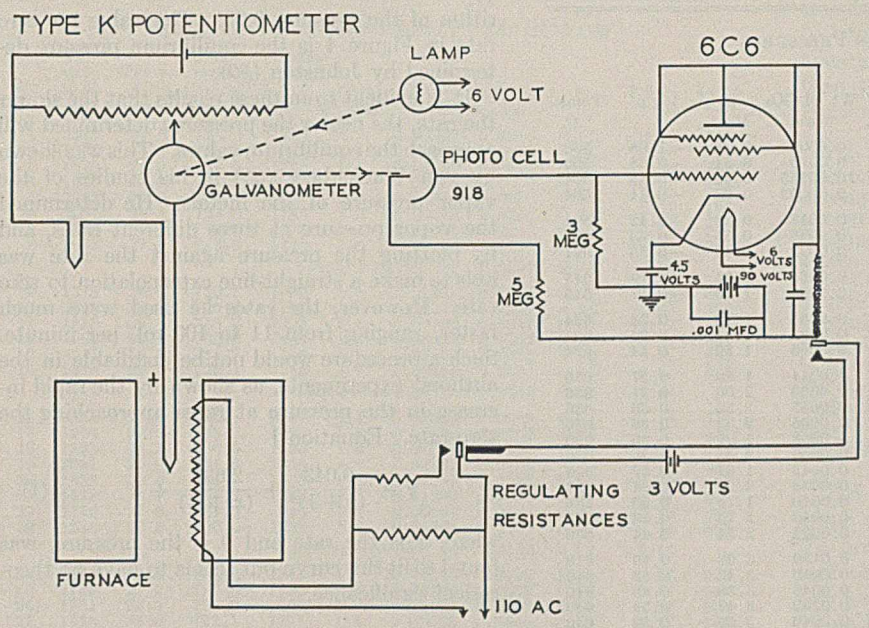


FIGURE 2. DIAGRAM OF REGULATOR

During preliminary runs, it was noted that the temperature of the furnace varied as much as 20° to 25° C. during a day, owing to variations in the line voltage. The furnace was therefore equipped with a temperature regulator with which it was possible to keep the temperature constant to within ±1.0° C. for any length of time. The regulator is shown in Figure 2. It was operated by a chromel-alumel thermocouple, the hot junction of which was placed outside the reaction tube directly beneath the boat containing the sample. The thermocouple was connected in the usual manner to a Leeds & Northrup Type K potentiometer, balance being indicated by a Leeds & Northrup Type R No. E reflecting wall galvanometer. The beam of light reflected from the galvanometer struck a photoelectric cell which, by means of amplification and two relays, switched on an auxiliary heating current. By adjusting the main heating current so that it was not quite sufficient to maintain the desired temperature, and by having the photoelectric cell in such a position that as the temperature fell the light beam struck the cell and turned on the auxiliary current, it was possible to keep the temperature practically constant. The proper key of the potentiometer was kept permanently depressed by a clamp.

The operating current was supplied by a 3-cell, 2-volt storage battery and was adjusted frequently with a standard cell. The cold junction was kept at room temperature, adjusting for any changes. The temperature readings of the chromel-alumel thermocouple were calibrated by a platinum, platinum-rhodium thermocouple, calibrated by the National Bureau of Standards, the junction of which was placed in the reaction tube in the same position that the platinum boat occupied in the experiment. The temperatures recorded in all the experiments are believed to be accurate within ±2.0° C. It was found that a space about 4 cm. long in the tube was at the same temperature and for a distance of 1 cm. on each side of this space it was not more than 1° C. lower. Therefore, in all experiments, the entire contents of the boat were within 1° C. of the same temperature.

## Decomposition of Calcium Carbonate

Pure calcium carbonate was prepared from Baker and Adamson reagent grade calcium carbonate by double precipitation as the oxalate, drying, and then igniting for 12 hours in an electric muffle at 500° C. This temperature converts (*40*) all the oxalate to carbonate. The calcium carbonate was pulverized in an agate mortar and kept in a desiccator. Spectroscopic analysis of this calcium carbonate showed that it contained less than 0.01 per cent of magnesium and no appreciable amounts of any other impurity.

The platinum boat containing 0.5 gram of calcium carbonate was placed in the center of the reaction tube where the temperature was constant to within ±1° C. After replacing the stopper in the mouth of the tube, the flow of nitrogen through the tube and the by-pass was started by opening the stopcock at the bottom of the buret. Generally 500 ml. were used for flushing and increasing this volume seemed to have no effect. It is only necessary to flush sufficiently to remove all air and to give time enough for the sample to begin to dissociate and to saturate the gas with carbon dioxide. When the level of the water in the buret reached the top graduation, the stopcocks were turned to pass the gas through the absorption train. After the desired volume of gas had been aspirated through the system, the flow of water from the buret was stopped and the absorption train disconnected. In many cases several runs were made with the same sample by merely refilling the buret to the top graduation, inserting another absorption train, and omitting any flushing.

At the end of the run the temperature of the nitrogen was noted, the barometric pressure was read, and corrections for gravity, temperature, and capillary depression were applied, and the time required to carry out the run was recorded. The weighings were made as previously described. Any changes in barometric pressure during the experiment were neglected. The pressure in the tube was found to be within 0.2 mm. of the barometric pressure. Since this difference in pressure would have no significant influence on the results, it was also neglected.

The results for the determination of the dissociation pressures at various temperatures are tabulated in Table I and are shown in Figure 3 by plotting the logarithm of the pressure against the reciprocal of the absolute temperature. Only the values obtained at the faster rates were used and for comparison the values obtained by Johnston (*20*) and Andrussow (*7*) have also been plotted on the same graph.

To show the effect of varying rates of gas flow on the dissociation pressures, a series of experiments at 636° C. with different rates was made. The results are shown in Table II and Figure 4. The results at the slowest rate at 640° C. were

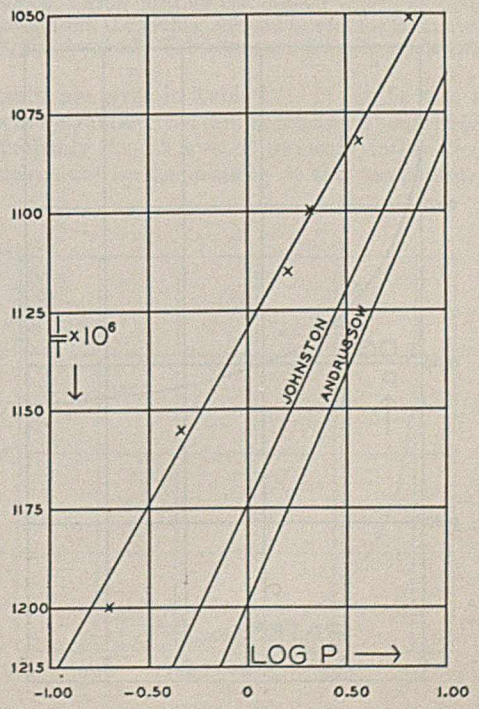


FIGURE 3. EFFECT OF TEMPERATURE

TABLE I. DISSOCIATION PRESSURES

| No. | Vol. | Temp. | Barometric Pressure | Volume at S. T. P. | Rate | Wt. of $CO_2$ | $p$ | log $p$ | Temp. |
|---|---|---|---|---|---|---|---|---|---|
| | *Ml.* | *° C.* | *Mm. Hg* | *Ml.* | *Ml./min.* | *Gram* | *Mm.* | | *° C.* |
| 1 | 2031 | 22.8 | 727.3 | 1744 | 14 | 0.0004 | 0.08 | −1.08 | 560 |
| 2 | 2031 | 23.0 | 727.3 | 1741 | 17 | 0.0010 | 0.21 | −0.68 | 560 |
| 3 | 2031 | 23.3 | 733.2 | 1754 | 10 | 0.00155 | 0.33 | −0.48 | 564 |
| 4 | 2031 | 24.0 | 737.3 | 1755 | 10 | 0.00092 | 0.20 | −0.71 | 564 |
| 5 | 2031 | 22.3 | 728.7 | 1750 | 14 | 0.0018 | 0.38 | −0.42 | 591 |
| 6 | 2031 | 22.8 | 724.2 | 1735 | 17 | 0.0026 | 0.55 | −0.26 | 591 |
| 7 | 2031 | 23.5 | 723.3 | 1736 | 9 | 0.0021 | 0.45 | −0.35 | 591 |
| 8 | 1811 | 21.8 | 733.2 | 1575 | 1.5 | 0.0065 | 1.54 | 0.19 | 615 |
| 9 | 1011 | 22.4 | 733.7 | 877 | 5.8 | 0.0035 | 1.49 | 0.17 | 615 |
| 10 | 2031 | 23.5 | 739.4 | 1765 | 17 | 0.0081 | 1.72 | 0.23 | 624 |
| 11 | 2031 | 23.8 | 739.0 | 1762 | 17 | 0.0085 | 1.81 | 0.26 | 624 |
| 12 | 2031 | 24.0 | 738.9 | 1759 | 23 | 0.0065 | 1.39 | 0.14 | 624 |
| 13 | 1011 | 21.0 | 723.0 | 870 | 10 | 0.0044 | 1.86 | 0.27 | 636 |
| 14 | 1011 | 20.8 | 724.1 | 872 | 10 | 0.0050 | 2.06 | 0.31 | 636 |
| 15 | 1011 | 20.8 | 725.1 | 873 | 10 | 0.0055 | 2.32 | 0.36 | 636 |
| 16 | 1011 | 20.7 | 727.9 | 877 | 10 | 0.0055 | 2.32 | 0.36 | 636 |
| 17 | 1256 | 20.7 | 727.9 | 1092 | 10 | 0.0058 | 1.97 | 0.29 | 636 |
| 18 | 1026 | 20.4 | 751.4 | 922 | 5.7 | 0.0059 | 2.45 | 0.39 | 636 |
| 19 | 1011 | 20.9 | 751.7 | 905 | 5.7 | 0.0045 | 1.87 | 0.27 | 636 |
| 20 | 1011 | 21.2 | 751.1 | 903 | 5.7 | 0.0052 | 2.20 | 0.34 | 636 |
| 21 | 1011 | 21.8 | 739.7 | 886 | 5.7 | 0.0040 | 1.70 | 0.23 | 636 |
| 22 | 1011 | 22.3 | 739.5 | 884 | 5.7 | 0.0050 | 2.13 | 0.33 | 636 |
| 23 | 1011 | 20.8 | 750.8 | 905 | 3.5 | 0.0065 | 2.74 | 0.44 | 636 |
| 24 | 1856 | 22.5 | 735.3 | 1613 | 1.7 | 0.0130 | 3.08 | 0.49 | 640 |
| 25 | 506 | 22.8 | 735.3 | 439 | 1.8 | 0.0040 | 3.40 | 0.53 | 640 |
| 26 | 506 | 22.0 | 734.5 | 440 | 1.5 | 0.0046 | 3.88 | 0.59 | 640 |
| 27 | 1624 | 21.6 | 741.9 | 1431 | 1.7 | 0.0132 | 3.47 | 0.54 | 640 |
| 28 | 575 | 20.5 | 739.4 | 508 | 1.5 | 0.0046 | 3.32 | 0.53 | 640 |
| 29 | 1011 | 24.0 | 746.2 | 885 | 18 | 0.0074 | 3.16 | 0.51 | 651 |
| 30 | 1011 | 24.0 | 746.2 | 885 | 18 | 0.0092 | 3.93 | 0.59 | 651 |
| 31 | 1011 | 24.3 | 745.4 | 883 | 13 | 0.0093 | 3.97 | 0.60 | 651 |
| 32 | 1011 | 24.7 | 745.2 | 881 | 13 | 0.0083 | 3.56 | 0.55 | 651 |
| 33 | 2031 | 22.0 | 732.5 | 1762 | 17 | 0.0320 | 6.70 | 0.83 | 679 |
| 34 | 2031 | 22.5 | 735.6 | 1765 | 17 | 0.0280 | 5.38 | 0.73 | 679 |
| 35 | 2031 | 22.5 | 737.8 | 1770 | 16 | 0.0293 | 6.16 | 0.79 | 679 |
| 36 | 2031 | 22.8 | 736.0 | 1773 | 14 | 0.0338 | 7.07 | 0.85 | 679 |
| 37 | 2031 | 24.5 | 740.6 | 1759 | 14 | 0.0324 | 6.87 | 0.83 | 679 |
| 38 | 2031 | 24.9 | 738.7 | 1750 | 12 | 0.0364 | 7.83 | 0.89 | 679 |
| 39 | 2031 | 25.0 | 738.7 | 1750 | 14 | 0.0288 | 6.20 | 0.79 | 679 |

corrected graphically to 636° C. In Figure 3 the rate of change of the logarithm of the pressure with the temperature is 0.0137 per degree, which agrees well with the corresponding values obtained from the curves of Johnston and Andrussow. Thus a change of 4° C. would change the logarithm of the pressure 0.05. The value for zero rate in Figure 4 is the equilibrium pressure determined by Johnston (*20*).

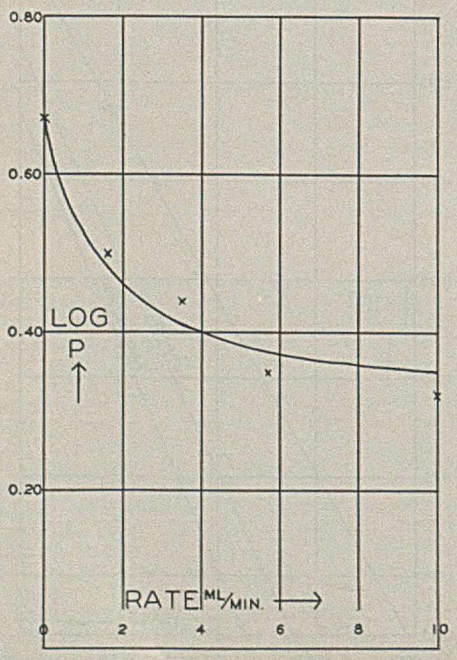


FIGURE 4. EFFECT OF RATE OF GAS FLOW

It is evident from these results that the slower the rate, the nearer the pressures determined will approach the equilibrium values. This was shown by von Wartenberg (*39*) in his studies of the vapor pressure of the metals. He determined the vapor pressure at three different rates, and by plotting the pressure against the rate was able to make a straight-line extrapolation to zero rate. However, the rates he used were much faster, ranging from 11 to 100 ml. per minute. Such a procedure would not be justifiable in the authors' experiments, as shown by the rapid increase in the pressure at rates approaching the zero rate. Equation 1

$$y = \frac{0.043}{(x+1)^2} + \frac{2.637}{(x+1)} + 2 \qquad (1)$$

where $x$ is the rate and $y$ is the pressure, was found to fit this curve but seems to have no theoretical significance.

## Decomposition of Lead Sulfate

Pure lead sulfate was prepared by adding, slowly and simultaneously, a solution of thrice recrystallized lead nitrate and dilute sulfuric acid to 5 liters of hot water, the lead nitrate being kept in slight excess. After thorough digestion, the lead sulfate was separated, washed, dried, and ignited at 500° C. Spectroscopic analysis showed that the lead sulfate contained no significant amount of impurity.

The decomposition of sulfates has two complicating factors. First, the decomposition product, sulfur trioxide, dissociates into sulfur dioxide and oxygen.

$$2SO_3 \longrightarrow 2SO_2 + O_2 \qquad (2)$$

Thus both oxides of sulfur must be absorbed and determined. Although it would be possible to determine these separately, it is not convenient and it would have no significance, as the reverse reaction

$$2SO_2 + O_2 \longrightarrow 2SO_3 \qquad (3)$$

may occur as the gases are cooled down on leaving the furnace. In one experiment at the highest temperature, after

TABLE II. EFFECT OF RATE ON DISSOCIATION PRESSURES

(Temperature 636° C.)

| Run No. | Rate *Ml./min.* | log $p$ |
|---|---|---|
| 13 | 10 | 0.27 |
| 14 | 10 | 0.31 |
| 15 | 10 | 0.36 |
| 16 | 10 | 0.36 |
| 17 | 10 | 0.29 |
| | | Av. 0.32 |
| 18 | 5.7 | 0.39 |
| 19 | 5.7 | 0.27 |
| 20 | 5.7 | 0.34 |
| 21 | 5.7 | 0.23 |
| 22 | 5.7 | 0.33 |
| | Av. (of runs 18, 20, and 22) | 0.35 |
| 23 | 3.5 | 0.44 |
| 24 | 1.7 | 0.44 |
| 25 | 1.8 | 0.48 |
| 26 | 1.5 | 0.54 |
| 27 | 1.7 | 0.49 |
| 28 | 1.5 | 0.48 |
| | Av. (of runs 25, 26, 27, and 28) | 0.50 |
| | Johnston's value for equilibrium or zero rate | 0.67 |

TABLE III. DECOMPOSITION OF LEAD SULFATE

| No. | Volume of $N_2$ | Temp. of $N_2$ | Barometric Pressure | Volume at S.T.P. | Rate | Wt. of $BaSO_4$ | | $p$ | log $p$ | Temp. |
|---|---|---|---|---|---|---|---|---|---|---|
| | *Ml.* | *° C.* | *Mm. Hg* | *Ml.* | *Ml./min.* | *Gram* | *Liter* | | | *° C.* |
| 1 | 2031 | 24.5 | 734.6 | 1745 | 4 | 0.00265 | 0.0015 | 0.11 | −0.96 | 762 |
| 2 | 2031 | 23.5 | 740.5 | 1770 | 4 | 0.00264 | 0.0015 | 0.11 | −0.96 | 762 |
| 3 | 1528 | 23.0 | 739.5 | 1333 | 1.4 | 0.00252 | 0.0019 | 0.14 | −0.86 | 762 |
| 4 | 1518 | 23.5 | 737.2 | 1316 | 1.4 | 0.00268 | 0.0020 | 0.14 | −0.86 | 762 |
| 5 | 2031 | 24.0 | 736.6 | 1755 | 1.3 | 0.00305 | 0.0017 | 0.12 | −0.92 | 762 |
| 6 | 2031 | 24.8 | 734.8 | 1743 | 1.3 | 0.00277 | 0.0016 | 0.11 | −0.96 | 762 |
| 7 | 1868 | 26.6 | 733.8 | 1535 | 1.1 | 0.00261 | 0.0017 | 0.12 | −0.92 | 762 |
| 8 | 1668 | 27.0 | 733.0 | 1410 | 1.4 | 0.00220 | 0.0016 | 0.11 | −0.96 | 762 |
| | | | | Averages at 762° C. | | 0.0017 | 0.12 | −0.92 | | |
| 9 | 1578 | 26.8 | 730.2 | 1330 | 1.5 | 0.00432 | 0.0032 | 0.23 | −0.64 | 809 |
| 10 | 2031 | 26.2 | 730.4 | 1719 | 4 | 0.00512 | 0.0030 | 0.21 | −0.68 | 809 |
| 11 | 1518 | 25.6 | 735.8 | 1298 | 1.4 | 0.00486 | 0.0037 | 0.27 | −0.57 | 809 |
| 12 | 1908 | 25.0 | 736.0 | 1639 | 1.3 | 0.00531 | 0.0032 | 0.23 | −0.64 | 809 |
| 13 | 2031 | 25.0 | 733.7 | 1740 | 4 | 0.00551 | 0.0032 | 0.22 | −0.66 | 809 |
| 14 | 1618 | 25.0 | 728.1 | 1375 | 1.4 | 0.00486 | 0.0035 | 0.25 | −0.61 | 809 |
| | | | | Averages at 809° C. | | 0.0032 | 0.23 | −0.64 | | |
| 16 | 3355 | 23.0 | 731.7 | 2897 | 2.5 | 0.02866 | 0.0099 | 0.69 | −0.16 | 857 |
| 17 | 1956 | 22.5 | 743.7 | 1720 | 1.3 | 0.01850 | 0.0108 | 0.77 | −0.11 | 857 |
| 18 | 1883 | 24.8 | 736.5 | 1620 | 1.6 | 0.01586 | 0.0098 | 0.69 | −0.16 | 857 |
| 19 | 2031 | 25.0 | 734.5 | 1740 | 4 | 0.01851 | 0.0106 | 0.75 | −0.12 | 857 |
| 20 | 1688 | 25.5 | 739.7 | 1453 | 1.4 | 0.01537 | 0.0106 | 0.75 | −0.12 | 857 |
| 21 | 1518 | 26.0 | 734.2 | 1298 | 1.3 | 0.01233 | 0.0095 | 0.67 | −0.17 | 857 |
| 22 | 1110 | 26.0 | 734.2 | 946 | 1.4 | 0.00991 | 0.0105 | 0.74 | −0.13 | 857 |
| 23 | 1518 | 26.8 | 730.9 | 1282 | 1.3 | 0.01311 | 0.0102 | 0.72 | −0.14 | 857 |
| | | | | Averages at 857° C. | | 0.0102 | 0.72 | −0.14 | | |
| 24 | 1478 | 27.3 | 730.6 | 1245 | 4 | 0.06625 | 0.0532 | 3.72 | 0.57 | 904 |
| 25 | 1728 | 27.2 | 726.6 | 1446 | 1.5 | 0.07772 | 0.0533 | 3.72 | 0.57 | 904 |
| 26 | 1981 | 26.4 | 726.9 | 1666 | 4 | 0.09160 | 0.0550 | 3.81 | 0.58 | 904 |
| 27 | 1518 | 25.0 | 732.1 | 1297 | 1.4 | 0.07002 | 0.0540 | 3.78 | 0.58 | 904 |
| 28 | 518 | 24.6 | 732.9 | 444 | 1.2 | 0.02232 | 0.0503 | 3.52 | 0.55 | 904 |
| 29 | 1201 | 24.5 | 735.8 | 1034 | 4 | 0.05999 | 0.0581 | 4.07 | 0.61 | 904 |
| 30 | 1036 | 23.0 | 742.1 | 908 | 1.2 | 0.04974 | 0.0548 | 3.89 | 0.59 | 904 |
| 31 | 1056 | 23.5 | 742.5 | 922 | 4 | 0.04999 | 0.0543 | 3.85 | 0.59 | 904 |
| 32 | 1011 | 23.5 | 742.2 | 882 | 4 | 0.04831 | 0.0548 | 3.89 | 0.59 | 904 |
| 33 | 1011 | 23.4 | 742.7 | 883 | 4 | 0.04795 | 0.0543 | 3.85 | 0.59 | 904 |
| | | | | Averages at 904° C. | | 0.0550 | 3.81 | 0.58 | | |

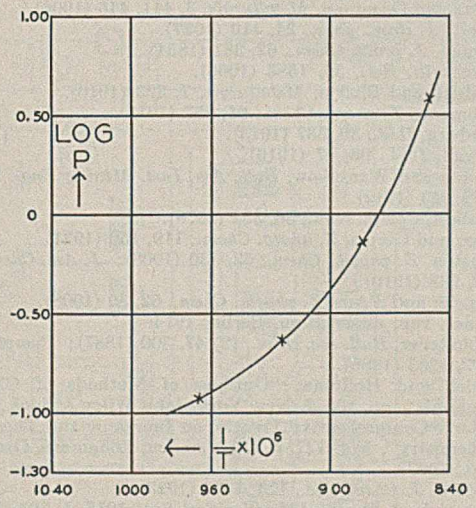


FIGURE 5

the alkali used to absorb the oxides of sulfur was made just neutral to methyl red and hydrogen peroxide was added to oxidize the sulfur dioxide, it required almost as much alkali as had been originally used to bring the solution back to neutral. This indicates that practically all of the sulfur trioxide was converted to sulfur dioxide and that the reverse reaction (Equation 3) is unimportant.

The other disturbing factor is the possibility of the sulfur trioxide condensing on the cooler parts of the apparatus before reaching the absorption flask. To prevent this the absorption flask was placed as near the reaction tube as conveniently possible. No visible signs of condensation were noted; but after a series of ten to fifteen runs, a small amount of sulfur trioxide fumes was visible on heating the part of the tube usually outside the furnace. Since this could not be prevented and as indicated previously, the amount of sulfur trioxide was probably much less than that of sulfur dioxide, this was neglected. However, it is a possible source of error.

The determinations were made the same as with calcium carbonate except for the absorption of the decomposition products. The oxides of sulfur were absorbed in dilute sodium hydroxide and determined gravimetrically, so no desiccant was needed. An excess of sodium hydroxide was used, about 10 to 15 ml. of 0.01 *N* at the lower temperatures and double this amount at the highest temperature. The alkali was placed in a 50-ml. Erlenmeyer flask, fitted with two delivery tubes. The tip of the inlet tube was drawn out to a capillary and dipped below the surface of the liquid. A second flask showed that all the oxides of sulfur were absorbed in this flask. The analysis for the oxides of sulfur was made by the standard microprocedure.

The results are given in Table III and a graph of the averages, plotting the logarithm of the pressure against the reciprocal of the absolute temperature, is shown in Figure 5. Only two rates were used and, as is evident from Table III, no significant differences are apparent at the two rates in this case.

Figure 6 is a graph of the same results showing the weight of sulfur trioxide per liter of nitrogen plotted against the centigrade temperature. It would seem that below 710° C. no appreciable loss in weight should occur when lead sulfate is heated in a muffle furnace, while at 730° C. a small but definite loss should occur. This prediction was tested by determining the loss in weight of lead sulfate on heating.

A platinum-wound muffle furnace (*28*) of about 700-ml. volume was used. The temperature regulator previously described was used and the temperature was measured with a platinum, platinum-rhodium thermocouple. The temperatures are believed to be accurate to within ±5° C.

Two samples of lead sulfate were weighed into small 8-ml. platinum crucibles and placed in the center of the muffle. One crucible was covered with a loose-fitting lid while the other was left uncovered. After heating the desired length of time, they were removed from the muffle, allowed to cool for 20 minutes in a desiccator, and weighed. The same samples were used repeatedly.

The results are given in Table IV. It can be seen that a small loss in weight occurred during the first 2 hours at 680° C. This is probably due to loss of moisture rather than decomposition, since further heating at this temperature and

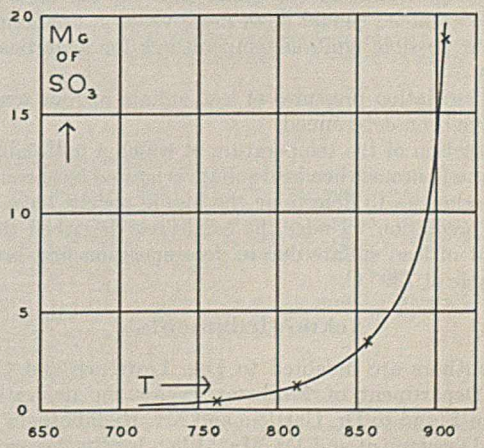


FIGURE 6

TABLE IV. LOSS IN WEIGHT OF LEAD SULFATE

| Temp. | Time: Each ignition | Time: Total at each temperature | Sample 1 (Covered). Original Weight, 1.5469 Mg. Weight after each ignition | Loss in Weight: Loss in each ignition | Loss in Weight: Total at each temperature | Loss per hour (av.) | Sample 2 (Uncovered). Original Weight, 1.1671 Mg. Weight after each ignition | Loss in Weight: Loss in each ignition | Loss in Weight: Total at each temperature | Loss per hour (av.) |
|---|---|---|---|---|---|---|---|---|---|---|
| ° C. | *Hours* | *Hours* | *Mg.* | *Mg.* | *Mg.* | % | *Mg.* | *Mg.* | *Mg.* | % |
| 680 | 2 | 2 | 1.5465 | 0.4 | 0.4 | | 1.1668 | 0.3 | 0.3 | |
| | 4.5 | 6.5 | 1.5465 | 0.0 | 0.4 | | 1.1669 | (0.1) | 0.2 | |
| | 12 | 18.5 | 1.5464 | 0.1 | 0.5 | 0.0018 | 1.1668 | 0.1 | 0.3 | 0.0019 |
| 700 | 3 | 3 | 1.5463 | 0.1 | 0.1 | | 1.1667 | 0.1 | 0.1 | |
| | 3 | 6 | 1.5463 | 0.0 | 0.1 | | 1.1667 | 0.0 | 0.1 | |
| | 5 | 11 | 1.5463 | 0.0 | 0.1 | | 1.1667 | 0.0 | 0.1 | |
| | 11 | 22 | 1.5463 | 0.0 | 0.1 | 0.0003 | 1.16665 | 0.05 | 0.15 | 0.0008 |
| 720 | 12 | 12 | 1.5463 | 0.0 | 0.0 | | 1.1665 | 0.15 | 0.15 | |
| | 10.5 | 22.5 | 1.5462 | 0.1 | 0.1 | | 1.1664 | 0.1 | 0.25 | |
| | 29 | 51.5 | 1.5462 | 0.0 | 0.1 | 0.0001 | 1.1660 | 0.4 | 0.65 | 0.0011 |
| 730 | 16 | 16 | 1.5460 | 0.2 | 0.2 | | 1.1655 | 0.5 | 0.5 | |
| | 7 | 23 | 1.5460 | 0.0 | 0.2 | | 1.1653 | 0.2 | 0.7 | |
| | 42 | 65 | 1.5453 | 0.7 | 0.9 | 0.0009 | 1.1635 | 1.8 | 2.5 | 0.0033 |
| 800 | 1 | 1 | 1.5449 | 0.4 | 0.4 | | 1.1628 | 0.7 | 0.7 | |
| | 1 | 2 | 1.5449 | 0.0 | 0.4 | | 1.1626 | 0.2 | 0.9 | |
| | 1 | 3 | 1.5447 | 0.2 | 0.6 | | 1.1621 | 0.5 | 1.4 | |
| | 1 | 4 | 1.5445 | 0.2 | 0.8 | | 1.1618 | 0.3 | 1.7 | |
| | 1 | 5 | 1.5444 | 0.1 | 0.9 | 0.019 | 1.1616 | 0.2 | 1.9 | 0.033 |

at 700° C. caused no further appreciable loss. At 720° C. the sample in the open crucible showed a slight loss in weight on each ignition while the weight of the sample in the covered crucible remained almost the same. At 730° C. the decomposition is slightly more marked, although heating for 1 or 2 hours at this temperature would not introduce a noticeable error when igniting samples as large as 1 gram. The loss in weight of 800° C. is appreciable in the course of 1 hour. It is of interest to note that both samples turned slightly yellow at the temperature at which the loss in weight became noticeable. To be certain that the loss in weight reported is not due to volatilization of the platinum, the weights of the crucibles before use were compared with those after all the ignitions were completed. The total loss in weight of the covered and uncovered crucibles after 162 hours' heating was 0.1 and 0.2 mg., respectively.

## Summary

The dissociation of calcium carbonate has been studied by the gas saturation method of determining vapor pressures and the results have been compared with the values obtained by Johnston with a static method.

The effect of the rate of gas flow in this method has been studied and the results indicate that equilibrium values are not attainable at rates which are convenient to use. The slower the gas rate, the nearer the pressures approach equilibrium values.

Since this method more nearly duplicates the conditions in an actual ignition than the static method, the dissociation pressures obtained should be of more value in predicting the maximum possible ignition temperatures for analytical precipitates.

The dissociation pressures of lead sulfate at four temperatures have been determined.

A prediction of the temperature at which a noticeable loss in weight will occur when lead sulfate is ignited has been made and experiments to determine the loss in weight have borne out the prediction. Under the conditions described the loss in weight of lead sulfate due to decomposition first becomes appreciable at 730° C.

## Acknowledgments

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## Literature Cited

(1) Bäckström, *Z. physik. Chem.*, **121**, 289 (1926).
(2) Bailey, *J. Chem. Soc.*, **51**, 676 (1887).
(3) Berthier, *Ann. chim. phys.*, (1) **20**, 275 (1822).
(4) Bito *et al.*, *J. Soc. Chem. Ind. Japan*, Suppl. Binding, **34**, 145 (1931); **35**, 191 (1932); **36**, 152–7 (1933).
(5) Boussingault, *J. prakt. Chem.*, **102**, 90 (1867); *Ann. chim. phys.*, (4) **12**, 419 (1867).
(6) Brill, *Z. anorg. Chem.*, 45, 275 (1905).
(7) Centnerszwer and co-workers, *Z. physik. Chem.*, **111**, 79 (1924); **114**, 237 (1924); **116**, 81 (1925).
(8) Debray, *Compt. rend.*, 64, 603 (1867).
(9) Doeltz and Grauman, *Metallurgie*, 3, 441, 446 (1906).
(10) Dutoit, *J. chim. phys.*, 24, 110 (1927).
(11) Erdman, *J. prakt. Chem.*, 62, 381 (1854).
(12) Finkelstein, *Ber.*, 39, 1585 (1906).
(13) Friedrich and Blickle, *Metallurgie*, 7, 323 (1910).
(14) Grahmann, *Z. anorg. Chem.*, 81, 257 (1913).
(15) Grünberg, *Ibid.*, 80, 337 (1913).
(16) Hedvall, *Ibid.*, 98, 47 (1916).
(17) Hofman and Wanjukow, *Bull. Am. Inst. Mining Eng.*, No. 69, 889–943 (1912).
(18) Isambert, *Compt. rend.*, 86, 332 (1878).
(19) Jaeger and Germs, *Z. anorg. Chem.*, **119**, 150 (1921).
(20) Johnston, *Z. physik. Chem.*, 62, 330 (1908); *J. Am. Chem. Soc.*, 32, 938 (1910).
(21) Keppeler and d'Ans, *Z. physik. Chem.*, 62, 89 (1908).
(22) Kohner, von, dissertation, Berlin, 1914.
(23) Le Chatelier, *Bull. soc. chim.*, (2) 47, 300 (1887); *Compt. rend.*, 102, 1243 (1886).
(24) Lundell and Hoffman, "Outlines of Methods of Chemical Analysis", pp. 105–7, New York, John Wiley & Sons, 1938.
(25) Mellor, "Comprehensive Treatise on Inorganic and Theoretical Chemistry", Vol. VII, p. 807, London, Longmans, Green and Co., 1927.
(26) Mitchell, *J. Chem. Soc.*, 123, 1055 (1923).
(27) Mostovich, *J. Russian Metallurgical Soc.*, **1915**, I, 303–27.
(28) Nichols and Schempf, IND. ENG. CHEM., Anal. Ed., **11**, 278 (1939).
(29) Regnault, *Ann. chim. phys.*, (3), 15, 129 (1845).
(30) Riesenfeld, *J. chim. phys.*, 7, 561 (1909).
(31) Sagortschew, *Z. physik. Chem.*, A182, 31 (1938); **183**, 153 (1938).
(32) Saito, *Sci. Repts. Tôhoku Imp. Univ.*, 16, 37–200 (1927).
(33) Schenck and Rassbach, *Ber.*, 41, 2917 (1908).
(34) Smyth and Adams., *J. Am. Chem. Soc.*, 45, 1157 (1923).
(35) Sosman, Hostetter, and Mervin, *J. Wash. Acad. Sci.*, 5, 563–9 (1915).
(36) Southard and Royster, *J. Phys. Chem.*, 40, 435 (1936).
(37) Tamaru, Siomi, and Adati, *Z. physik. Chem.*, A157, 447 (1931).
(38) Turner, *J. Soc. Glass Tech.*, 14, 409 (1930); 16, 80 (1932).
(39) von Wartenberg, *Z. Elektrochem.*, 19, 482 (1913).
(40) Willard and Boldyreff, *J. Am. Chem. Soc.*, 52, 1888 (1930).
(41) Zavriev, *Compt. rend.*, 145, 428 (1907); *J. chim. phys.*, 7, 31 (1909).
(42) Zimens, *Z. physik. Chem.*, B37, 231, 241 (1937).

PRESENTED before the Division of Physical and Inorganic Chemistry at the 99th Meeting of the American Chemical Society, Cincinnati, Ohio.

# A Precision Cryostat for the Range −35° to +25° C.

## An Improved Electrical Circuit and a New Expansion Valve

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IN A PREVIOUS article (*1*), a description was given of a precision cryostat which continuously maintained temperatures to within a few thousandths of a degree within the range −35° to +25° C. Two important improvements in the system have since been made and are described herein.

The electrical circuit shown in the previous article (*1*) can be considered to consist of two main parts, the only connection between them being established through the grid circuits of the electron tubes; one part, containing the automatically controlled autotransformer (Variac), supplied modulated energy continuously to one heating coil immersed in the bath, called the steady heater, and the other part, containing the Thyratron tube, supplied on-off energy to a second heating coil, designated as the intermittent heater. This circuit had been so arranged in order to eliminate the undesirable effects of large thermoregulator current, interaction of grid currents, and failure of the Thyratron to "fire" if the voltage drop across it became too low. The use of a separate heating coil for the on-off energy input to the bath led to frosting inside the heating coil sheath, owing to the "breathing" effect, to the minute amount of energy being dissipated, and to the fact that the coil was energized but approximately half of the time. At times this frosting led to undesirable stray currents in the bath.

A simpler circuit with several advantageous features has been developed and is shown in Figure 1, the lettering being made to correspond to Figure 3 (*1*), and additional parts being assigned new designations.

Only one electron tube, the Thyratron, is employed, resulting in a simplified grid circuit and somewhat smaller grid currents. The modulator remains the same as before, as does the supply to the steady heater from the automatically controlled output of the Variac, but in the present circuit the intermittent energy input is superimposed upon the steady current, employing only one heating coil immersed in the bath. The resultant operation is somewhat more satisfactory than the previous circuit, the thermal lag in the intermittent heating is slightly decreased, and no frosting takes place in the heating coil sheath. The manner of intercoupling the Variac and the Thyratron is apparent from Figure 1, the small transformer, *P*, serving to induce a small additional current in the main circuit, *M4-H1-R5-E*. There is no danger that the voltage drop across the Thyratron will attain a low value, for it is not appreciably influenced by the main circuit. The proper voltage drop across *R4* is secured by adjusting *R8*. Adjusting the taps at *X* on the secondary of transformer *P*, and if necessary changing the values of *R6* and *R7*, the intermittent current may be made as small as desired. A very desirable characteristic of such a type of control, although not required for the present installation, is that by interchanging leads *Y* and *Z* on *P*, a decrease in the energy supplied to heater *H1* is secured when Thyratron *T2* passes current.

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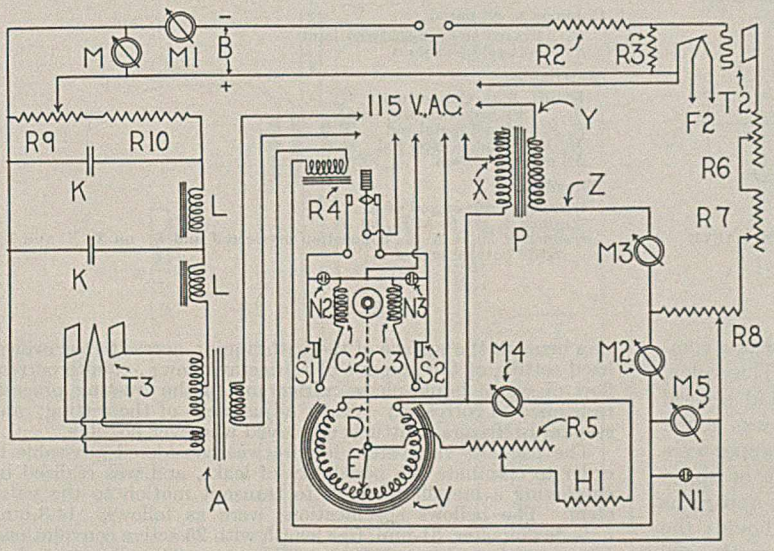


FIGURE 1. IMPROVED ELECTRICAL CONTROL CIRCUIT

*A.* Power supply transformer for rectifier
*T3.* Type 80 rectifier electron tube
*L.* Choke coils, 30 henries, 15-milliampere capacity
*K.* Capacitances, 4 mfd., 300 volts
*R9.* Variable resistor, 5000-ohm, 2-watt size
*R10.* 10,000-ohm fixed resistor, 2-watt size
*M.* Direct current voltmeter, 0 to 50 volts
*M1.* Direct current microammeter in thermoregulator circuit, 0 to 50 microamperes
*B.* Rectifier-filter output, 0 to 25 volts
*T.* Thermoregulator connections
*R2.* Resistor, 0.5 megohm
*R3.* Grid leak resistor, 2.0 megohms
*T2.* Type FG-57 Thyratron tube
*F2.* Heater connections for *T2*, 5.0 volts
*R6.* Variable resistor, 30 ohms, 3 amperes
*R7.* Variable resistor, 100 ohms, 1.5 amperes
*R8.* Variable resistor, 65 ohms, 2 amperes
*M3.* Ammeter, 0 to 3 amperes
*P.* 150-watt toy transformer, primary *YZ* in Thyratron circuit, secondary in Variac circuit
*X.* Variable selector switch on secondary of *P*
*R4.* Mechanical relay, 11,000 ohms, contact in position shown for cooling part of cycle when relay is not energized—that is, Thyratron not passing current
*C2.* Telechron stator coil, energized on heating part of cycle to rotate movable arm *E* on Variac clockwise, increasing amount of energy supplied to heater, *H1*
*C3.* Telechron stator coil, energized on cooling part of cycle to rotate *E* counterclockwise, decreasing amount of energy supplied to *H1*
*N2.* Neon glow lamp across leads to *C2*, 2 watts
*N3.* Neon glow lamp across leads to *C3*, 1 watt
*S1, S2.* Safety contact devices, normally closed as shown; a special arm on *E* prevents it from passing beyond end of normal path, by opening *S1* or *S2*.
*D.* Mechanical coupling between Telechron rotor and *E*
*E.* Variable rotating contact switch on Variac
*V.* Variac, a variable autotransformer
*M4.* Ammeter in heater circuit, 0 to 3 amperes
*R5.* Variable resistor in heater circuit, 80 ohms, 2 amperes
*H1.* Heater coil immersed in bath liquid, 26 ohms
*N1.* Neon glow lamp across intermittent action leads, installed directly above thermometer-galvanometer scale
*M5.* Voltmeter across output from *R8*, 0 to 50 volts
*M2.* Milliammeter in output leads of *R8*, 0 to 50 milliamperes

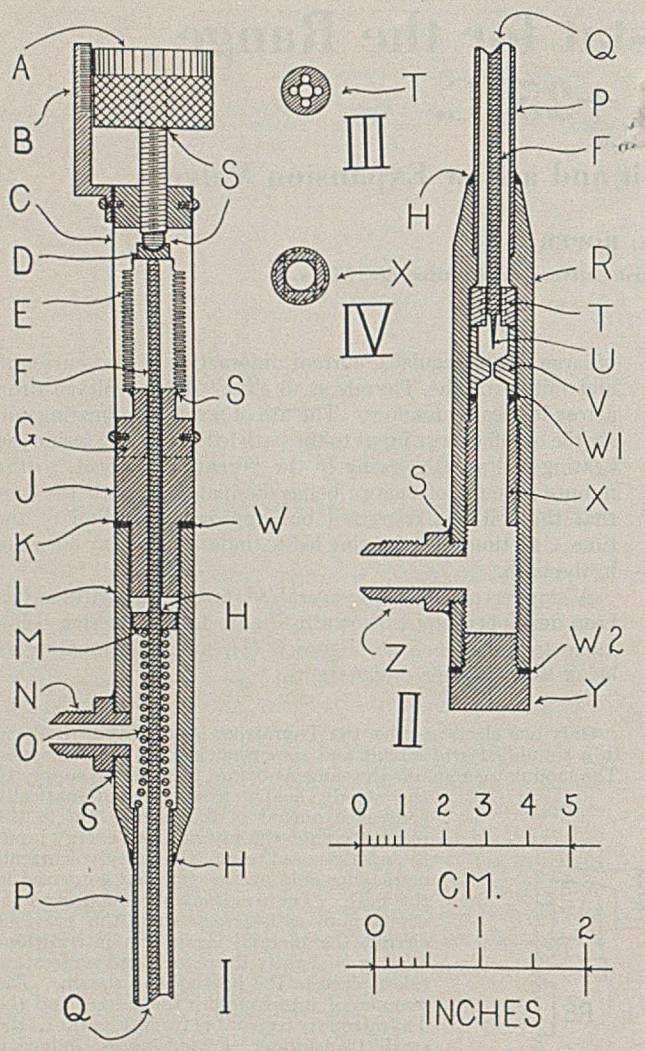


FIGURE 2. CROSS-SECTIONAL VIEW OF EXPANSION VALVE

Part I.

*A*. 50-division circular vernier engraved on top portion of knurled adjusting wheel
*B*. Turn counter, employed in connection with vernier
*C*. Tubing sheath for protection of bellows and for support of *A*, *B*
*D*. Thrust washer, soldered to *A*, free contact with *E*
*E*. Flexible circular bellows
*F*. Valve stem rod
*G*. Circular portion turned on end of hexagonal bar piece *J* to engage and support *C*
*J*. Hexagonal bar piece for support of upper structure, for providing a bearing for *F* and for engaging *L*
*K*. Joint made in assembly of all pieces
*L*. Hexagonal bar piece containing spring chamber and liquid refrigerant entry port
*M*. Thrust collar for *O*, soldered to *F*, slides freely in *L*
*N*. Standard refrigeration flare union for 0.63-cm. (0.25-inch) copper tubing, modified by cutting off portion of one end
*O*. Spring to keep *F* thrust against inside top part of *E*
*P*. Extension tube, connecting upper part of valve as shown in Part I with lower part as shown in II
*Q*. Joined to *Q* in Part II
*W*. Washer gasket

Part II.

*F*, *P*, *Q*. See Part I
*R*. Hexagonal bar piece forming body of expansion chamber
*T*. Centering bearing for *F*, drilled with holes to permit passage of liquid refrigerant
*U*. End of valve stem rod turned down to fit into orifice in *V*; regulation of vertical position permits varying amounts of liquid refrigerant to vaporize from chamber about *U* into chamber below *V*.
*V*. Orifice-containing piece
*W1*, *W2*. Washer gaskets
*X*. Thrust nut, to force gasket *W1* tightly against sliding-fit pieces *T* and *V*, making a seal against liquid refrigerant
*Y*. End nut formed of hexagonal bar; removal allows ready access to orifice chamber.
*Z*. Standard refrigeration flare union for 0.78-cm. (0.3125-inch) copper tubing, modified by cutting off a portion of one end; subsequently coupled onto bottom end of 0.9375-cm. (0.375-inch) copper tubing expansion coil through intermediary of a reducing union.

Part III.

*T*. End view of centering bearing, *T*, showing position of holes for liquid flow with respect to bearing hole

Part IV.

*X*. Bottom-end view of thrust nut, *X*, showing portion of center hole scraped out square; a special square-end wrench allows this nut to be inserted or removed from threading in *R*.

Common to all parts.

*H*. Brazed or hard-soldered joint
*S*. Soft-soldered joint

Material list.

*W*, *W1*, *W2*. Copper plate
*O*. Phosphor-bronze wire
*F*, *V*. Monel metal rod
*M*, *X*. Stainless steel rod
All others. Brass

Thread sizes.

1/4-inch × 40, on rod of *A*
7/16-inch × 20, S. A. E., on *N*
1/2-inch × 20, S. A. E., connection between *J* and *L*; on *X*, *Y*, and *Z*; inside bottom end of *R*

In the previous description of the cryostatic system, a commercial type of expansion valve was mentioned. This valve, although exhibiting good enough performance, had several undesirable features. The manual adjustment was much too critical; the size and shape of the instrument proper were such that it required a relatively large amount of space above the cryostat, this space being required for apparatus which was to be partially immersed in the bath liquid; the expansion of the refrigerating liquid took place above the surface of the bath liquid, the size and construction of the valve precluding direct immersion in the bath; an objectionable amount of frosting took place on the valve, in spite of heavy insulation; and the valve was of the diaphragm type, so constructed as to give a constant pressure in the evaporator coil. Since the pressure in the evaporator coil did not appreciably vary, owing to the action of the constant-pressure valve, *Q*, (Figure 2, *I*), this function was unnecessary, but the diaphragm was influenced by changes in barometric pressure and in the pressure of the liquid phase on the compressor high side due to changes in the room temperature; this latter change acted in contradistinction to that required for good modulation.

A new valve incorporates the following features: absence of a packed valve stem; small, compact size, with expansion occurring beneath the surface of the bath liquid; access to the orifice; fixed setting of the amount of refrigerant flow; countercurrent flow of warm liquid-phase refrigerant to the frosting process; resistance to corrosion; vernier adjustment of the setting; and standard refrigerant fittings employed wherever possible.

The packless valve-stem feature was considered advisable in order to eliminate the possibility of leaks, and was realized by employing a metallic bellows to transmit motion to the valve stem. The bellows specifications were as follows: 14.3-mm. inside diameter, 31-mm. free length with 25 active convolutions, fabricated from brass (80 per cent copper, 20 per cent zinc) of 0.114-mm. wall thickness. A motion of 7 mm. was obtainable on the compressive motion and the maximum internal pressure allowable was 14.3 atmospheres, a value somewhat in excess of that to be expected when in actual use. One end of the bellows was closed, and the open end was soldered onto a machined part that screwed into the valve assembly. (This bellows was a standard item fabricated by the Clifford Manufacturing Co., Boston, Mass., which soldered the bellows to the machined brass part and tested the subsequent joint.)

Hexagonal brass bar was employed for the main body parts of the valve, since it was then easy to use standard wrenches in assembling the constituent parts. Figure 2 shows a cross-sectional view of the valve, broken in two to avoid an unduly long drawing. The connection between the upper and the lower parts was about 25 cm., but any suitable length may be employed; for an exceptional length, it would appear necessary to have a centering bearing similar to *T*, in the connecting tube. In operation, the liquid refrigerant enters at *N* and passes through the interior of tube *P*, effectively preventing much frosting from taking place

on the exterior of the tube. The centering bearing, $T$, has four holes in it, in addition to the bearing hole for $F$, for the passage of the liquid refrigerant through the bearing piece. The actual expansion from liquid to gas, or to mist, takes place through the orifice in $V$.

A commercial refrigeration halide leak tester has been found an invaluable adjunct for working with such an experimental system.

### Acknowledgments

This work was done for Arthur B. Lamb in his laboratory and the author wishes to thank him for permission to publish this description. W. W. Rice aided in testing the initial operation of the new expansion valve. The actual construction of the valve was effected by Alfred Gedies, the laboratory technician, to whom credit is due for his careful execution of the task and for certain suggestions, both factors contributing materially to the successful functioning of the instrument.

### Literature Cited

(1) Roper, Ind. Eng. Chem., Anal. Ed., 12, 113 (1940).

# An Improved Electrodialyzer

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ELECTRODIALYZERS on the market consist of three glass cells, clamped together in a specially designed stand, and in which two parchment or cellophane membranes separate the middle cell from the outer cells. In addition to the fact that the middle cell has only a small capacity, the apparatus is somewhat difficult to handle, the dialyzing surface is small, and leaks may occur between the membranes and the cells. It was desired, therefore, to design an electrodialyzer which would not only allow one to dialyze larger quantities of liquor, but would require little wash water for the electrodes—a distinct advantage when the material that dialyzes out is to be recovered by evaporation of the wash water.

An electrodialyzer which permits dialysis of larger amounts of liquids in a relatively short time and has been found to work satisfactorily is shown in Figure 1.

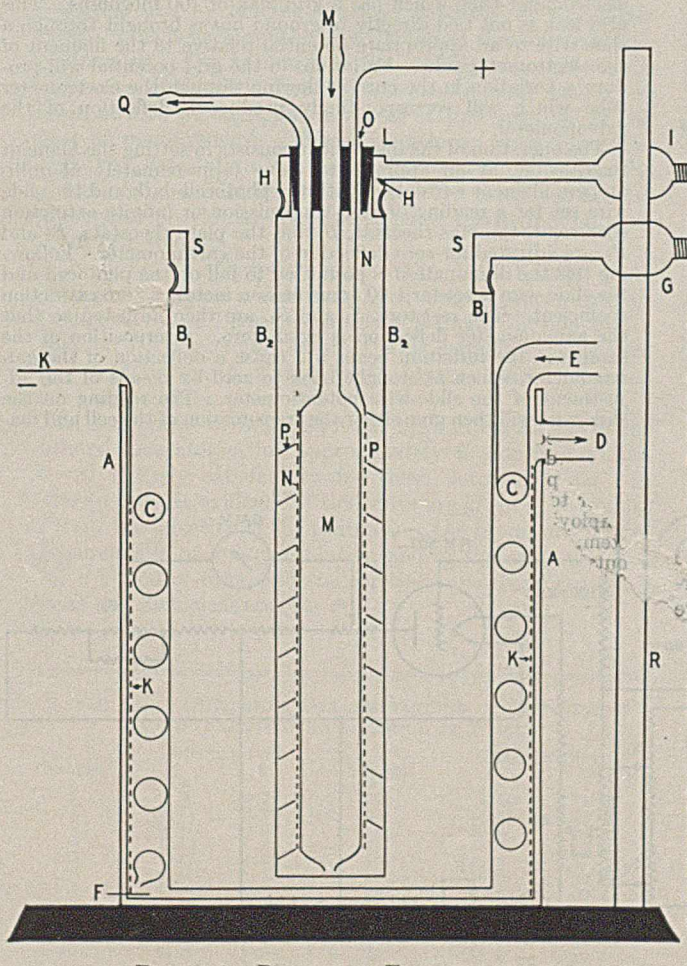


FIGURE 1. DIAGRAM OF ELECTRODIALYZER

The three cells required in any electrodialysis apparatus are formed by the glass vessel, $A$, and the two dialyzing bags, $B_1$ and $B_2$. The outside cell, formed by $A$ and $B_1$, has a platinum gauze electrode, $K$, which is laid against the glass wall of $A$ and is used as a cathode. $K$ is separated from $B_1$ by the glass tube spiral, $C$, which serves a double purpose. It prevents $B_1$, after it has been filled with the liquor to be dialyzed, from coming into contact with $K$ and thus soon rendering the bag defective; further, the wash water for $K$ is passed into the outside cell through this spiral. The water enters the spiral at $E$ and the outside cell at $F$. Inasmuch as the spiral lies close to $K$ on one side and $B_1$ on the other, the water is forced to pass through the outside cell along the spiral between $K$ and $B_1$ and, in this way, causes a thorough washing of the electrode. The water leaves the outside cell at the overflow, $D$. The small distance between $B_1$ and $K$ reduces the resistance of the wash water. The spiral fills a considerable amount of the space between the electrode and the bag, thus reducing the amount of wash water and increasing the concentration of the mineral content of the outside wash water. $B_1$ is suspended on a ring, $S$, which, in turn, is fastened to a ringstand, $R$, by means of the screw clamp, $G$.

The inner cell is formed by the dialyzing bag, $B_2$, which is tightly fastened to the ring, $H$, which, in turn, is screwed to the ringstand, $F$, at $I$. The glass tube, $M$, which is widened at its lower half, is inserted in $B_2$ and held in place by a three-hole rubber stopper, $L$. Around the outerside of the wider part of $M$, the electrode, $N$, is wound, the lead wire of which is led through [illegible] led in the capillary tube, $O$. In order to prevent any [illegible] tact between $B_2$ and $N$, the latter is protected by the glass ribbon sp[illegible] $P$, the ribbon of which forms an angle of about 70° with the spiral axis. The wash water enters the inner cell at the bottom of $M$, is forced by the glass spiral to rinse $N$, and leaves the cell through the glass tube at $Q$.

When hot water is passed through the outer and inner cells, the dialysis can be carried out at higher temperatures. The dialyzer can be used as an ordinary dialysis apparatus by taking out the inner cell at $I$. On account of its relatively large dialyzing surface, the dialysis proceeds rapidly and may be further accelerated by stirring the liquor in the middle cell.

Cotton cloth bags impregnated with viscose have proved most satisfactory as containers.

# Photoelectric Photometer for Vitamin A Estimation

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VARIOUS methods have been devised for physically determining the amount of preformed vitamin A present in materials. One satisfactory type of measurement is based on the determination of the spectral absorption at 328 millimicrons, since Morton and Heilbron (*3*) found that the absorption of vitamin A–containing materials at that wave length was directly related to the potency of the material.

Numerous instruments have been devised for determining this absorption. One instrument, the Hilger Vitameter, employs visual estimation of the intensity of radiation transmitted by a solution of the vitamin A–containing material, the radiation being allowed to fall on a fluorescent screen. Absorption spectrograms have been used with densitometric determinations of the blackening of the photographic plate. These methods, while essentially correct, are approximate in the first case and tedious in the second and the latter requires relatively expensive equipment.

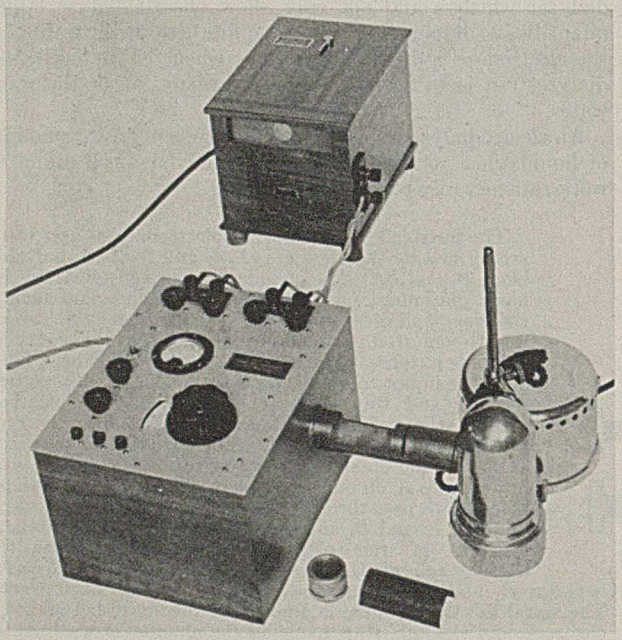

FIGURE 1. ASSEMBLED EQUIPMENT

Two simple instruments have been devised, one by Bills and Wallenmeyer (*1*) and the other by McFarlan, Reddie, and Merrill (*2*), which employ radiation in the vicinity of 328 millimicrons.

The instrument of Bills and Wallenmeyer utilizes an argon lamp as the source of radiation which with suitable filters isolates two bands, one slightly above and the other slightly below 328 millimicrons. This instrument requires calibration with materials of known potency, as readings are not strictly linear. The instrument designed by McFarlan, Reddie, and Merrill employs a sodium arc together with a red-purple Corex filter isolating radiation at 330 millimicrons. The radiation from this source is split into two halves, one of which passes through the test sample. In the case of this instrument it is necessary to balance the responses of the two photocells and amplify it. The adjustments determining the absorption of the material are based on the openings of diaphragms before the photocells.

The present paper describes the results of another investigation having for its object the construction of an economical instrument for this purpose, which would be portable, accurate, and simple in operation. Figure 1 shows a photograph of the equipment as it was finally assembled.

The sodium Lab-arc is shown, together with the controls and the tube in which the absorption cells are placed. The light transmitted through this cell chamber passes through a red-purple Corex A filter, No. 986.

Figure 2 is a schematic drawing of the circuit involved. A sodium photocell (G. E. No. FJ 405) sensitive to ultraviolet radiation is supplied with 90 volts and connected to the grid of the electrometer tube which has a grid leak of 100 megohms. This grid leak is not tied directly to ground but is brought through a slide wire to an appropriate potential relative to the filament of the electrometer tube. Variations in the grid potential will produce a variation in the current flowing through the electrometer tube which will correspondingly produce a deflection of the galvanometer.

The operation of the instrument consists in setting the filament rheostat, $F$, at an appropriate value (approximately 55-milliampere filament current). With the photocell dark and the slide wire set for a reading of zero transmission or infinite extinction coefficient, the bias rheostat, $B$, and the plate rheostats, $P_1$ and $P_2$, are adjusted for zero deflection of the galvanometer. Following this the illumination is permitted to fall on the photocell and the slide wire is set for 1.00 transmission factor or zero extinction coefficient. The resistors, $S_1$ and $S_2$, are then adjusted so that the galvanometer deflection is again zero. Interposition of the sample in the radiation beam will cause a deflection of the galvanometer, which is brought back to zero by means of the adjustment of the slide-wire potentiometer. The reading on the slide wire will then give either the transmission of the cell and ma-

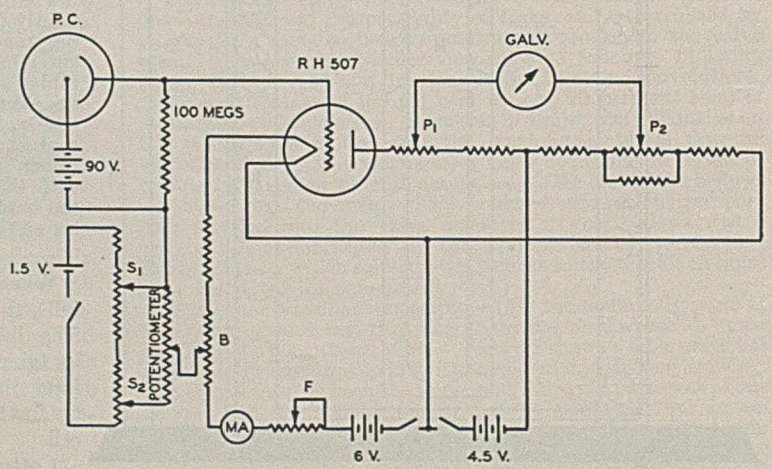


FIGURE 2. VITAMIN A PHOTOMETER CIRCUIT DIAGRAM

TABLE I. EXTINCTION COEFFICIENTS WITH VARIOUS DILUTIONS OF FISH LIVER OILS

(Cell of 1-mm. depth)

| Ranges of potentiometer drum scale | 1.5–3 | 4–6 | 6–8 | 8–10 |
|---|---|---|---|---|
| Dilution number | 1 | 2 | 3 | 4 |
| Shark liver oil | 19.0 | 18.7 | 18.7 | 18.7 |
| Swordfish liver oil | 20.8 | 21.0 | 20.4 | 21.2 |
| Halibut liver oil | 65.2 | 64.4 | 64.9 | 66.1 |
| Tuna liver oil | 66.7 | 66.6 | 66.4 | 66.7 |
| Swordfish liver oil | 94.0 | 98.0 | 98.5 | 98.2 |

TABLE II. COMPARATIVE *E* VALUES OF SOLUTION AT TWO CELL THICKNESSES

| Sample | *E* Value 1-mm. cell | *E* Value 10-mm. cell |
|---|---|---|
| 216 | 1.12 | 0.99 |
| 215 | 1.17 | 1.18 |
| 222 | 38.5 | 38.9 |
| 363 | 51.0 | 50.2 |
| 338 | 58.3 | 59.6 |
| 353 | 59.3 | 59.4 |
| 360 | 65.1 | 65.0 |

terial or their extinction coefficient. If a reading is first taken on the extinction coefficient scale, of the cell and solvent, subsequent readings taken of the cell, solvent, and active material give the extinction coefficient of the cell and solution in question. Subtraction of the former from the latter gives the extinction coefficient of the material at the concentration employed.

Summarizing this procedure of operation, the principles involved are the maintenance of static conditions in the electrometer tube by holding the grid potential at a constant value. This is accomplished by the use of the slide-wire potentiometer. The accuracy of the readings depends on the calibration of the slide wire and this can be made with a precision of 0.25 per cent, far in excess of that required in this type of measurement. These operations, while somewhat lengthy of description, can be carried out extremely rapidly; in fact, only 5 minutes are required for three consecutive readings to provide an average value of the extinction coefficient of a solution.

The use of an absorption cell of either 1- or 10-mm. depth enables one to cover a wide range of vitamin A concentrations. A range of 150 to 1 can be covered in a single dilution with a precision of 2 per cent or better—that is, samples ranging from 1000 to 150,000 units of vitamin A per gram can be measured in a single dilution.

An instrument of this design has been in use at one of the authors' laboratories for approximately 9 months and has proved entirely satisfactory for these determinations. In order to provide evidence of this, data are given in the tables to show the linearity of the calibration, the reproducibility of measurements of a single dilution and of the same material over a period of time, and the correlation with other instruments and biological assays.

TABLE III. DUPLICATE DETERMINATIONS OF EXTINCTION COEFFICIENTS OVER A PERIOD OF WEEKS

| Sample | Determination 1 | Determination 2 | Sample | Determination 1 | Determination 2 |
|---|---|---|---|---|---|
| 1 | 0.39 | 0.37 | 12 | 4.70 | 4.88 |
| 2 | 0.71 | 0.68 | 13 | 14.46 | 14.75 |
| 3 | 0.91 | 0.92 | 14 | 28.4 | 29.6 |
| 4 | 1.30 | 1.29 | 15 | 37.4 | 37.3 |
| 5 | 1.43 | 1.36 | 16 | 56.3 | 57.0 |
| 6 | 1.47 | 1.43 | 17 | 58.2 | 56.7 |
| 7 | 1.55 | 1.60 | 18 | 63.6 | 62.9 |
| 8 | 1.62 | 1.64 | 19 | 66.8 | 66.5 |
| 9 | 2.00 | 1.97 | 20 | 67.1 | 66.8 |
| 10 | 2.04 | 2.09 | 21 | 86.8 | 86.2 |
| 11 | 2.86 | 2.78 | 22 | 137.2 | 140.4 |

Table I contains a series of typical readings on samples of fish liver oils originating from different species. Various dilutions of the materials were made and the extinction coefficients were determined from readings made at four widely separated positions on the potentiometer. The extinction coefficients obtained indicate that regardless of the type of fish liver oil or of the dilution, there is a linear relationship between potentiometer settings and concentration.

With respect to the reproducibility of the measurements, settings on the transmission scale may be duplicated to within ±2 parts of the scale which is divided into 1000 parts.

When determinations are made on a sample for cell depths of 1 and 10 mm., accurate linearity is found (Table II). Results are those obtained by reading the same solution at the two cell thicknesses.

TABLE IV. *E* VALUES OF OILS DETERMINED INDEPENDENTLY ON TWO INSTRUMENTS

| Sample | Authors' Instrument | Monochromator Assembly |
|---|---|---|
| 391 | 7.04 | 6.74 |
| 394 | 21.1 | 20.3 |
| 722 | 42.5 | 42.5 |
| 580 | 68.9 | 68.6 |
| 581 | 76.7 | 72.4 |
| 582 | 77.0 | 76.2 |
| 584 | 84.4 | 84.2 |
| U. S. P. reference oil (whole) | 1.56 | 1.45 |

TABLE V. COMPARATIVE POTENCY DETERMINATIONS

[Potencies (International units per gram) as determined by this instrument, by bioassay, and the average obtained by a group of laboratories (A. D. M. A. collaborators) employing physical methods of measurement]

| Sample | Parker and Oser[a] | Average of Laboratories | Bioassay |
|---|---|---|---|
| 1 | 3,000 | 3,074 | 3,000 |
| 2 | 1,500 | 1,713 | 1,500 |
| 3 | 61,800 | 61,595 | 70,000 |
| 4 | 31,200 | 31,401 | 35,000 |
| 5 | 298,000 | 285,027 | 300,000 |
| 6 | 143,000 | 143,740 | 150,000 |

[a] *E* value × 2120.

The reproducibility of results obtained at different times by an operator unacquainted with the duplication of the samples is shown in Table III, which lists the results obtained for samples varying in *E* value from 0.38 to 139.

Numerous interchecks have been carried out between this instrument and equipment consisting of a tungsten lamp as a light source, a monochromator set at a wave length of 328 millimicrons (10-millimicron slit width), and a photocell amplifier combination comparable to the one described above.

TABLE VI. VITAMIN A UNITAGE ESTIMATES FROM BIOASSAY DATA AND *E* VALUES

| Sample | Assay Level | Average Gains Assay | Average Gains Reference | Estimated Unitage[a] | $E^{1\%}_{1cm.}$ | Calculated Unitage |
|---|---|---|---|---|---|---|
| | *Units/g.* | *Grams* | *Grams* | | | *E* × 2120 |
| 605 | 2,250 | 39.5 | 35.9 | 2,500 | 1.17 | 2,480 |
| 617 | 1,750 | 41.0 | 29.4 | 2,100 | 1.20 | 2,540 |
| 668 | 20,000 | 25.0 | 24.9 | 20,000 | 11.1 | 23,500 |
| 689 | 48,000 | 39.9 | 34.4 | 50,000 | 23.4 | 49,500 |
| 816 | 60,000 | 36.3 | 34.3 | 63,000 | 30.5 | 64,600 |
| 607 | 122,000 | 30.0 | 28.2 | 124,000 | 58.4 | 124,000 |
| 798 | 130,000 | 31.7 | 29.3 | 133,000 | 64.1 | 138,000 |
| 748 | 160,000 | 31.3 | 30.2 | 168,000 | 78.3 | 166,000 |
| | 168,000 | 32.2 | 32.5 | | | |
| 750 | 170,000 | 28.3 | 30.7 | 168,000 | 80.1 | 170,000 |
| | 180,000 | 25.7 | 30.7 | | | |
| 725 | 150,000 | 29.4 | 26.9 | 155,000 | 80.2 | 170,000 |
| 749 | 175,000 | 34.6 | 32.9 | 180,000 | 87.1 | 184,000 |
| | 185,000 | 30.4 | 33.1 | | | |
| 650 | 600,000 | 14.8 | 29.2 | 400,000[b] | 337 | 715,000[b] |

[a] Based on average dose-response curve from which unitage ratio corresponding to relative weight gains in assay and reference groups was obtained.

[b] This sample was a concentrate which may have undergone partial oxidation. It is not uncommon in such cases to observe lack of correlation between bioassay and physical measurement.

These checks (Table IV) show satisfactory agreement between the extinction coefficients determined by the two instruments.

At the meeting of the American Drug Manufacturers' Association in May, 1939, a report was presented of determinations of the extinction coefficients of six samples of oil, which were circulated among a number of collaborating laboratories. It had been hoped that the instrument described here would be available for use in the survey, but such was not the case. The authors therefore report the values subsequently obtained on this instrument for these samples. Table V lists the data obtained, together with the average values as determined physically by eight of these laboratories which were most consistent and the bioassay figures supplied to the collaborators.

Additional data bearing on the same point based on bioassays conducted at one of the authors' laboratories on a number of oils, together with the vitamin A potencies computed from measurements with this instrument, are given in Table VI.

## Conclusion

The instrument here described makes possible rapid and accurate estimation of the amount of preformed vitamin A. It has been economically constructed, is readily portable, and does not require a high degree of technical skill in its operation or in the evaluation of the results. By the use of other light sources and filters, the instrument can be employed for making transmission measurements in other ranges of the ultraviolet; hence its scope is greater than for vitamin A assay alone.

## Literature Cited

(1) Bills and Wallenmeyer, paper presented before American Society of Biological Chemists, March, 1938.
(2) McFarlan, Reddie, and Merrill, IND. ENG. CHEM., Anal. Ed., 9, 342 (1937).
(3) Morton and Heilbron, *Nature*, 122, 10 (1928); *Biochem. J.*, 22, 987 (1928).

PRESENTED before the Division of Biological Chemistry at the 99th Meeting of the American Chemical Society, Cincinnati, Ohio.

# A Simple Vacuum Tube Relay

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THE successful operation of many recently developed laboratory and industrial instruments depends in a large part upon the operation of a sensitive relay control circuit. The control of heating elements or refrigeration equipment for the regulation of temperature is the outstanding example of the use of vacuum tube relays. Pressure-regulating manometers (*1*) and liquid-level controls (*4*) also require control circuits that operate with minute current flow or with a change in high resistance.

Several vacuum tube relay circuits have been recently offered (*2, 3, 5, 6*) and for each is claimed one or more of the following advantages: simplicity of construction, low control current, wide range, ease of adjustment, continuous service, low cost, high degree of sensitivity, large plate current, power line operation, and alternating and direct current operation.

The relay described here has been designed to incorporate all these advantages as well as one or two others. The obvious advantage afforded by small size is realized in this circuit, which consists of one vacuum tube, four resistors, a condenser, and a commercial relay.

Schematic diagrams of the relay circuit connected for two

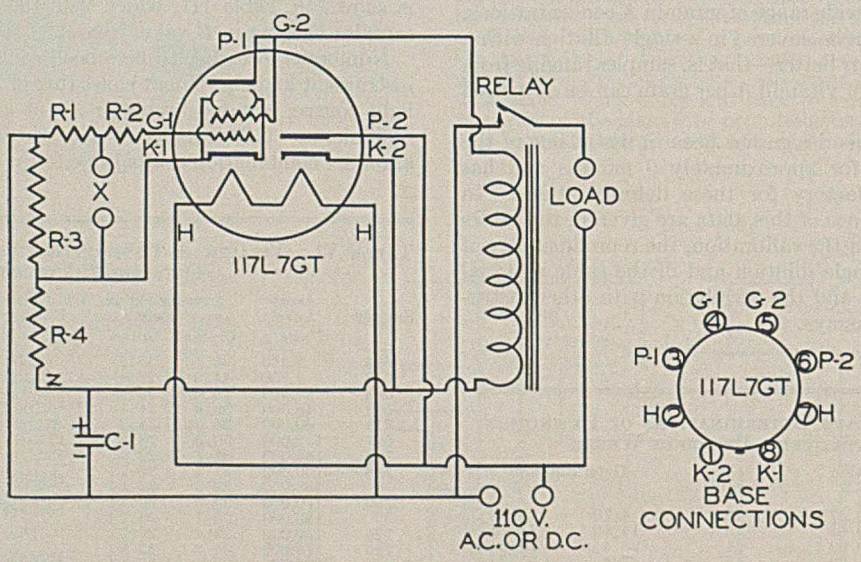


FIGURE 1. SCHEMATIC DIAGRAM OF SENSITIVE RELAY CIRCUIT

Make contacts to close load circuit
*C*-1. 8-mfd. electrolytic condenser, 250 working volts
*R*-1. 2-megohm, 0.5-watt resistor
*R*-2. 1-megohm, 0.5-watt resistor
*R*-3. 1000-ohm, 5-watt resistor
*R*-4. 15,000-ohm, 2-watt resistor
RELAY. Struthers-Dunn, Model ABTX1, 32-volt direct current coils (normally open)

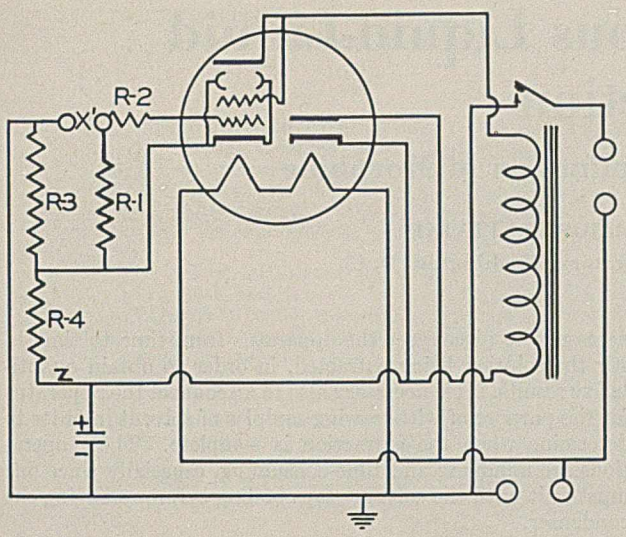


FIGURE 2. SCHEMATIC DIAGRAM OF SENSITIVE RELAY CIRCUIT
Make contacts to open load circuit

types of operation are shown in Figures 1 and 2. Connection of the circuit as shown in Figure 1 causes the relay magnet to become energized when the control contacts close or when the resistance between the contacts becomes less than a certain value. The alternative connection in which the relay magnet opens when the contacts close is shown in Figure 2. Both types of circuits will operate with equal efficiency on either alternating or direct current power supply lines.

The use of the rectifier-beam power amplifier type tube (type 117L7GT) serves the dual purpose of rectifying an alternating current supply and amplifying the minute control current which flows through the contact points, *X*. Since rectified and partially filtered alternating current is supplied to the beam power amplifier section of the tube, maximum tube life and efficiency are assured. The direct current operation of the relay, obtained by this method, eliminates the necessity of placing a condenser across the relay coil to prevent chattering of the relay contacts. Instantaneous positive operation of the relay is thereby assured. The usual condenser may, however, be inserted across the relay coil if delayed action is desired.

One of the outstanding features of this circuit is the use of a vacuum tube, the filament of which operates directly from the 110-volt power line. The absence of the usual filament transformer, lamp bulb, or other limiting resistor in the filament circuit further simplifies construction and eliminates one of the major sources of trouble in many relay circuits.

The very high plate current supplied by the beam power amplifier (45 to 50 milliamperes) eliminates the necessity for the use of a sensitive relay. The inexpensive and rugged commercial type of relay suggested for this circuit assures positive operation at all times for loads up to 1000 watts. Although high plate current flow is obtained, the current flow across the control circuit contacts, *X*, is limited to a maximum of about 5 microamperes. If an external resistance is inserted in the control circuit, this control current may be decreased, although the necessity for this precaution seems unlikely.

Since fixed resistors are used in the construction of the relay circuit, no electrical adjustments need be made. Mechanical variation of the spring tension on the relay contacts may be desired for different types of operation. Should a wider range of adjustment be desired for operation with a different type of relay, resistor *R*-3 may be replaced by a 1000-ohm wire-wound potentiometer.

Either relay circuit may be adapted to uses which require the control circuit to operate the relay with a change in control resistance. The relay of the circuit shown in Figure 1, for example, will remain energized so long as the resistance in the control circuit (across *X*) does not exceed approximately 500,000 ohms. If the control circuit resistance exceeds this value the relay magnet will open the load circuit. Variation of the critical resistance may be obtained by adjustment of the relay contact spring tension.

The simple and compact construction which may be realized with this type of relay is shown in Figure 3. The total cost of parts necessary for this model is less than $7. Standard radio replacement parts are used throughout.

The circuit shown in Figure 1 may be adapted for operation as a photoelectric cell relay. The cathode of a high-

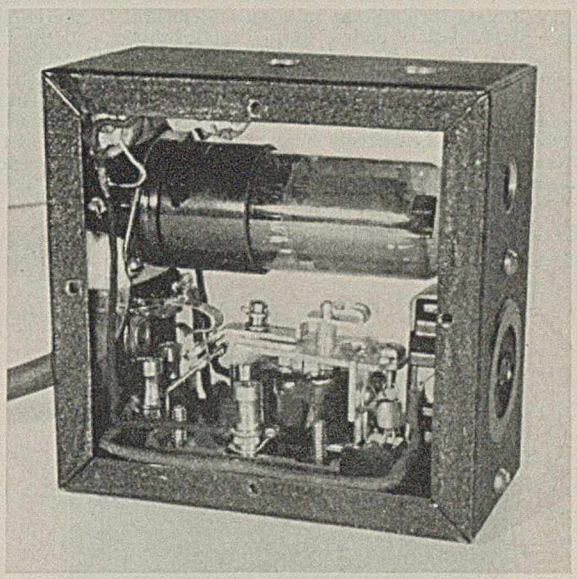

FIGURE 3. COMPLETED RELAY UNIT IN 4 × 4 × 2 INCH CASE

resistance photocell is connected to *G*-1 and the anode is connected at point *Z* in Figure 1. Light striking the photoelectric cell will cause the relay to become energized, providing that contact points *X* are open.

Since the circuits described are not isolated from the power supply line, care must be exercised in handling the control circuit contacts. If one side of the regulator is to be grounded, the grounded side of the power line and regulator must be connected as shown in Figure 2.

About twenty of these relays have been constructed and installed for various types of operation. A number of these units have been subjected to continuous 24-hour operation for 10 months with no apparent change in sensitivity. Not a single failure of any tube or part has been reported during this time.

## Literature Cited

(1) Ferry, C. W., IND. ENG. CHEM., Anal. Ed., 10, 647 (1938).
(2) Hawes, R. C., *Ibid.*, 11, 222 (1939).
(3) Heisig, G. B., and Gernes, D. C., *Ibid.*, 6, 155 (1934).
(4) Hersh, Frey, and Fenske, IND. ENG. CHEM., 30, 363 (1938).
(5) Huntress, E. H., and Hershberg, E. B., *Ibid.*, Anal. Ed., 5, 144, 344 (1933).
(6) Waddle, H. M., and Saeman, W., *Ibid.*, 12, 225 (1940).

# Device for Continuous Liquid-Liquid Extraction

## Adaptation for the Determination of Morphine

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CONTINUOUS liquid-liquid extraction devices for use with immiscible solvents lighter than water have been described in numerous publications.

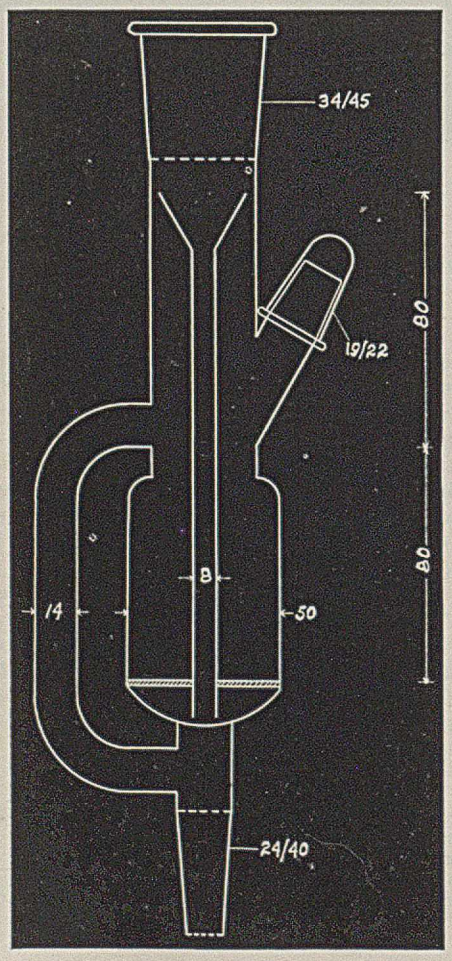
The most useful of these for the quantitative extraction of small samples are patterned after the general design of Palkin, Murray, and Watkins (*1*), in which solvent is conveyed from a condenser to the bottom of the solution being extracted, from whence it rises through the solution and returns to the boiling flask. Sintered-glass plates, spirals, small orifices, and the like are used for distributing the solvent throughout the solution. These distributing devices are generally separate from the body of the extractor and, being fragile, are consequently subject to breakage; but more serious disadvantages are that solvent is distributed from a relatively small area and a space exists at the bottom of the extractor where solvent and solution do not come into contact.

Under these conditions, much time is required for complete extraction and, in this laboratory, it has been found necessary to disconnect the apparatus from time to time to stir the solution being extracted, in order to obtain quantitative results. It is necessary also to disconnect the apparatus for the purpose of withdrawing samples of solvent in order to determine when the extraction is complete. These operations are annoying and time-consuming, especially since one must wait until the solvent stops boiling before removing the condenser.

The device described herein has been designed to obviate these difficulties. It has been found both quick and convenient for quantitative extraction of alkaloids, and is equally well suited for other substances.

The sintered-glass solvent-distributing plate is permanently sealed in the extraction tube where it covers the entire cross section and is protected from breakage. Solvent is distributed throughout the entire volume of the solution being extracted rather than in a narrow stream of droplets. There is no space under the distributing plate where solvent and solution do not come into contact.

The apparatus is filled and emptied through a side arm closed by a ground joint which also provides an opening through which to pipet occasional samples of solvent to test for completeness of extraction. The opening is sufficiently small to permit removing samples without interrupting the boiling. A drawn-out glass tube provided with a rubber bulb is convenient for this purpose.




### Apparatus

The diagram is self-explanatory. In operation a boiling flask for the solvent is attached by the ground joint at the bottom and a good condenser is attached in similar fashion at the top. The solvent vapors distilling from the boiling flask are condensed, passed down the inner funnel, and distributed throughout the aqueous solution by means of the sintered-glass plate. Solvent returns from the upper layer in the extractor to the boiling flask through the same tube which carries the vapors to the condenser. The device is charged and emptied through the side opening. The dimensions can be modified to almost any desired extent.

For most satisfactory operation it is important that the sintered plate be as coarse as possible, that the funnel tube extend as nearly as possible to the bottom of the extraction tube, and that the space between the plate and the bottom be small.

### Determination of Morphine

The device has been found particularly useful for the determination of morphine, where it presents a number of advantages over the generally used method of extracting the alkaloid from an ammoniacal solution by a mixture of chloroform and isopropanol. This operation is not readily adaptable to a continuous process where the solvent is recycled by boiling and condensation, because chloroform hydrolyzes under these conditions and the resulting acids unite with the extracted morphine, thus vitiating the results. The hand operation, employing separatory funnels, requires much solvent, is extremely tedious, and permits extraction of only small samples.

A mixture of benzene and isopropanol (2 volumes to 1 volume) serves admirably as extracting solvent in the device

described. Only small volumes are required and relatively large samples (about 0.20 gram of alkaloid) are readily extracted.

Place sufficient solvent to cover the sintered plate in the device, introducing it through the inner funnel. Accurately weigh a sample equivalent to approximately 0.20 gram of morphine alkaloid and dissolve it in approximately 50 cc. of dilute hydrochloric or sulfuric acid. Quantitatively transfer the solution to the extractor through the side opening, using approximately 25 cc. of wash water.

Place 50 to 75 cc. of solvent and a few Carborundum chips in the boiling flask and connect flask to the extractor. Drop sufficient ammonia to render the solution faintly alkaline through the inner funnel and connect the condenser. Distill the solvent rapidly through the solution until a sample of solvent removed (through the side tube by means of a pipet) from the upper layer in the extractor is found by a suitable test (Marquis reagent) to be free of morphine. The presence of sufficient ammonia in the solution may be confirmed at the same time by holding a piece of wet litmus paper in the vapor escaping from the side tube. When extraction is complete (0.5 to 1 hour) disconnect the boiling flask, evaporate the solvent, and titrate the alkaloid in the usual way.

Under the conditions described there is a volume increase of 20 per cent in the aqueous layer due to solubility of isopropanol. The addition of ammonia in the prescribed manner renders the solution alkaline at the same time that solvent is available to extract the liberated alkaloid. This is of consequence in morphine extraction and presumably also in other cases.

The simple analytical procedure outlined is, of course, useful only where interfering substances are absent. When this is not the case, the extractor becomes even more useful. Morphine can be separated from many other alkaloids, for example, by dissolving the mixture in dilute acid, rendering the solution distinctly alkaline with sodium hydroxide, and extracting with benzene to exhaustion. The solution is acidified, then made alkaline with ammonia, and the morphine removed in fresh mixed solvent (benzene-isopropanol). A method for opium assay based on this procedure is now under consideration in this laboratory.

### Literature Cited

(1) Palkin, Murray, and Watkins, IND. ENG. CHEM., 17, 612 (1925).

# Perforated Plate Columns for Analytical Batch Distillations

C. F. OLDERSHAW, Shell Development Company, Emeryville, Calif.

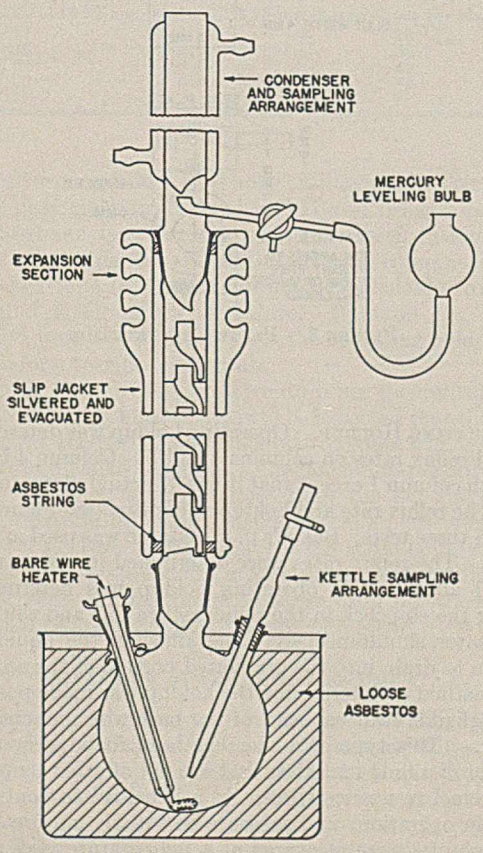



FIGURE 1. PERFORATED PLATE COLUMN

ACCURATE analytical batch distillations have become increasingly important during the past few years. Of the many different columns tested in these laboratories for this purpose, the perforated plate column described in this paper has been found to be of especial value for the analysis of hydrocarbon mixtures. It combines a low operating holdup per theoretical plate and a negligible static, or nondrainable holdup, with a high capacity or reflux rate. It has a low H. E. T. P., is easy to operate, and since it is constructed entirely of glass, may be used for corrosive liquids.

### Description of Column

The column (Figures 1 and 2) consists of a series of perforated glass plates sealed into a tube. Each plate is equipped with a baffle to direct the flow of liquid, a weir to maintain a liquid level on the plate, and a drain pipe. The first plate in a series serves as a small reservoir which is necessary in order to maintain a liquid seal for the drain pipe from the first regular plate.

### Construction

The plates are constructed in the following manner: A tube which later will form the baffle is placed in a lathe and rapidly rotated. The protruding end of the tube is heated in an oxygen flame and flared to form the disklike portion of the plate. The perforations are then drilled with a red-hot tungsten wire. This operation, which is ordinarily somewhat time-consuming when done by hand, is now entirely performed by an automatic drilling device designed in these laboratories for the purpose. The hot tungsten wire forms a small burr on the plate which is removed with an ordinary file. After the drilling operation the plates are examined and only those having perforations of uniform diameter and spacing are selected for use. The selected plates are ground to size; if they have been correctly flared, the grinding is a minor operation.

The drain pipes are sealed in place and shaped as shown in Figure 3, and a bead 0.5 mm. high is made on the lower end of each to regulate the distance between the drain and the plate below. The baffle tube is cut off at the desired height above the plate, and two longitudinal cuts are made with small high-speed

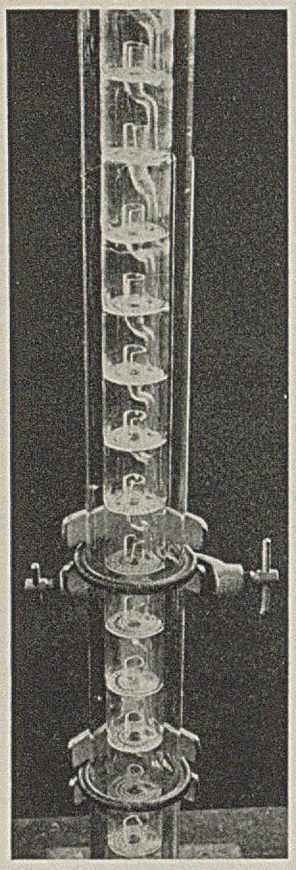


FIGURE 2. SECTION OF PERFORATED PLATE COLUMN SHOWING DETAILS OF PLATE CONSTRUCTION

emery wheels correctly spaced on a shaft. The intervening segment of the baffle is then cut off 1 mm. above the plate to form the weir. The plates are made up in large batches and carefully annealed between operations, when necessary, to remove strains. The finished plates are examined for imperfections.

The selected plates are then carefully sealed into a tube, so that each plate is level and the bead on the bottom of each drain pipe rests firmly on and in the center of the unperforated section of the plate below. The bottom of the pipe is placed sufficiently close to the plate below to ensure a liquid seal under normal operating conditions. The drain pipe is tapered from top to bottom to prevent vapor bubbles from being trapped by the overflowing liquid.

The column may be insulated in a number of ways. A slip jacket of the type shown in Figure 1 has been developed and is generally preferred in these laboratories. The jacket has a narrow window running its entire length through which the operation of the column may be observed.

## Testing the Column

PLATE EFFICIENCY. Plate efficiencies were determined at several reflux rates on four columns, Nos. 1, 2, 3, and 5.

Columns 1, 2, and 3 are 25 mm. in diameter. Each plate in column 1 has 42 perforations, 0.85 mm. in diameter; those of column 2 have 44 perforations, 1.1 mm. in diameter; and those of column 3 have 44 perforations, 1.35 mm. in diameter. The plate spacing is 25 mm. in columns 1, 2, and 3. Column 5 is 32 mm. in diameter. Its plates have 81 perforations, 0.85 mm. in diameter, arranged in three concentric circles. The plates are spaced 30 mm. apart in the column to give greater flexibility of operation. A carbon tetrachloride–benzene mixture prepared from redistilled Baker's C. P. chemicals was used in these tests.

In the plate-efficiency tests, the compositions of still-head and kettle samples were determined by the method of refractive index.

Still-head samples were withdrawn by means of the special still head shown in Figure 1. The mercury leveling bulb was lowered, thereby drawing some liquid through the three-way stopcock, the stopcock was then turned, and a few drops of sample were forced out by raising the mercury level. The remainder of the liquid in the line was then forced back into the column by displacing it with mercury. Kettle samples for the tests on columns 1, 2, and 3 were withdrawn by means of the pipet shown in Figure 1. However, in testing column 5, samples were taken from the bottom plate of the column instead of the kettle by means of the reflux rate and holdup measuring device shown in Figure 5. This apparatus is similar to that described by Bragg (*1*).

The plate efficiencies were determined by the method of McCabe and Thiele. In order to determine when equilibrum was obtained, still-head samples were analyzed at intervals. When successive samples showed constant composition, still-head and kettle samples were taken simultaneously and analyzed. The number of theoretical plates was determined from a diagram constructed from the vapor-liquid equilibrium data of Rosanoff and Easley (*5*). The kettle was taken as equal to one theoretical plate; therefore, the numbers of plates in columns 1, 2, and 3 are given as one less than the total number found. The method of sampling used in testing column 5 gave directly the number of plates in the column.

REFLUX RATE. The reflux rates at which the plate-efficiency tests were made were determined on columns 1, 2, and 3 at the end of each test by simply replacing the sampling still head with the total take-off head shown in Figure 4 and measuring the liquid collected in a given time. The reflux rate and holdup measuring device shown in Figure 5 was used in all tests on column 5. Therefore, the reflux rates at which plate efficiencies were determined on column 5 were measured by simply closing the stopcock in the reflux return line. In this case the volume was determined at the boiling point of the liquid. The results of these tests are given in Table I.

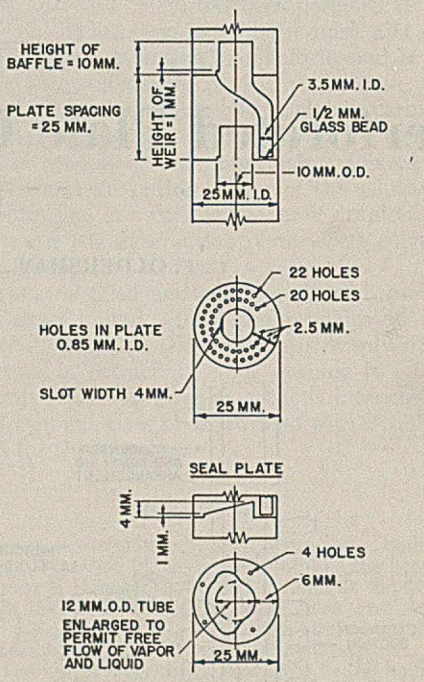



FIGURE 3. PLATE CONSTRUCTION

OPERATING HOLDUP. Operating holdup was determined at several reflux rates on columns 4 and 5. Column 4 is identical with column 1 except that it has 37 actual plates instead of 10. The reflux rate and holdup apparatus (see Figure 5) was used in these tests; Baker's C. P. benzene was used as the test liquid. The reflux rates were determined in the manner described above. The operating holdup was determined by closing the stopcock in the reflux return line and shutting off the heater simultaneously, thus allowing the liquid in the column to drain into the graduated portion of the apparatus. This method of determining the holdup was made possible by the negligible heat capacity of the bare wire immersion type heater. (This type of heater has been found very satisfactory for distilling materials that are not affected by platinum or chromel resistance wires. It is sensitive to control, constant in operation, and presents essentially no fire hazard.) The volumes were measured at a temperature very near the boiling point of the liquid. The results are given in Table II.

TABLE I. EFFICIENCY TESTS

| Reflux Rate Ml./min. | Reflux Rate Moles/min. | CCl4 Kettle Mole % | CCl4 Still head Mole % | No. of Theoretical Plates in Rectifying Section (Total Plates − 1) | Plate Efficiency % | H. E. T. P. Inches |
|---|---|---|---|---|---|---|
| Column 1, 10 25-mm. plates, 42 0.85-mm. perforations per plate | | | | | | |
| 6 | 0.065 | 15.4 | 44.0 | 10.0 | 100 | 0.98 |
| 13 | 0.141 | 16.7 | 42.5 | 8.9 | 89 | 1.10 |
| 18 | 0.195 | 19.0 | 45.2 | 8.8 | 88 | 1.12 |
| 24 | 0.260 | 16.7 | 42.0 | 8.6 | 86 | 1.14 |
| Column 2, 10 25-mm. plates, 44 1.1-mm. perforations per plate | | | | | | |
| 15 | 0.162 | 18.0 | 43.0 | 8.4 | 84 | 1.17 |
| 26 | 0.282 | 18.0 | 41.0 | 7.7 | 77 | 1.28 |
| 28 | 0.304 | 18.0 | 40.5 | 7.5 | 75 | 1.31 |
| Column 3, 10 25-mm. plates, 44 1.35-mm. perforations per plate | | | | | | |
| 12.5 | 0.136 | 14.8 | 37.5 | 7.8 | 78 | 1.26 |
| 23.5 | 0.255 | 14.8 | 34.5 | 6.7 | 67 | 1.47 |
| Column 5, 32-mm. plates[a], 81 0.85-mm. perforations per plate | | | | | | |
| 18.3 | 0.190 | 18.6 | 57.4 | 15.3 | 102 | 1.16 |
| 36.8 | 0.381 | 21.2 | 55.1 | 13.0 | 87 | 1.36 |
| 39.0 | 0.404 | 17.0 | 50.3 | 12.7 | 85 | 1.39 |
| 52.0 | 0.540 | 17.0 | 48.0 | 11.7 | 78 | 1.51 |
| 55.5 | 0.575 | 17.0 | 47.4 | 11.5 | 77 | 1.53 |
| 70.5 | 0.730 | 19.2 | 46.8 | 10.3 | 69 | 1.71 |

[a] Plates spaced 3.0 cm. apart in column.

TABLE II. TESTS OF HOLDUP AND REFLUX RATES

| Reflux Rate Ml./Min. | Reflux Rate Moles/min. | Holdup in Column Ml. |
|---|---|---|
| Column 4, 37 25-mm. plates, 42 0.85-mm. perforations per plate | | |
| 9 | 0.094 | 20 |
| 14 | 0.146 | 26 |
| 22 | 0.229 | 53 |
| 28 | 0.292 | 58 |
| 40 | 0.417 | 67 |
| Column 5, 15 32-mm. plates, 81 0.85-mm. perforations per plate | | |
| 17 | 0.177 | 14 |
| 28 | 0.292 | 38 |
| 52 | 0.542 | 40 |
| 75 | 0.782 | 45 |

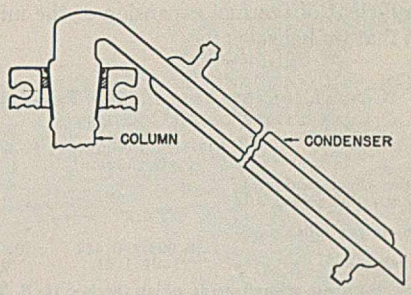



FIGURE 4. TOTAL TAKE-OFF STILL HEAD

instead of the holdups were determined by extrapolation.

The reflux rates in moles per minute were calculated from the molar volumes of the liquids and the reflux rates in milliliters per minute. The molar volume of benzene at 80° C., the approximate temperature at which the volumes were measured, was calculated from the molar volume at 20° C. by means of the data given by Perry (*4*) on the coefficient of thermal expansion. For mixtures of carbon tetrachloride and benzene and of ethylene dichloride and benzene, the molar volumes were determined from graphs of molar volume *vs.* molal composition. In calculating the molar volumes at the approximate boiling points of the mixtures it was assumed

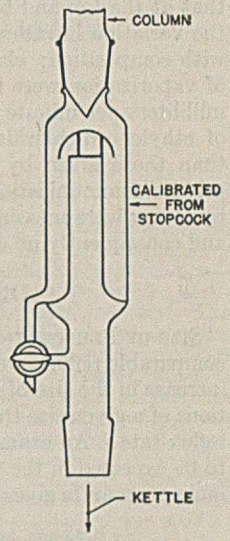



FIGURE 5. REFLUX RATE AND HOLDUP TEST APPARATUS

The performance data in Table III have been calculated from the data given in Tables I and II and Bragg's data (*1*) on the Stedman type packing which has been included for purposes of comparison. Similar data on columns of the helix-packed type (*3*, *7*, *8*) could not be calculated from the information given in the literature. Data on the Bruun column (*2*) were also omitted, since the operating holdup was not given for a specified reflux rate.

PRESSURE DROP. The pressure drop per theoretical plate was not measured; however, it is to be expected that the pressure drop per theoretical plate in a plate column will be higher than that in a packed column. The maximum pressure drop may be calculated approximately from the density of the refluxing mixture, the plate spacing, and the plate efficiency. Thus, for a mixture whose density is 0.8 at its boiling point, and which has a plate spacing of 25 mm. and a plate efficiency of 80 per cent, the maximum pressure drop per theoretical plate would be approximately 1.8 mm. of mercury.

## Methods of Calculating Data

The plate efficiencies given for column 4 in Table III were determined by extrapolating and interpolating those found for column 1 to the molal reflux rates at which operating holdups were determined. The operating holdups given for column 5 in Table III were determined by interpolating those given in Table II. In the case of the highest and lowest reflux rates given for column 5 in Table III, the plate efficiencies

TABLE III. PERFORMANCE OF PERFORATED PLATE AND STEDMAN TYPE PACKED COLUMNS

| Reflux Rate Ml./min. | Reflux Rate Mole/min. | No. of Theoretical Plates in Column | Plate Efficiency % | H. E. T. P. Inches | Holdup Total Ml. | Holdup Per theoretical plate Ml. | Holdup Per theoretical plate per 100 ml. of reflux per min. Ml. |
|---|---|---|---|---|---|---|---|
| Column 4, 37 25-mm. plates, 42 0.85-mm. perforations per plate | | | | | | | |
| 9 | 0.094 | 35.1 | 95 | 1.04 | 20 | 0.57 | 6.4 |
| 14 | 0.146 | 32.9 | 89 | 1.10 | 26 | 0.79 | 5.7 |
| 22 | 0.229 | 32.2 | 87 | 1.13 | 53 | 1.65 | 7.5 |
| 28 | 0.292 | 31.8 | 86 | 1.14 | 58 | 1.83 | 6.5 |
| 40[a] | 0.417 | 30.0 | 81 | 1.21 | 67 | 2.23 | 5.6 |
| | | | | | | | Av. 6.3 |
| 25-mm. Stedman column, 24 inches of packing 112 | | | | | | | |
| 3.33 | 0.0394 | 43.8 | ... | 0.55 | 13 | 0.30 | 8.5 |
| 6.67 | 0.0790 | 34.8 | ... | 0.69 | 22.4 | 0.64 | 9.3 |
| 8.33 | 0.0985 | 31.1 | ... | 0.77 | 24.0 | 0.77 | 8.8 |
| 11.65 | 0.138 | 29.5 | ... | 0.81 | 27.0 | 0.92 | 7.6 |
| 15.0 | 0.177 | 26.3 | ... | 0.91 | 33.0 | 1.25 | 8.0 |
| 16.7 | 0.197 | 24.6 | ... | 0.98 | 34.0 | 1.38 | 8.0 |
| 18.0[a] | 0.213 | 24.2 | ... | 0.99 | 35.8 | 1.48 | 7.9 |
| | | | | | | | Av. 8.3 |
| Column 5, 15 32-mm. plates[b], 81 0.85-mm. perforations per plate | | | | | | | |
| 17.0 | 0.177 | 15.5 | 103 | 1.14 | 14 | 0.90 | 5.3 |
| 18.3 | 0.190 | 15.3 | 102 | 1.16 | 17 | 1.11 | 6.2 |
| 36.8 | 0.381 | 13.0 | 87 | 1.36 | 39 | 3.00 | 8.1 |
| 52.0 | 0.542 | 11.7 | 78 | 1.51 | 40 | 3.42 | 6.6 |
| 70.5 | 0.730 | 10.3 | 69 | 1.71 | 44 | 4.37 | 6.2 |
| 75.0[a] | 0.782 | 10.0 | 67 | 1.76 | 45 | 4.50 | 6.0 |
| | | | | | | | Av. 6.4 |

[a] Maximum reflux rate.
[b] Plates spaced 30 mm. apart in column.

that the coefficient of thermal expansion of the mixtures was the same as that for benzene.

TABLE IV. COMPARISON OF PERFORATED PLATE WITH STEDMAN TYPE PACKED COLUMNS

| | 25-Mm. Perforated Plate Column | 25-Mm. Stedman Column |
|---|---|---|
| Holdup ml. per theoretical plate per 100 ml. of reflux per minute (average) | 6.3 | 8.3 |
| Reflux rate, mole per min., maximum | (0.094)[a]–0.417 | (0.039)[a]–0.213 |
| H. E. T. P., inches | 1.04–1.21 | 0.55–0.99 |

[a] This figure represents reflux rate at which the low H. E. T. P. was determined and not minimum reflux rate of column.

In calculating the holdup per theoretical plate per 100 ml. of reflux per minute, the reflux rate was taken as an average of that at the top and bottom of the column by assuming that the variation in rate was due to the change in molar volume with composition; changes in rates due to variation in heats of vaporization were neglected. The average reflux rates in milliliters per minute calculated in this manner for mixtures of ethylene dichloride and benzene were 4 per cent higher than those given by Bragg. It has been learned through private communication that the volumes of holdup reported by Bragg were measured near the boiling point of the liquid, and consequently no correction for expansion has been made.

## Discussion of Data

SIZE OF PERFORATIONS. The data in Table I show that at comparable reflux rates the plate efficiencies decrease with an increase in the size of the perforations, and that with perforations of a given size the plate efficiencies vary inversely as the reflux rate. An examination of the data on plate efficiencies to be expected in the 25-mm. columns at a reflux rate of 0.25 mole per minute gives the following results:

0.65-mm. perforations, 90% by extrapolation
0.75-mm. perforations, 88% by extrapolation
0.85-mm. perforations, 86%
1.1-mm. perforations, 79%
1.35-mm. perforations, 68%

The 0.65-mm. perforations could be expected to increase the plate efficiency by 4 per cent over that of the recommended 0.85-mm. perforations, but on the other hand, the pressure required to force vapor through smaller perforations against the surface tension of the liquid is higher. Therefore it is necessary to increase the spacing of the plates and consequently to increase the H. E. T. P. The use of perforations larger than 0.85 mm., however, was found to decrease the flexibility in reflux rate.

The plate spacing of 25 mm. recommended for column 1 represents actually a compromise between flexibility and H. E. T. P. In the case of column 5, which was built for continuous as well as batch distillations, a plate spacing of 30 mm. was used because flexibility was considered to be of greater importance than a low H. E. T. P.

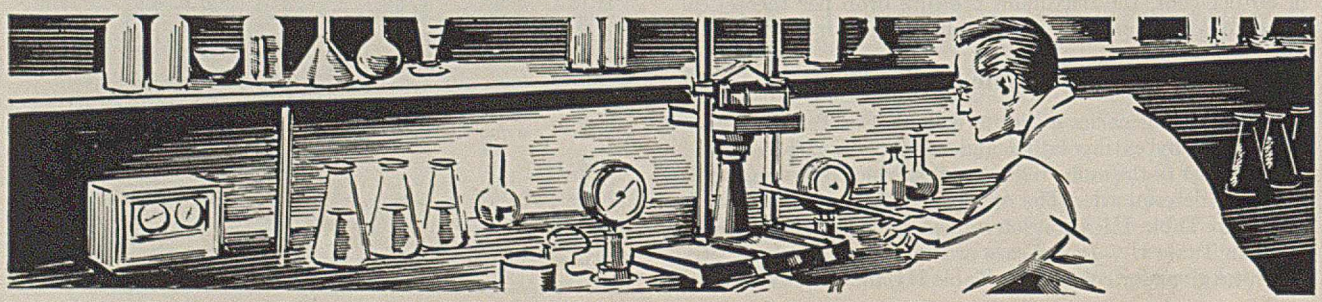
HOLDUP AND COMPARISON OF PERFORATED PLATE WITH OTHER TYPES OF COLUMNS. It may be seen from Tables II and III that the operating holdup per theoretical plate varies with the reflux rate. However, if the holdup per theoretical plate is divided by the reflux rate in milliliters of liquid per minute, a practically constant value is obtained. Now if the capacity or maximum reflux rate of the columns is expressed in moles per minute, a useful basis for the comparison of batch distilling columns of different design is established. For example, a comparison of the perforated plate column with the Stedman column by this method gives the results of Table IV.

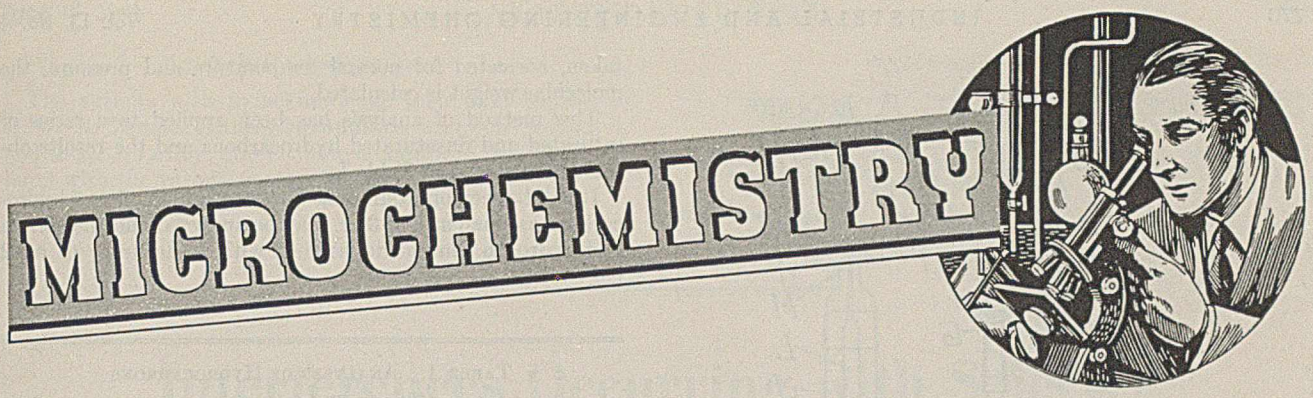
Of the three factors given above, the holdup per theoretical plate at a fixed reflux rate is the most important in choosing a column for analytical batch distillations, since it determines the minimum volume of liquid which must occur as intermediate fractions between two essentially pure components. Thus the minimum intermediate fraction which must exist in the Stedman column would be approximately 32 per cent larger than that in the perforated plate column if both were operated at the same reflux rate. It follows that, if intermediate fractions of equal size are desired in both cases, the perforated plate column could be operated at a higher reflux rate, thus decreasing the time required to effect the separation. The actual volume of the intermediate cut could be decreased in both the perforated plate and the Stedman columns by decreasing the reflux rates. A detailed discussion of the effect of holdup and also of the effect of reflux ratio in batch distillations is given by Rose (*6*).

The figures obtained for reflux rate and H. E. T. P. show that the 25-mm. perforated plate column has a somewhat greater H. E. T. P. than the 25-mm. Stedman column and that the perforated plate column has a higher maximum reflux rate or capacity. Comparison with the helix-packed type of column (*8*) shows that the perforated plate column has a lower average holdup per theoretical plate per 100 ml. of reflux per minute. Comparison with the Bruun column (*2*) shows that the perforated plate column has a higher plate efficiency at high reflux rates. Since the liquid drains readily and practically completely from the perforated plate column, it is superior in this respect to the Bruun column.

## Literature Cited

(1) Bragg, L. B., IND. ENG. CHEM., Anal. Ed., 11, 283 (1939).
(2) Bruun, J. H., *Ibid.*, 8, 224 (1936).
(3) Fenske, M. R., Lawroski, S., and Tongberg, C. O., IND. ENG. CHEM., 30, 297 (1938).
(4) Perry, J. H., "Chemical Engineers' Handbook", Smithsonian Tables, p. 434, New York, McGraw-Hill Book Co., 1934.
(5) Rosanoff and Easley, *J. Am. Chem. Soc.*, 31, 980 (1909).
(6) Rose, A., Welshans, L. M., and Long, H. H., IND. ENG. CHEM., 32, 673 (1940).
(7) Tongberg, C. O., Lawroski, S., and Fenske, M. R., *Ibid.*, 29, 957 (1937).
(8) Whitmore, F. C., Fenske, M. R., Quiggle, D., Bernstein, H., Carney, T. P., Lawroski, S., Popkin, A. H., Wagner, R. B., Wheeler, W. R., and Whitaker, J. S., *J. Am. Chem. Soc.*, 62, 795 (1940).






# Microanalysis of Gaseous Hydrocarbons

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IN THE study of gaseous reactions the gaseous products are often obtained in such small quantities that ordinary methods of gas analysis may not readily apply. Furthermore, these methods do not render it possible to differentiate between a homogeneous gaseous hydrocarbon and a mixture of hydrocarbons.

A procedure has therefore been developed applying Pregl's microanalytical method for the determination of carbon and hydrogen to the analysis of gaseous hydrocarbons obtained in such small quantities. By introducing about 2 cc. of the gas into the combustion tube by means of a buret inserted into the Pregl train and slightly modifying the well-known procedure, extremely good results are obtained. As in the ordinary microdetermination (*1*, *2*) of carbon and hydrogen in organic substances, the water and carbon dioxide are weighed. From these the weights of carbon and of hydrogen are calculated and the sum of the two is obviously equal to the weight of the sample taken. This weight makes it possible not only to determine the percentage composition but to calculate the molecular weight of the hydrocarbon from the relation of the weight of the sample to the volume of gas taken.

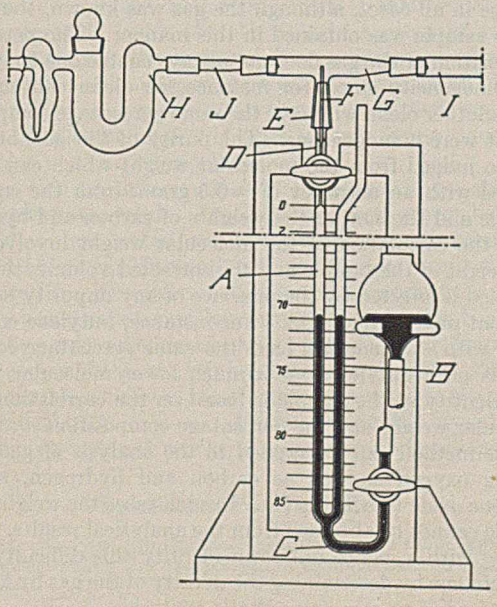


FIGURE 1

## Apparatus

The gas buret designed for this purpose is illustrated in Figure 1. It consists of a glass tube, *A* (3-mm. bore), approximately 1 meter in length, branched at its lower end into a parallel compensating tube, *B*, and a short tube, *C*, which is closed with a stopcock and connected to a leveling bulb filled with mercury. The upper end of *A* is sealed to a capillary, *D* (1-mm. bore), carrying a stopcock and terminating in a ground tip, *E*, inside of which there extends a fine capillary tube, *F* (0.5-mm. bore), which is sealed to *D*. A piece of glass tubing, *G*, of the same diameter as the Pregl combustion tube, *I*, is drawn at one end to a tip of the size of the thermometer capillary, *J*, prescribed by Pregl (*2*, p. 26) to join the bubble counter, *H*, to *I*. *G* is connected to *E* by means of a ground-glass joint and can be secured in place with springs held by small glass projections on the two pieces of the joint. *F* should be sufficiently long to reach the center of *G* as shown. The buret is mounted on a supporting board on which a strip of graph paper has been pasted and is inserted in the combustion train between *J* and *I*. The buret is calibrated and the relation of centimeter length to volume is plotted on a graph which can be used to determine the volume of gas between any two levels of mercury in the buret.

To fill the buret with a sample of gas requires special precautions in order to exclude the possibility of contaminating the sample with air. The sample is best introduced into the buret by the apparatus illustrated in Figure 2.

It includes a manifold, *A*, one end of which, *B*, is connected to a vacuum pump while the opposite end, which is equipped with a stopcock, *C*, is joined to a mercury blow-off, *D*, and to a gas cylinder, when such is the source of the sample. *A* branches at *K* into a parallel tube, *E*, which is connected by means of ground-glass joints, *F* and *G*, to the gas buret and to a receiver, *H*. The end of *E* carries a large hollow-barreled stopcock, *I*, which is sealed to a cap with a ground-glass lip fitting over a ground-glass container, *J*.

## Procedure

When a gas sample is to be taken from a steel cylinder the latter is joined to the manifold, *A*, and the buret, after removal of its T-shaped head (*G*, Figure 1), is connected at *F*. After the system, including the buret, has been evacuated, *P* is closed and stopcock *C* set to allow but a small opening through which the gas is then allowed to enter the system while the surplus escapes through the mercury blow-off. More than the required quantity of hydrocarbon is condensed into the receiver, *H*, by cooling the latter in liquid air. *C* is then closed, the liquid air removed, and the pump allowed to run while the solidified hydrocarbon liquefies. In order to remove noncondensable impurities completely the process of alternate solidifying and liquefying of the gas under the action of the pump is repeated once or twice. After the final removal of the liquid air from *H*, *P* is opened, *K* is closed, and the hydrocarbon is allowed to distill into the buret until atmospheric pressure has been reached.

If the sample to be analyzed is in a sealed glass ampoule, *L*, the latter is placed in *J* and enough mercury added to bring the

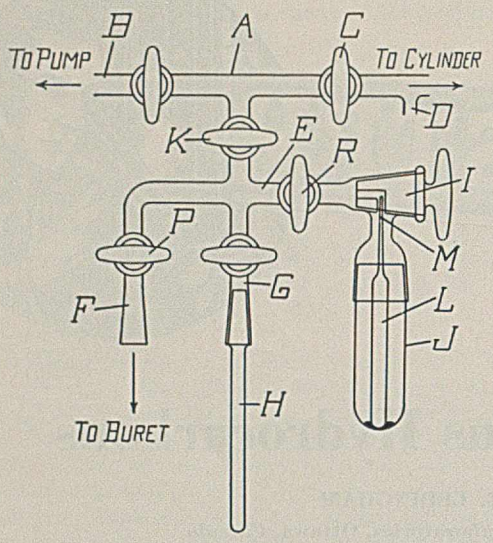


FIGURE 2

tip, $M$, of the ampoule into the bore of $I$. $D$ is cut off at $C$ and the system, including the buret, is evacuated. $P$ is then closed, $J$ is immersed in liquid air to condense the gas, and $K$ is closed. After the gas has solidified, $M$ is broken by a sharp turn of $I$. The liquid air flask is removed from $J$ and raised around $H$ and the gas is allowed to distill over and condense in $H$. Stopcock $R$ is closed, $K$ is opened, and the system is pumped while the hydrocarbon liquefies. As in the case mentioned above, the alternate process of freezing and pumping is repeated once or twice. Finally, the liquid air is removed, the system is cut off from the pump at $K$, $P$ is opened, and the gas is allowed to distill into the buret until atmospheric pressure has been reached.

After removal of the buret from the filling apparatus the T-shaped head ($G$, Figure 1) is attached to it and the buret is inserted in the combustion train between $J$ and $I$, as shown in Figure 1. The usual precautions prior to combustion are followed closely, except as outlined below. For the combustion of solids Pregl found that the optimum speed of the stream of oxygen going through the train as measured in the bubble counter should be 5 cc. per minute. In the case of gases it is essential to slow this down to 3 cc. per minute. It is also preferable to insert an empty platinum boat in the combustion tube and to keep it hot with the movable burner, exactly as for the combustion of solids.

To carry out the combustion the weighed absorption tubes are connected into the train, the flow of gas is adjusted, and the barometric pressure, temperature, and buret reading are recorded. The lower stopcock is then opened and the leveling bulb adjusted so that the mercury in $B$ shows a head of 2 to 3 mm. The upper stopcock is now opened and the gas forced into the combustion tube by the gradual and careful raising of the leveling bulb at such a rate that the time required to introduce the sample is from 15 to 20 minutes, depending on the volume of gas. After the sample has been introduced into the combustion tube, the upper stopcock is closed, the level of the mercury adjusted, and the reading recorded. The stream of oxygen is kept up at the same speed for 5 minutes more and the sweeping operation is effected by a stream of air forced through the train at the usual rate of 5 cc. per minute. In the course of this sweeping operation 200 cc. of water are collected from the Mariotte flask in 40 minutes. After the products of the combustion have been so swept out from the combustion tube the absorption tubes are disconnected, wiped, and weighed as usual.

The volume of gas used in a combustion will obviously vary with the hydrocarbon and should be such as to correspond to a weight of 3.5 to 4.5 mg., as in the ordinary microanalytical technique.

From the weights of carbon dioxide and water the weights of carbon and of hydrogen in the sample of hydrocarbon are obtained and their sum is equal to the weight of the sample. From these the percentage composition is determined. Furthermore, from the weight of the sample thus obtained (sum of carbon and hydrogen) and the volume of hydrocarbon taken, corrected for normal temperature and pressure, the molecular weight is calculated.

This method of analysis has been applied to a series of saturated and unsaturated hydrocarbons and the results obtained are shown in Table I.

It will be seen on examining Table I that samples of hydrocarbons as small as 2 to 3 cc. may be analyzed and the carbon and hydrogen content determined with an accuracy of ±0.2 per cent.

TABLE I. ANALYSES OF HYDROCARBONS

| Gas Sample | Volume (Corrected) | Wt. of Hydrogen | Wt. of Carbon | Hydrogen | Carbon | Mol. Wt. | Ratio of C to H |
|---|---|---|---|---|---|---|---|
| | *Cc.* | *Mg.* | *Mg.* | % | % | | |
| Ethane | ... | ... | ... | 20.00 | 80.00 | 30.00 | 4.00 |
| | 1.862 | 0.491 | 1.970 | 19.95 | 80.05 | 29.63 | 4.01 |
| | 2.263 | 0.599 | 2.427 | 19.79 | 80.21 | 29.95 | 4.03 |
| Propane | ... | ... | ... | 18.18 | 81.81 | 44.00 | 4.50 |
| | 1.974 | 0.707 | 3.177 | 18.22 | 81.78 | 44.08 | 4.49 |
| | 1.981 | 0.705 | 3.192 | 18.11 | 81.89 | 44.08 | 4.52 |
| Butane | ... | ... | ... | 17.24 | 82.76 | 58.00 | 4.80 |
| | 1.524 | 0.669 | 3.245 | 17.09 | 82.91 | 57.53 | 4.85 |
| | 1.392 | 0.625 | 3.000 | 17.25 | 82.75 | 58.37 | 4.80 |
| Cyclopropane | ... | ... | ... | 14.29 | 85.71 | 42.00 | 6.00 |
| | 2.176 | 0.568 | 3.466 | 14.08 | 85.92 | 41.52 | 6.10 |
| | 2.112 | 0.564 | 3.398 | 14.24 | 85.76 | 42.03 | 6.02 |
| Acetylene | ... | ... | ... | 7.69 | 92.31 | 26.00 | 12.00 |
| | 3.232 | 0.280 | 3.447 | 7.52 | 92.48 | 25.84 | 12.28 |
| | 3.138 | 0.278 | 3.374 | 7.63 | 92.37 | 26.08 | 12.10 |
| Ethylene | ... | ... | ... | 14.29 | 85.71 | 28.00 | 6.00 |
| | 2.241 | 0.400 | 2.401 | 14.28 | 85.72 | 28.00 | 6.00 |
| | 2.186 | 0.394 | 2.338 | 14.42 | 85.58 | 28.00 | 5.94 |

TABLE II. ANALYSES OF ETHYLENE OXIDE

| Volume (Corrected) | Wt. of Sample Calcd. from Mol. Wt. | Wt. of $H_2O$ | Wt. of $CO_2$ | $H_2$ | C |
|---|---|---|---|---|---|
| *Cc.* | *Mg.* | *Mg.* | *Mg.* | % | % |
| ... | ... | ... | ... | 9.09 | 54.54 |
| 1.986 | 3.901 | 3.152 | 7.774 | 9.04 | 54.35 |
| 1.939 | 3.808 | 3.122 | 7.589 | 9.17 | 54.34 |

With an unknown gas, the weight of the sample is obtained from the sum of the weights of carbon and hydrogen and thus the percentage composition will always add up to 100 per cent. This method, however, does not seem to involve any appreciable error, as evidenced by the results given in Table I, since in all cases, although the gas was known, the weight of the sample was obtained in this manner. The percentage composition of the gas does not always enable one to judge of its homogeneity since, for instance, an olefin contaminated with another olefin will have the same percentage composition as if it were homogeneous. The purity of the gas, however, can be judged from the molecular weight which can be calculated with an accuracy of ±0.5 gram, from the corrected volume and the sum of the weights of carbon and hydrogen. Since the calculation of the molecular weight involves both the weight of the sample and the corrected volume, the value obtained is affected by the presence of any impurity having a different molecular weight. For instance, butylene contaminated with ethylene will have the same percentage composition as pure butylene but a much lower molecular weight. The identity of the sample is based on the correlation of the molecular weight and the percentage composition.

This method can be applied to the analysis of gases containing oxygen as well as carbon and hydrogen, such as ethylene oxide (see Table II). In such cases the weight of the sample cannot be obtained from the analytical results. When dealing with a gas of unknown identity this difficulty could be overcome by determining the density of the gas by a micromethod prior to carrying out the analysis.

### Acknowledgment

The authors wish to acknowledge their indebtedness to E. W. R. Steacie, of these laboratories, who built the buret, and to D. J. LeRoy, also of these laboratories, who filled the buret with the various gases analyzed.

### Literature Cited

(1) Niederl, J. B., and Niederl, V., "Micromethods of Quantitative Organic Elementary Analysis", New York, John Wiley & Sons, 1938.
(2) Pregl, F., "Quantitative Organic Microanalysis", Philadelphia, P. Blakiston's Son & Co., 1930.

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# Interferences Occurring with Selected Drop Reactions

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AS A GENERAL rule drop tests and other color reactions are developed especially for use for specific tests. There are, however, few truly specific reactions and there is need for information concerning such interferences as may occur and steps that may be taken to remove them. Systems of semimicroqualitative analysis have been developed (*1*, *2*, *8*, *10*) in which spot tests are used for the identification of the different ions after the usual group separations. Gutzeit (*5*), Krumholz (*4*), and Heller (*6*) have proposed schemes of qualitative analysis based on the more simple separations. The interests of the authors have been more closely allied to those of the latter group of workers, and a system of analysis has been developed (*9*). Special consideration has been given to the adaptation of this system to use in portable kits.

In the system employed by the authors, a two-group separation of the elements under consideration is used. The first step is either fusing the sample with sodium carbonate and sodium peroxide, or adding sodium carbonate and then sodium peroxide to a solution of the unknown. In either case, the water-soluble carbonates and hydroxides are separated from the insoluble oxides, carbonates, hydroxides, etc., by filtration. This accomplishes an almost equal division of the more common elements into two major groups. One of the main advantages of this procedure is that oxidation by sodium peroxide yields the elements in a constant state of oxidation, usually the highest. This adjustment of valence has two main advantages: (1) since all forms of an element are converted to one common valence, many tests are eliminated; (2) the elimination of extra valence forms reduces the number of possible interferences.

When this procedure was first investigated, no study of interferences was made. Instead, the interferences listed by Feigl (*3*, *4*) were noted, and the effect of the proposed separation procedure in eliminating these interferences was taken into account. Throughout 4 years of observation and use the two-group separation has proved to be of great value in conducting microchemical analyses by means of drop tests. In the course of this work it has been observed that there are a considerable number of interferences not listed in the general literature on drop reactions. For this reason the investigation on which the present report is based was undertaken.

## Solutions and Reagents

For the study of interferences, solutions were made up to contain approximately 5.0 mg. per ml. of each element to be studied. In the preparation of each such solution, an appropriate amount of the C. P. chemical was fused in a platinum crucible with sodium carbonate and sodium peroxide. The melt was then dissolved in water, the pH was adjusted to a value as near 7.0 as possible, and the solution was boiled to decompose the remaining hydrogen peroxide. After being cooled, the solutions were made up to the required volume and placed in convenient Pyrex dropping tubes.

By this procedure, solutions were prepared for lithium, sodium, potassium, copper, silver, gold, beryllium, magnesium, calcium, zinc, strontium, cadmium, barium, mercury, boron, aluminum, carbon, silicon, titanium, zirconium, tin, lead, thorium, nitrogen, phosphorus, vanadium, arsenic, antimony, bismuth, sulfur, chromium, selenium, molybdenum, tungsten, uranium, fluorine, chlorine, manganese, bromine, iodine, iron, cobalt, and nickel. Only one solution was needed for each element, irrespective of the number of variations in valence in which it occurs, since the action of the sodium peroxide in the preparation of the solution leaves each element (except manganese) in a single state of oxidation, usually the highest. In the case of manganese, a fresh known solution was made up each day, since the solution gradually decomposed to give variable mixtures of manganese dioxide, manganates, and manganous salts.

The reagents and apparatus used in this work have been described previously (*9*).

## Method of Studying Interferences

The actual investigation of interferences was carried out according to a definite scheme. Because of the varied forms of phosphate interference met with, special attention was paid to the behavior of this ion under each set of conditions studied. The following series of solutions was prepared and used in studying the interferences with each test.

1. Blank
2. Element under consideration
3. Element plus phosphate
4. Individual members of periodic group I, in the absence of the element to be studied
   Individual members of periodic group II, in the absence of the element to be studied, etc.
5. Individual members of periodic group I plus element
   Individual members of periodic group II plus element, etc.
6. Individual members of periodic group I plus phosphate, in the absence of the element
   Individual members of periodic group II plus phosphate, in the absence of the element, etc.
7. Individual members of periodic group I plus phosphate plus element
   Individual members of periodic group II plus phosphate plus element, etc.

The interferences found were reported as positive if a false test was obtained in the absence of the element sought, and negative if the test failed to indicate presence of the element.

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TABLE I. ANALYTICAL PROCEDURES AND FINDINGS

| Element | Procedure | Interferences Listed: Feigl (3, 4) | Interferences Listed: Lange (7) | Interferences Found[a] | Remarks |
|---|---|---|---|---|---|
| | | Aqueous Group | | | |
| Al | Spot plate<br>1. 1 drop of soln.<br>2. 1 drop of 0.1 *N* NaOH<br>3. 1 drop of alizarin S<br>4. 2 drops of acetic acid<br>Orange color | Fe, Cu, Co, alkaline earths, Cr | Fe, Cr, Co, Mn | Cu(+), Ag(+), Ba(+)[b], Zr(+), Pb(+), Th(+), V(+), Bi(+), Mo(+), Cr(+), Fe(+), Ni(+), Co(+), Ca(+), Be(+) | Test drop should be alkaline prior to addition of alizarin S. Final pH should be less than 4.0. After $Na_2CO_3$–$Na_2O_2$ separation. V, Mo, and Cr are only interfering elements present. |
| Sb | A. Watch glass<br>1. 2 drops of soln.<br>2. 1 drop of $(NH_4)_2S$<br>3. 1 drop of concd. HCl<br>4. Remove excess liquid<br>5. 1 drop of concd. HCl Warm<br>B. Filter paper<br>1. 1 drop of phosphomolybdic acid<br>2. 1 drop of soln. A<br>3. Hold in steam<br>Blue stain | $Sn^{++}$ | Not listed | $Sn^{++}$(+) | $(NH_4)_2S$ separation yields Sn in stannic condition which does not interfere, thus making test specific. |
| As | Gutzeit apparatus<br>A. Place in body of apparatus<br>1. A few grains of Zn<br>2. 1 ml. of 0.1 *N* HCl<br>3. 4 drops of $SnCl_4$<br>4. 2 to 4 drops of test soln.<br>5. Put reaction stopper in place<br>B. Reaction stopper should be filled with PbAc paper and covered with $HgBr_2$ paper.<br>Yellow to black stain | Sb, $S^{--}$, Hg, $PO_4^{---}$ | Not listed | Au(−), Ag(−), Ba(−), Hg(−), Pb(−), Bi(−) | Interfering ions slow up reaction but by adding fresh Zn at intervals and allowing reaction to proceed 20 minutes or more test is specific for qualitative work. |
| Be | Casserole<br>1. 1 drop of soln.<br>2. 1 drop of concd. HCl<br>3. Evap. to dryness<br>4. Dissolve in 1 drop 5 *N* NaOH<br>5. 1 drop of quinalizarin<br>Blue color | $NH_4^+$, Cu, Ni, Fe, Mg, tartrates in presence of aluminum | Fe, $PO_4^{---}$, Mg | Ag(−), Au(−), Cu(−), Mg(+), Cd(+), Ba(+), $NH_4^+$(+) | 1 drop of KCN will mask Cu, Ag, and Au interferences. 1 ml. of $Br_2$ water fades color due to Mg but not that due to Be. Test is inconclusive in presence of Cd and Ba. |
| B | Glass spot plate<br>1. 1 drop of turmeric<br>2. 1 drop of soln.<br>3. 1 drop of 0.1 *N* HCl<br>4. Evaporate to dryness<br>5. 1 drop of 5 *N* NaOH<br>Greenish-blue color (momentary) | Oxidizing agents, $I^-$ | $PO_4^{---}$, $SiO_3^{--}$, oxidizing agents | $PO_4^{---}$(−),Au(+),Be(−), Si(−), Zn(−), Ti(−), V(−), Cr(−), Mo(−), Fe(−), Co(−) | Many ions give red colors with turmeric but only B and Au stains turn blue upon addition of NaOH. $Na_2CO_3$–$Na_2O_2$ separation eliminates all interferences except those due to $PO_4^{---}$, Si, Be, V, Cr, and Mo, all of which tend to inhibit test |
| Cl | Chloride apparatus<br>A. Place in ignition tube of apparatus<br>1. Suitable amount of dry sample<br>2. Few grains of $K_2CrO_4$<br>3. 1 drop of concd. $H_2SO_4$<br>B. Place 1 drop of fresh diphenylcarbazide in reaction capillary<br>C. Warm ignition tube<br>Pink color in reaction capillary | Ag, Hg, $NO_2^-$, Br, I, F | Not listed | Ag(−), Hg(−), I(−), Br(−), F(+) | Test is inconclusive in presence of Ag, Hg, and F. Very large amts. of I and Br mask test. |
| Cr | Spot plate<br>1. 1 drop of soln.<br>2. Acidify ($H_2SO_4$)<br>3. 1 drop of diphenylcarbazide<br>Pink color | Hg, V, Mo | Fe does not interfere | Hg(+), Zr(+), V(+), Mo (blue), Fe(+) | V and Mo are only interfering elements appearing in aqueous group. Mo interference may be eliminated by adding 1 drop of oxalic acid to test drop. |
| F | Spot plate<br>1. 1 drop of soln.<br>2. 1 drop of 0.1 *N* HCl<br>3. 1 drop of Zr-alizarin soln.<br>Pink reagent fades to yellow | $SO_4^{--}$, $S_2O_3^{--}$, $PO_4^{---}$, $AsO_4^{---}$, $C_2O_4^{--}$ | $SO_4^{--}$, $PO_4^{---}$ | Be(−), Al(−), Si(−), Th(−), Sb(−), S(+), Cr (masks) | Zr and Th appear in other group. S ($SO_4^{--}$) may be removed by adding 1 drop of $BaCl_2$ and filtering off $BaSO_4$ ppt. Be, Al, Si, and Sb suppress test if present in large amounts. |
| I | Spot plate<br>1. 1 drop of soln.<br>2. 1 drop of 0.1 *N* HCl<br>3. 1 drop of fresh starch paste (cold)<br>4. 1 drop of $KNO_2$<br>Blue color | Test inhibited by alum, $MgSO_4$, alkali sulfate, cyanides and certain organic compounds | Interferences not mentioned | Ag(−), Hg(−), Pb(−) | Specific within acid group. |
| Mo | Filter paper<br>1. 1 drop of concd. HCl<br>2. 1 drop of soln.<br>3. 1 drop of KCNS<br>4. 1 drop of $SnCl_2$<br>Red stain on edge of spot (not faded by $SnCl_2$) | Sensitivity reduced by $HCHO$, $H_3PO_4$, organic acids, Hg salts, nitrites | Not listed | Au (red color), Se(+), Fe(+), W (blue stain) | Stain due to Au turns purple when $SnCl_2$ is added. Red stain due to Mo is carried past any blue Au or W stain by capillary action. This capillary movement of Mo stain also differentiates it from red Se stain which is insoluble in HCl and therefore remains at center of spot. A specific test. |
| P | Spot plate<br>1. 2 drops of $(NH_4)_2MoO_4$ | Si, As | Interferences not given | Si(+), As(+) | All interferences are eliminated by adding 1 drop of concd. HCl, evaporating to dryness, and extracting with $HNO_3$. |

TABLE I (*Continued*)

| Element | Procedure | Interferences Listed: Feigl (*3*, *4*) | Interferences Listed: Lange (*7*) | Interferences Found[a] | Remarks |
|---|---|---|---|---|---|
| | | Aqueous Group(*Con't*) | | | |
| P (*Con't*) | 2. 1 drop of soln.<br>3. 2 drops of $HNO_3$<br>4. 1 drop of $SnCl_2$<br>Blue color | | | | Extract will contain only $PO_4^{---}$ as As distills off as chloride and Si is converted to insoluble $SiO_2$. Si interference alone may be prevented by adding small crystal of tartaric acid to $(NH_4)_2MoO_4$ drop. |
| K | Spot plate (black)<br>1. 1 drop of soln.<br>2. Evaporate to dryness<br>3. 1 drop of acetic acid<br>4. Few grains of sodium cobaltinitrite<br>Yellow ppt. | Li, Tl, $NH_4^+$ | Not listed | Pb(+) soluble when heated, Li(+) | Lithium interferes. Lead gives yellow ppt. soluble when heated, thus differentiating it from Li ppt. |
| Se | Spot plate<br>1. 1 drop of soln.<br>2. 1 drop of thiourea<br>3. 1 drop of $HNO_3$<br>Red color | Cu, $NO_3^-$, Te (yellow ppt.), Bi (yellow ppt.) | $NO_3^-$, Cu | $PO_4^{---}$(−), N(−), Au (brown ppt. soluble in excess reagent), Cu(−), V (very light blue color, does not interfere), Bi (yellow color), As(−), Sb(−), Cr (blue, green, or brown color. Very large amounts mask the test) | Only interfering element in this group is Cr. |
| Si | Casserole<br>1. 2 drops of $(NH_4)_2MoO_4$<br>2. 1 drop of soln.<br>3. Warm, cool, and filter from any visible ppt. of $(NH_4)_3PO_4 \cdot 12MoO_3$<br>4. 2 drops of oxalic acid<br>5. 1 drop of $SnCl_2$<br>Blue color | $AsO_4^{---}$ | $PO_4^{---}$, $AsO_4^{---}$ | As(+) | Arsenic may be removed by evaporating test drop in presence of 1 drop of concd. HCl. |
| Na | A. Watch glass<br>1. 2 drops of soln.<br>2. 1 drop of $(NH_4)_2S$<br>3. 1 drop of $(NH_4)_2CO_3$<br>4. Filter<br>B. Spot plate (black)<br>1. Filtrate A<br>2. 2 drops of acetic acid<br>3. 6 to 8 drops of zinc uranyl acetate<br>Yellow ppt. (allow 5 minutes to form) | None, except in very concentrated solns. | $PO_4^{---}$, proteins, large amounts of K | Ti(−), Pb(−), As(+), $PO_4^{---}$ (white ppt.) | Sulfide separation removes As and Pb. Large amounts of Ti reduce sensitivity. $PO_4^{---}$ interferes, owing to masking effect of zinc phosphate ppt. |
| S | Casserole<br>1. 1 drop of soln.<br>2. 1 drop of $HNO_3$<br>3. 1 drop of $Hg(NO_3)_2$<br>4. Evaporate to dryness<br>5. 2 drops of $H_2O$<br>Yellow ppt. | None | Not listed | V(+), Sb and As gave ppts. which masked test, Cr (masks) | If V, Sb, As, or Cr is present alternate procedure such as $BaCl_2$ test (carried out on black spot plate) should be used. |
| Sn | A. Watch glass<br>1. 1 drop of soln.<br>2. 1 drop of $(NH_4)_2S$<br>3. Absorb excess liquid<br>4. 1 drop of concd. HCl<br>5. Add few grains of powdered Mg and heat until Mg dissolves<br>B. Filter paper<br>1. 1 drop of soln. A<br>2. 1 drop of phosphomolybdic acid<br>Blue stain | Sb | Not listed | Sb(+) | If Sb is absent proceed from B. Test is specific. |
| W | Filter paper<br>1. 1 drop of concd. HCl<br>2. 1 drop of soln.<br>3. 1 drop of KCNS<br>4. 1 drop of $SnCl_2$<br>Blue stain in center of spot | Mo, $H_3PO_4$, certain organic hydroxy acids | Not listed | Au (red stain changing to blue upon addition of $SnCl_2$), Mo (red stain on edge of drop), Fe (red stain; disappears upon addition of $SnCl_2$), Se (red color in center of spot) | Specific when attention is paid to color and position of stains. Au and W both give blue stains in center of spot but Au gives a preliminary red stain upon addition of KCNS while W does not. |
| V | Filter paper<br>1. 1 drop of aniline<br>2. 1 drop of $HNO_3$<br>3. 1 drop of soln.<br>4. Warm<br>Green stain | Not listed | Not listed | $PO_4^{---}$(−), Sn(−), Cr(+), Br (yellow-brown color), I(+) | Cr, Br, I, Sn, and large amounts of $PO_4^{---}$ interfere. Confirm by 1 drop of soln., 1 drop of concd. $H_2SO_4$, 1 drop of $H_2O_2$. Red color. |
| | | Acid Group | | | |
| Ba | A. Watch glass<br>1. 2 drops of soln.<br>2. 1 drop of $(NH_4)_2S$<br>3. Filter<br>B. Filter paper<br>1. 1 drop of filtrate A<br>2. 1 drop of 0.1 *N* HCl<br>3. 1 drop of sodium rhodizonate (fresh)<br>4. Let stand until color develops<br>5. 1 drop of 0.1 *N* HCl brown-red stain; HCl causes slow fading | Heavy metals, Sr | Sr | $PO_4^{---}$(slight −), Sr (red stain; faded by HCl), Pb (red stain; turns purple upon addition of HCl), As(−), Sb(−), Cr(−), Fe (green-blue stain) | Sr stain differentiated from Ba stain since it fades quickly upon addition of HCl. All other possible interferences are eliminated by pptn. as sulfides. |

(*Continued on pages 274 and 275*)

TABLE I (*Continued*)

| Element | Procedure | Interferences Listed: Feigl (3, 4) | Interferences Listed: Lange (7) | Interferences Found[a] | Remarks |
|---|---|---|---|---|---|
| | | | Acid Group (*Con't*) | | |
| Bi | Spot plate<br>1. 1 drop of soln.<br>2. 1 drop of thiourea<br>3. 1 drop of $HNO_3$<br>Yellow color | Not listed | Specific | $PO_4^{---}$(−), Au (brown ppt., soluble in excess reagent), V (blue color), As(−), Sb(−), Cr (masks), Se (red ppt., masks) | Specific. All possible interferences appear in aqueous group. |
| Cd | Spot plate<br>1. 1 drop of diphenylcarbazide thiocyanate<br>2. 1 drop of soln.<br>3. 1 drop of sodium acetate<br>4. 1 drop of $NH_4OH$<br>Violet-pink color | Only very large amounts of Hg, Cu, Pb, and Bi interfere | Specific within Group II | $PO_4^{---}$ (slow up reaction), Cu(+), Ag(+), Au(+), Hg(+), V (blood red color), As(−), Sb(−), Cr(+), Fe(+), Pb (purple color), Bi(+), Ni(+), Co(purple color) | All interferences except Cu and Fe are eliminated by $Na_2CO_3$–$Na_2O_2$ separation Fe reduced to ferrous state does not interfere. Cu does not interfere if crystal of KI is added to reagent prior to test. |
| Ca | Watch glass<br>1. 2 drops of soln.<br>2. 1 drop of $(NH_4)_2S$<br>3. Warm, filter<br>Spot plate (black)<br>1. Filtrate A<br>2. 1 drop of fresh saturated soln. of dihydroxy tartaric osazone<br>Yellow precipitate | Only alkali and ammonium salts may be present | Alkaline earth metals | Li(+), Cu(+), Au(+), Ag(+), Be(+), Zn(+), Sr(+), Ba(+), Al(+), Zr(+), Sn(+), Pb(+), Th(+), V(+), As(+), Sb(+), Bi(+), Mo(+), Mn(+), Fe(+), Ni(+), Co(+) | All interferences are removed except those due to Be, Sr, and Ba. Very large amounts of Mg may give false test. |
| Co | Filter paper<br>1. 1 drop of soln.<br>2. 1 drop of α-nitroso-β-naphthol<br>3. Hold over $NH_4OH$<br>Brown stain | Fe, U, Cu, Pd | None listed | Cu(+), Au(+), Zr(+), V(+), U(+), Cr(+), Fe(+) | V and Cr appear in aqueous group. Zr, Cu, U, and Fe interferences may be prevented by adding 1 drop of $H_3PO_4$ and 1 drop KI to 1 drop of soln. on watch glass, stirring, then adding 2 drops of $Na_2S_2O_3$. Test on resulting soln. is specific. |
| Cu | Casserole<br>1. 1 drop of soln.<br>2. 1 drop of $(NH_4)_2HPO_4$<br>3. Acidify (concd. $H_2SO_4$)<br>4. 1 drop of $ZnSO_4$<br>5. 1 drop of ammonium mercuric thiocyanate<br>Violet ppt. | Fe, Cd, Co, Ni | Not listed | Au (brick-red ppt., masks), Cd (powder-blue ppt.), Fe (red color), Co (green ppt.) Ni (green-blue ppt.) | Gold is only interference if a control is run since colors due to other elements do not mask nor resemble positive Cu test. |
| Au | Filter paper<br>1. 1 drop of soln.<br>2. 1 drop of $SnCl_2$<br>3. 1 drop of KCNS<br>4. Boil in water<br>Purple stain | Not listed | Not listed | W(+) | Stain given by W is distinguished from true test for Au by fact that W stain fades in boiling water while Au stain does not. |
| Fe | Spot plate<br>1. 1 drop of soln.<br>2. 1 drop of KCNS<br>3. 1 drop of 0.1 *N* HCl<br>Red color | $PO_4^{---}$, organic hydroxyl compounds, Hg, $NO_2^-$ | Not listed | $PO_4^{---}$(−), Cu (slight yellow color), Au(+), Hg(−), Ag(−), As(−), Cr (masks), Mo(+), F(−), Mn (masks) | $Na_2CO_3$–$Na_2O_2$ separation removes interferences due to $PO_4^{---}$, Hg, Ag, Cr, Mo, and F. As, oxalates, citrates may be removed prior to test by evaporating in presence of 1 drop concd. HCl and roasting. |
| Pb | Spot plate<br>1. 1 drop of soln.<br>2. 2 drops of KCN<br>3. 1 drop of $NH_4Cl$<br>4. 1 drop of dithizone<br>5. Compare at once with blank<br>Pink color in $CCl_4$ layer | Specific | $Sn^{++}$, Bi, Tl | Sb(+), Bi(+) | Specific if blank and control are run, since Sb gives off-color green-brown soln. Bi gives red color similar to that given by Pb. |
| Mg | A. Watch glass<br>1. 2 drops of soln.<br>2. 1 drop of $(NH_4)_2S$<br>3. Warm and filter<br>B. Spot plate (white)<br>1. 1 drop of soln. A<br>2. 1 drop of KCN<br>3. 1 drop of Titan yellow<br>4. 1 drop of 5 *N* NaOH<br>Red color | Al, Zn, Sn, Cd, Ni, Co | Al, Ni, Co, Mn, Zn, Sn, $NH_4^+$ | Au(−), Zn(+), Cd(+), Hg(−), Al(−), Mn(+), Fe(+), Ni(+), Co(+), Sn(+) | Test is specific when $Na_2CO_3$–$Na_2O_2$ and $(NH_4)_2S$ separations are used. |
| Mn | Casserole<br>1. Portion of original sample<br>2. 1 to 4 drops of $AgNO_3$ | Cl, I, Br | Not listed | Cl(−), Br(−), I(−) | Test is specific since interference due to halogens may be prevented by adding excess $AgNO_3$. |

when the element was actually present. Table I summarizes the analytical procedures used and the findings of the investigation.

## Conclusions

A systematic study of interferences occurring with selected drop reactions has been made. The investigation dealt with tests for 38 elements.

Special attention is given to the types of interferences encountered and the term "positive interferences" is applied to interferences giving false tests. Interferences which inhibit a true positive test are designated by the term "negative interference".

The data obtained in this investigation are tabulated and applied to a study of the two-group sodium peroxide–sodium carbonate system of analysis. The effect of this separation on the elimination of interferences is noted.

TABLE I (*Concluded*)

| Element | Procedure | Interferences Listed: Feigl (*3, 4*) | Lange (*7*) | Interferences Found[a] | Remarks |
|---|---|---|---|---|---|
| | | Acid Group (*Con't*) | | | |
| Mn (*Con't*) | 3. 1 drop of $H_3PO_4$<br>4. Few grains of $(NH_4)_2S_2O_8$<br>5. Warm<br>Pink color | | | | |
| Hg | Spot plate<br>1. 1 drop of soln.<br>2. 1 drop of sodium acetate<br>3. 1 drop of dimethylaminobenzal rhodanine<br>4. Run blank<br>Pink color | Cl, Cu | Ag | Au(+), Ag(+), Cu(+), Si(−), Cl(−) | pH of test soln. should be between 3 and 7. Acetic acid makes best medium. When blank is run Cu and Au are only interferences. Interferences due to Cu may be prevented by adding 1 drop of $Na_2HPO_4$ to test drop. |
| Ni | A. Watch glass<br>1. 1 drop of soln.<br>2. 1 drop of $H_2O_2$<br>3. 1 drop of $(NH_4)_2CO_3$<br>4. 1 drop of $NH_4OH$<br>B. Filter paper<br>1. Absorb soln. A on filter paper.<br>2. 1 drop of dimethylglyoxime<br>Pink stain | Pd, Pt, Co, $Fe^{+++}$ + Co, Cu, Mn, $Fe^{++}$ | $Fe^{++}$, Co, Pd | Mn(−), Fe(+), Co(+) | Test is specific when preliminary treatment A is carried out. |
| Ag | Spot plate<br>1. 1 drop of acetic acid<br>2. 1 drop of $K_2CrO_4$<br>3. 1 drop of test soln. containing $(NH_4)_2CO_3$<br>Red ppt. | None given | Not listed | $MnO_4^-$ (masks) | $MnO_4^-$ may be reduced with 1 drop of thiourea to $MnO_2.xH_2O$ and filtered off, making test specific. |
| Sr | A. Watch glass<br>1. 2 drops of soln.<br>2. 2 drops of $(NH_4)_2S$<br>B. Filter paper<br>1. Filtrate from A<br>2. 1 small drop of 0.1 *N* HCl<br>3. 1 drop of $K_2CrO_4$<br>4. 1 drop of sodium rhodizonate<br>Brown stain | Ba, heavy metals | Ba | Ag (masks), Pb(+), As(−), Sb(−), Fe(−) | Test drop should have pH between 5 and 7. Barium interference is prevented by pptn. as $BaCrO_4$. Other interferences are removed by sulfide pptn. |
| Ti | Spot plate<br>1. 1 drop of soln.<br>2. 1 drop of $H_3PO_4$<br>3. 1 drop of $H_2O_2$<br>Yellow color | F, Cl, Br, I, V, Cr, Fe, Acetate | Not listed | V(+), Cr (blue color), Mo(+), F(−), Cl(−), Br(−), I (masks) | All interfering substances appear in aqueous group, making test specific. |
| U | A. Watch glass<br>1. 1 drop of soln.<br>2. 4 drops of $Na_2S_2O_3$<br>B. Filter paper<br>1. Filtrate from A absorbed on filter paper<br>2. 1 drop of $K_4Fe(CN)_6$<br>3. 1 drop of 0.1 *N* HCl<br>Brown stain | Specific | Not listed | Be (blue stain), V (yellow stain), Fe (green stain) | Other stains are so light that test may be considered specific. |
| Zn | Casserole<br>1. 1 drop of soln.<br>2. 1 drop of $(NH_4)_2HPO_4$<br>3. 2 drops of $H_2SO_4$<br>4. 1 drop of $CuSO_4$<br>5. 1 drop of ammonium mercuric thiocyanate<br>6. Warm<br>Violet ppt. | Co, Fe, Ni | Not listed | Au (red ppt.), Cd (off-color blue ppt.), Fe (red color), Ni (green ppt.), Co (blue ppt.) | Test is specific if blank and control are run. |
| Zr | Glass spot plate<br>1. 1 drop of soln.<br>2. 1 drop of alizarin<br>3. Warm<br>4. 1 drop of HCl<br>Brown-pink color | F, $SO_4^{--}$, $PO_4^{---}$, Mo, W, organic hydroxyacids | Not listed | $PO_4^{---}$(−), Si(−), Sb(−), W(−), Mo(−), F(−) | All interferences appear in aqueous group, making test specific. |

[a] Interferences were studied without prior separations.
[b] −, negative interference (suppression of test). +, positive interference (false test).

## Literature Cited


(1) Arthur, Paul, and Smith, O. M., "Semimicro Qualitative Analysis", 1st ed., New York, McGraw-Hill Book Co., 1938.
(2) Engelder, C. J., Dunkelberger, T. H., and Shiller, W. J., "Semimicro Qualitative Analysis", New York, John Wiley & Sons, 1936.
(3) Feigl, F., "Qualitative Analyse mit Hilfe von Tüpfelreaktionen", 2nd ed., Leipzig, Akademische Verlagsgesellschaft, 1935.
(4) Feigl, F., "Spot Tests", New York, Nordeman Publishing Co., 1937.
(5) Gutzeit, G., *Helv. Chim. Acta*, **12**, 713, 839 (1929).
(6) Heller, K., *Mikrochemie*, **8**, 33 (1930).
(7) Lange, "Handbook of Chemistry", 2nd ed., Sandusky, Handbook Publishers, Inc., 1937.
(8) Nieuwenburg, C. J. van, and Dulfer, G., "Short Manual of Systematic Qualitative Analysis by Means of Modern Drop Reactions", 2nd ed., Amsterdam, D. B. Centen, 1935.
(9) West, P. W., and Smith, Lothrop, *J. Chem. Education*, **17**, 139–45 (1940).
(10) Winkley, J. H., Yanowski, L. K., and Haynes, W. A., *Mikrochemie*, **21**, 102–15 (1936).


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# Studies of the Methoxyl Determination

## Modification of Apparatus and Preparation of Hydriodic Acid

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IN THE course of an investigation, in which it was necessary to make a large number of methoxyl determinations, erratic results were encountered in running known samples, and were soon traced to the reagents. The importance of using highly purified reagents, especially hydriodic acid, is generally recognized (*6*, *10*); however, a review of the literature indicates that little has been published (*5*) regarding the nature of the interfering impurities or their probable origin. For this reason the following investigation was undertaken.

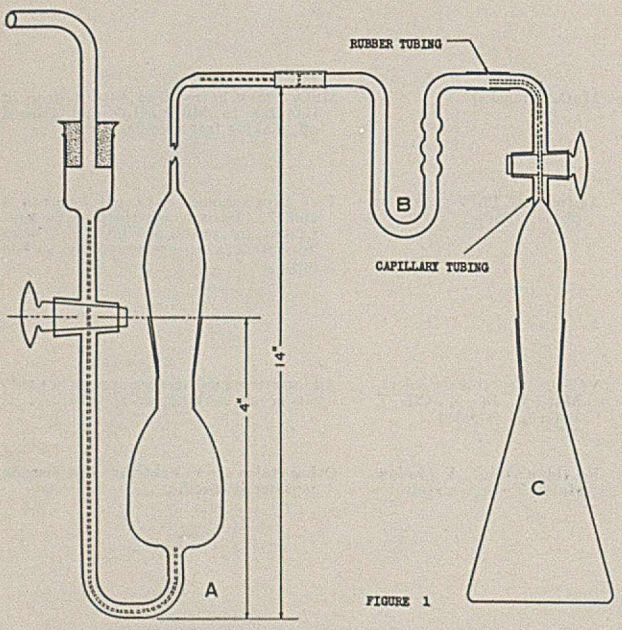


FIGURE 1

A modified Pregl apparatus was used. Many modifications (*1*, *2*, *3*, *15*, *16*, *17*) of the original methoxyl apparatus have been reported, and much attention has been given to the question of absorption (*7*, *9–12*, *15*, *18*). In this laboratory this difficulty was overcome by using an evacuated flask (containing the bromine solution) as a receiver for the alkyl iodide. Besides preventing loss of alkyl iodides, this arrangement provided an excellent control on the rate of gas flow.

### Apparatus

The apparatus, made of Pyrex glass, is illustrated diagrammatically in Figure 1. The reaction unit, *A*, was constructed from a 14/35 standard taper joint and a capillary stopcock, and had a volume of 7 ml. to the ground-glass joint. The condensing unit, made from 5-mm. and capillary Pyrex tubing was 35 cm. over-all in length. It was equipped with a water jacket (not shown) and was connected to U-tube *B*, which in turn was connected to absorption flask *C*. The absorption flask for the analysis of the alkyl iodides was constructed from a 250-ml. Erlenmeyer flask, a 24/40 standard taper joint, and a two-way capillary stopcock.

A phosphoric acid bath (*4*) served as a heating medium for the reaction vessel. The temperature was maintained by a microburner within ±5° C.

Approximately 5 mg. of material weighed in a glass boat were introduced into the reaction chamber, along with a few crystals of phenol and 4 or 5 drops of acetic anhydride, and the vessel was connected to the air-condensing unit. The U-tube was removed, charged with 0.5 cc. each of 5 per cent cadmium sulfate and sodium thiosulfate solutions, and then connected to the apparatus.

The absorption flask was charged with 2 ml. of 10 per cent sodium acetate–glacial acetic acid solution and 5 drops of bromine. The joint was lubricated with glycerol and the flask was evacuated by means of a water pump and connected to the open arm of U-tube *B*.

After making sure that the charge had dissolved, 2 ml. of hydriodic acid were introduced through the capillary stopcock, which was then connected to an open Kipp generator for carbon dioxide. The gas flow was regulated to approximately 10 to 30 bubbles per minute (depending on time of heating) and the reaction vessel brought to a temperature of 120° to 130° C. by means of a phosphoric acid bath.

After the heating operation (from 30 to 60 minutes) the flask was disconnected and the male joint sufficiently opened to permit the pipetting of 5 ml. of 20 per cent sodium acetate solution. The joint was again closed, and by inverting and shaking the flask, the entire inner surface was washed with solution. Formic acid was added dropwise until the solution was colorless, 4 to 6 drops in excess being used. Two milliliters of 10 per cent potassium iodide were introduced, and the solution was acidified with 5 ml. of 2 *N* sulfuric acid. The liberated iodine was then titrated with standard thiosulfate. From these data the methoxyl content was readily calculated.

TABLE I. BLANK RUNS

| Hydriodic Acid Sample | Blank, 0.02 *N* Thiosulfate *Ml.* |
|---|---|
| From $H_3PO_4$ | |
| 1 | 0.04 |
| 2 | 0.04 |
| 3 | 0.06 |
| Baker's, sp. gr. 1.5 (not redistilled) | 0.05 |
| After first redistillation (ordinary distilling unit) | 0.56 |
| After second redistillation (same unit) | 1.24 |
| After third redistillation (all-glass unit) | 0.08 |

TABLE II. PRECISION OF APPARATUS

| | Sample Weight *Mg.* | Methoxyl Found % | Methoxyl Calculated % |
|---|---|---|---|
| Anisic acid | 3.176 | 20.6 | 20.4 |
| | 4.380 | 20.3 | |
| Dibromo-*p*-dimethoxybenzene | 5.665 | 21.3 | 21.0 |
| | 4.159 | 21.1 | |
| Vanillin | 2.050 | 20.1 | 20.4 |
| | 3.030 | 20.2 | |
| | 2.775 | 20.1 | |
| | 4.048 | 20.2 | |
| | 3.731 | 20.3 | |

### Discussion and Results

Blank runs, made with hydriodic acid obtained from various sources, gave values varying from 0.04 to 1.5 cc. of 0.02 *N* thiosulfate. Initial experiments indicated that the magnitude of the blank was due mainly to the hydriodic acid used. It was thus evident that the interfering impurities must be both volatile and readily converted to an oxidizing agent by the bromine—probably arsenic compounds or alkyl iodides.

A Gutzeit determination showed the presence of only traces of arsenic. To prove the presence of alkyl iodide as an interfering impurity in the hydriodic acid, the gases from a blank determination (using carbon dioxide–free air as a carrier gas) were passed over a heated platinum spiral. Both iodine and carbon dioxide were found in sufficient quantities to account for most of the blank. These experiments gave

reasonable assurance that a volatile organic iodide must have been present in the hydriodic acid. Clark appears to have recognized this fact when he suggested refluxing in a stream of carbon dioxide as one step in preparation.

The nature of the impurities in hydriodic acid depends, of course, on the reagents used in making it. Various methods are reported for its preparation (*5*, *8*, *13*, *19*), some involving the use of organic materials. In order to avoid either sulfur or organic contaminants, the authors prepared a number of samples of hydriodic acid by distilling a mixture of 85 per cent phosphoric acid and potassium iodide (*14*) under reduced pressure (in an all-glass distilling unit). Constant-boiling acid obtained from this source gave the values noted in Table I. This method of preparing hydriodic acid proved to be rapid and simple.

In connection with this work the deleterious effect of cork on purity of hydriodic acid was observed. To measure the relative effect of cork, a sample of hydriodic acid was distilled several times in an ordinary distilling unit provided with two cork stoppers. The importance of keeping hydriodic acid out of contact with organic matter such as cork is indicated in Table I.

To illustrate the precision of the modified apparatus, a number of successive runs with it are tabulated in Table II.

## Literature Cited


(1) Asahina, Y., and Maeda, S., *J. Pharm. Soc. Japan*, 50, 660–5 (1930).
(2) Campbell, W. G., *Chemistry and Industry*, 51, 590 (1932).
(3) Chinoy, J. J., *Analyst*, 61, 602–3 (1936).
(4) Christensen, B. E., and King, A., Ind. Eng. Chem., Anal. Ed., 8, 194 (1936).
(5) Clark, E. P., *Ibid.*, 10, 677 (1938).
(6) Clark, E. P., *J. Assoc. Official Agr. Chem.*, 15, 136–40 (1932).
(7) Eaton, E. P., and West, E. A., *J. Biol. Chem.*, 75, 283 (1927).
(8) Ebaugh, W. C., *J. Sci. Lab. Denison Univ., Bull.* 21, 403–4 (1926).
(9) Elek, A., Ind. Eng. Chem., Anal. Ed., 11, 174–7 (1939).
(10) Friedrich, A., *Z. physiol. Chem.*, 163, 141–8 (1927).
(11) Gibson, D. T., and Caulfield, T. C., *J. Chem. Soc.*, 1935, 1419–20.
(12) Guillemet, R., *Bull. soc. chim.*, 51, 1547–51 (1932).
(13) Houben, J., Boedler, J., and Fischer, W., *Ber.*, 69B, 1766–88 (1936).
(14) Lepsius, B., *Ibid.*, 23, 1642 (1890).
(15) Nanji, H. R., *Analyst*, 59, 96–8 (1934).
(16) Rigakos, R. D., *J. Am. Chem. Soc.*, 53, 3903–4 (1931).
(17) Viebock, F., and Schroappoch, A., *Ber.*, 63, 2818–23 (1930).
(18) White, E. V., and Wright, G. F., *Can. J. Research*, 14B, 427–9 (1936).
(19) Whitehead, T. H., *J. Chem. Education*, 8, 541–2 (1931).


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# Micro- and Drop-Scale Titration of Oxalate

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SMALL amounts of oxalate have been titrated in the determination of calcium and other materials as a routine part of many methods. The common procedures in which permanganate is employed without the use of an additional indicator become increasingly less dependable as the amount of oxalate is lowered, because of the combination of a feeble end-point color, a proportionally large blank, and a relatively low stability of both titrating solution and oxalate in dilute, hot, acid solution (*2*).

Rappaport and Rappaport (*5*) utilized excess ceric sulfate for the determination of small amounts of calcium oxalate, determining the excess iodometrically. Larson and Greenberg (*3*) recorded the use of a similar procedure in which ferrous ammonium sulfate was used to determine the excess ceric sulfate. They reported no detailed study of the conditions necessary for the titration. Lindner and Kirk (*4*) studied the factors influencing the use of this procedure on the drop scale, while Ellis (*1*) employed ammonium hexanitrato cerate in perchloric acid solution for the same scale of titration. The use of various cerates including nitrato and perchlorato cerate on a macro scale has been studied by Smith and Getz (*6*). In their work, the reagents were 0.05 *N*, which is considerably stronger than can be employed conveniently in the type of titration reported here. Since all cerate solutions appear to become less stable with dilution, this point is of importance.

Extended use of these oxalate titrations on the micro and drop scale in this laboratory has shown various discrepancies in and between the different procedures which require control, and each method appears to have definite limitations in range which have never been adequately defined. In this communication are reported a study and definition of the conditions and range for each of these titrations, and an investigation of the little studied field of the use of permanganate with indicators in micro- and drop-scale titrations.

## Solutions

0.01 *N* Sodium Oxalate Solution, 0.1675 gram of pure sodium oxalate dissolved to make 250 ml. of solution.

Approximately 0.01 *N* Ceric Sulfate Solution. Approximately 6.7 grams of anhydrous ceric sulfate were heated gently with 28 ml. of redistilled sulfuric acid and 28 ml. of redistilled water. More water was added slowly to complete solution while warming, the volume being made to 400 to 500 ml. The solution was placed on a steam bath overnight and then diluted to 1 liter with redistilled water. The solution was standardized through ferrous ammonium sulfate (Mohr salt) solution against pure sodium oxalate, by the procedure of Smith and Getz (*6*).

0.01 *N* Mohr Salt Solution. Approximately 3.94 grams of pure ferrous ammonium sulfate were dissolved in approximately 0.1 *N* sulfuric acid to make 1 liter. This was standardized against pure sodium oxalate through ceric sulfate solution. This solution is relatively much more stable than the ferrous sulfate solution used by Smith and Getz.

0.01 *N* Hexanitrato Cerate in 1 *N* Perchloric Acid Solution, 20.21 grams of ammonium hexanitrato cerate dissolved in 200 to 300 ml. of water. To the solution were added 110 ml. of 60 per cent perchloric acid and water to make 1 liter. The resulting solution was heated on a steam bath for 24 hours, filtered, and standardized against pure sodium oxalate.

0.01 *N* Hexanitrato Cerate in 1 *N* Nitric Acid Solution, made as described above, except for the substitution of 65 ml. of concentrated nitric acid for the perchloric acid.

0.01 *N* Potassium Permanganate Solution. Approximately 3.5 grams of potassium permanganate were dissolved in water and diluted to 1 liter. This solution was allowed to stand for about 1 week and was filtered through asbestos which had previously been boiled in acid permanganate and washed. This solution, which was slightly more than 0.1 *N*, was carefully standardized against pure sodium oxalate and diluted as necessary to 0.01 *N* or 0.005 *N* with redistilled water. The diluted solution was in all cases rechecked against sodium oxalate solution.

Indicator Solutions. *o*-Phenanthroline–ferrous sulfate was prepared dissolving 1.485 grams of *o*-phenanthroline in 100 ml. of 0.025 *M* ferrous sulfate solution, to yield a 0.025 *M* stock solution. Small amounts of this solution were diluted with 9 parts of water before use. The indicator, either in stock or dilute solution, could be kept indefinitely. Setopaline C solution, made in 0.1 per cent aqueous solution, slowly deteriorated so that after a week it yielded a poor end point. It has been found in this laboratory that it can be regenerated as often as necessary by placing it on the steam bath for an hour with occasional shaking.

TABLE I. CONDITIONS FOR MICROTITRATION OF OXALATE WITH EXCESS CERIC SULFATE AND MOHR SALT

(5 ml. of 2 $N$ $H_2SO_4$ added in all cases. 2.99 ml. of 0.0133 $N$ $Ce(SO_4)_2$ used.)

| 0.0100 $N$ Oxalate Taken | Titer, 0.0107 $N$ Mohr Salt | Treatment |
|---|---|---|
| *Ml.* | *Ml.* | |
| None | 3.706 | Direct titration |
| | 3.707 | |
| | 3.705 | |
| 1.003 | 2.826 | 30 minutes at room temperature |
| | 2.811 | |
| | 2.825 | |
| 1.003 | 2.798 | 3 hours at room temperature |
| | 2.801 | |
| | 2.803 | |
| 1.003 | 2.785 | 10 minutes on steam bath |
| | 2.785 | |
| | 2.775 | 15 minutes on steam bath |
| | 2.772 | 30 minutes on steam bath |
| | 2.770 | |
| | 2.771 | |
| | 2.771 | 45 minutes on steam bath |
| | 2.770 | |
| | 2.771 | |

## Microtitrations with Ceric Sulfate

Since the determination of calcium rests directly on the titration of oxalate in the calcium oxalate precipitate, the titration of the oxalate in sodium oxalate solution with ceric sulfate was studied. Larson and Greenberg (*3*) implied that a short period of standing of the oxalate with excess ceric sulfate sufficed to complete the oxidation. Numerous trials of their procedure showed low and variable results. They did not employ a catalyst and in this study none is used, though the rate of the reaction of ceric sulfate with oxalate is increased markedly by use of iodine chloride or osmic acid. In Table I is shown the effect of different times of standing and of heat on the completeness of the reaction. To the sample was added a measured excess of ceric sulfate solution, the mixture was treated as indicated, and the excess cold ceric sulfate was titrated with standard ferrous ammonium sulfate solution, using *o*-phenanthroline–ferrous sulfate or Setopaline C as indicator. When no catalyst is used, the reaction does not reach completion unless the reactants are warmed on the steam bath for 30 minutes. The same length of time of standing in the cold gives about 98 per cent complete oxidation.

It is important that the acidity of the final solution be kept to at least 0.5 $N$ and usually it was made about 1 $N$ with sulfuric acid. At lower acidities several effects could be observed, all of which prevented an accurate determination. The usual first effect was a failure to observe a good color change, sometimes with fading. At still lower acidities hydrolysis of ceric sulfate was obtained with precipitation of the oxide.

The range of utility of the excess method with ceric sulfate is shown in Table II. By use of 0.005 $N$ ceric sulfate it was found possible to determine the oxalate corresponding to as little as 0.02 mg. of calcium with an error in titration of not more than 1 per cent. Part I represents complete calcium analyses in which the errors arise from all sources inherent in the method. Parts II and III represent only the titration of sodium oxalate in accurately known amounts, the calcium equivalent being given in each case. In these parts, the errors listed represent only those of the titration itself. It is readily apparent that the titration performed in the manner specified involves errors which are usually negligible in comparison with those of the entire calcium determination, down to about 0.02 mg. of calcium, and using only standard microtechnique. Smaller quantities require drop-analysis methods (*4*) if an accuracy of about ±1 per cent is desired.

## Microtitration with Hexanitrato Cerate

Ammonium hexanitrato cerate dissolved in 1 or 2 $N$ nitric or perchloric acid (perchlorato cerate) may be used for direct titration of oxalate, owing to the high oxidation potential of these reagents (*6*) which causes an increased rate of oxidation. Perchlorato cerate was successfully used as a drop-scale reagent by Ellis (*1*). Application of these reagents for microtitration have been rare.

Sodium oxalate samples equivalent to the amounts of calcium indicated were acidified with nitric or perchloric acid and titrated directly with the corresponding cerate. Table III gives the results of the titration with nitrato cerate solution at two values of oxalate concentration. The data indicate a reasonable accuracy but are somewhat misleading in that the reaction was too slow for convenience and the end point (with *o*-phenanthroline–ferrous sulfate) was difficult to read, particularly in the presence of asbestos. The reaction was of about equal utility to the permanganate titration described below, but was slower.

The use of perchlorato cerate for direct titration of oxalate was considerably better, because of the satisfactory speed of oxidation. The sample was made about 1 $N$ in perchloric acid and titrated in the cold with the perchlorato cerate solution, using Setopaline C as indicator. *o*-Phenanthroline–ferrous sulfate was less satisfactory, because of precipitation of the perchlorate of phenanthroline when the acidity was in excess of about 0.5 $N$. The end point with Setopaline C consisted in a final change from yellow to red, proceeding through a bronze which faded to yellow as the end point was approached. The final red color was permanent for several minutes and was readily visible in the presence of asbestos if about 30 seconds were allowed for full color development. Table IV shows a satisfactory accuracy in the titration with

TABLE II. RANGE AND ACCURACY OF OXALATE TITRATION WITH CERIC SULFATE

| Calcium Taken | Mohr Salt: Blank | Mohr Salt: Titer | Mohr Salt: Difference | Calcium Found | Error |
|---|---|---|---|---|---|
| *Mg.* | *Ml.* | *Ml.* | *Ml.* | *Mg.* | % |
| I. Calcium analysis, 4.96 ml. of 0.01 $N$ ceric sulfate used | | | | | |
| 0.540 | 5.959 | 3.415 | 2.544 | 0.545 | 0.9 |
| 0.540 | 5.959 | 3.406 | 2.553 | 0.546 | 1.1 |
| 0.540 | 5.959 | 3.412 | 2.547 | 0.545 | 0.9 |
| 0.526 | 5.963 | 3.507 | 2.456 | 0.525 | 0.2 |
| 0.526 | 5.963 | 3.512 | 2.451 | 0.526 | 0.0 |
| II. Oxalate titrations, 2.99 ml. of 0.01 $N$ ceric sulfate used | | | | | |
| Calcium Equivalent | | | | | |
| *Mg.* | | | | | |
| 0.200 | 3.705 | 2.772 | 0.933 | 0.199 | 0.5 |
| 0.200 | 3.705 | 2.770 | 0.935 | 0.200 | 0.0 |
| 0.200 | 3.705 | 2.771 | 0.934 | 0.200 | 0.0 |
| 0.200 | 3.705 | 2.771 | 0.934 | 0.200 | 0.0 |
| 0.200 | 3.705 | 2.771 | 0.934 | 0.200 | 0.0 |
| 0.200 | 3.705 | 2.770 | 0.935 | 0.200 | 0.0 |
| 0.200 | 3.705 | 2.772 | 0.933 | 0.199 | 0.5 |
| III. Oxalate titrations, 2.99 ml. of 0.005 $N$ ceric sulfate used | | | | | |
| 0.0199 | 3.686 | 3.334 | 0.352 | 0.0197 | 1.0 |
| 0.0199 | 3.686 | 3.333 | 0.353 | 0.0198 | 0.5 |
| 0.0199 | 3.686 | 3.333 | 0.353 | 0.0198 | 0.5 |

TABLE III. MICROTITRATIONS OF OXALATE WITH AMMONIUM HEXANITRATO CERATE

| Ca Equivalent of Oxalate in Sample | Titer, 0.0085 $N$ Cerate | Oxalate Found, Ca Equivalent | Error |
|---|---|---|---|
| *Mg.* | *Ml.* | *Mg.* | % |
| 0.598 | 3.526 | 0.599 | 0.2 |
| 0.598 | 3.529 | 0.600 | 0.3 |
| 0.598 | 3.534 | 0.601 | 0.5 |
| 0.198 | 1.166 | 0.198 | 0.0 |
| 0.198 | 1.168 | 0.199 | 0.5 |
| 0.198 | 1.164 | 0.198 | 0.0 |

TABLE IV. MICROTITRATIONS OF OXALATE WITH AMMONIUM HEXAPERCHLORATO CERATE

| Ca Equivalent of Oxalate in Sample | Titer, 0.0044 *N* Cerate | Oxalate Found, Ca Equivalent | Error |
|---|---|---|---|
| *Mg.* | *Ml.* | *Mg.* | % |
| 0.200 | 2.287 | 0.201 | 0.5 |
| 0.200 | 2.274 | 0.200 | 0.0 |
| 0.200 | 2.285 | 0.201 | 0.5 |
| 0.200 | 2.282 | 0.201 | 0.5 |
| 0.200 | 2.288 | 0.201 | 0.5 |
| 0.200 | 2.282 | 0.201 | 0.5 |

oxalate corresponding to 0.2 mg. of calcium, but the method did not show itself to be so accurate as the back-titration using ceric sulfate oxidation, and was not found useful for the lower ranges of sample size.

TABLE V. MICROTITRATION OF OXALATE WITH POTASSIUM PERMANGANATE

| Titer, $KMnO_4$ | Blank | Oxalate Found, Calcium Equivalent | Error |
|---|---|---|---|
| *Ml.* | *Ml.* | *Mg.* | % |
| I. 0.0166 *N* $KMnO_4$ serving as its own indicator, oxalate equivalent to 0.200 mg. of Ca | | | |
| 1.046 | 0.05 | 0.199 | 0.5 |
| 1.058 | | 0.202 | 1.0 |
| 1.041 | | 0.198 | 1.0 |
| 0.055 | | 0.201 | 0.5 |
| II. 0.0166 *N* $KMnO_4$, ferrous phenanthroline as indicator, oxalate equivalent to 0.200 mg. of Ca | | | |
| 0.968 | 0.022 | 0.198 | 1.0 |
| 0.970 | | 0.199 | 0.5 |
| 0.963 | | 0.192 | 4.0 |
| 0.967 | | 0.198 | 1.0 |
| III. 0.0058 *N* $KMnO_4$, ferrous phenanthroline as indicator, oxalate equivalent to 0.200 mg. of Ca | | | |
| 1.914 | 0.040 | 0.199 | 0.5 |
| 1.911 | | 0.198 | 1.0 |
| 1.916 | | 0.199 | 0.5 |
| 1.913 | | 0.199 | 0.5 |
| IV. 0.0116 *N* $KMnO_4$ Setopaline C as indicator, oxalate equivalent to 0.200 mg. of Ca | | | |
| 0.906 | 0.006 | 0.200 | 0.0 |
| 0.899 | | 0.198 | 1.0 |
| 0.898 | | 0.198 | 1.0 |
| 0.898 | | 0.198 | 1.0 |
| 0.900 | | 0.199 | 0.5 |
| 0.894 | | 0.197 | 1.5 |
| 0.896 | | 0.198 | 1.0 |
| V. 0.005 *N* $KMnO_4$, Setopaline C as indicator, oxalate equivalent to 0.040 mg. of Ca | | | |
| 0.399 | 0.009 | 0.039 | 2.5 |
| 0.419 | | 0.041 | 2.5 |
| 0.398 | | 0.039 | 2.5 |
| 0.410 | | 0.040 | 0.0 |
| 0.400 | | 0.039 | 2.5 |

## Drop-Scale Titration with Perchlorato Cerate

Many attempts to utilize perchlorato cerate for direct drop-scale titration of oxalate were unsatisfactory. In most of these the indicator was either precipitated (phenanthroline) or consumed by the oxidant (Setopaline C). If the end point was exactly known and the indicator added directly before reaching it, good results were obtained. Since this was impractical with unknowns, it was concluded that the method was not successful in the authors' hands. This is contrary to the experience of Ellis and probably indicates some differences in reagents or technique. Confirmation of Ellis' method (*1*) by other laboratories may be expected to clarify the discrepancy. No such difficulties have been encountered in the method of Lindner and Kirk (*4*), which is being used successfully in various laboratories including that of the authors.

## Direct Microtitration with Permanganate

The use of permanganate for titrating small amounts of oxalate has long been a standard procedure but suffers from a progressively less distinct end point as the amounts are reduced. Internal indicators to sharpen the end point have not been much employed and seemed to offer an improvement. Since indicators such as *o*-phenanthroline–ferrous sulfate and Setopaline C are not stable in hot acid solution, a little manganous sulfate had to be added as a catalyst. The resulting cold catalyzed permanganate titration was definitely more precise than the usual hot titration and was probably more accurate as well, since the known errors of the hot titration (*2*) were eliminated. *o*-Phenanthroline–ferrous sulfate was not a very favorable indicator, because of its change from red to light blue and the obscuring of the latter color by the purple

TABLE VI. DROP-SCALE TITRATIONS OF OXALATE WITH POTASSIUM PERMANGANATE

| Calcium Equivalent of Oxalate Taken | Titer, 0.0213 *N* $KMnO_4$ | Oxalate Found, Calcium Equivalent | Error |
|---|---|---|---|
| γ | λ | γ | % |
| 9.57 | 22.40 | 9.48 | 0.9 |
| 9.57 | 22.49 | 9.56 | 0.5 |
| 9.57 | 22.40 | 9.48 | 0.9 |
| 7.23 | 16.97 | 7.18 | 0.7 |
| 7.23 | 16.90 | 7.15 | 1.1 |
| 7.23 | 16.85 | 7.13 | 1.4 |
| 5.49 | 12.92 | 5.46 | 0.5 |
| 5.49 | 12.87 | 5.44 | 0.9 |
| 5.49 | 12.90 | 5.45 | 0.7 |

of the permanganate. It could be used if added immediately before the end point, followed by very careful permanganate additions. Setopaline C was found satisfactory, but could not be added until the slow disappearance of the permanganate indicated approach to the end point. The color change was from blue green through lemon yellow to bronze, the latter change being most readily observed and taken as the end point. It was easily possible to use permanganate as dilute as 0.005 *N*.

Table V shows the results of titration with two strengths of permanganate, using both *o*-phenanthroline–ferrous sulfate and Setopaline C as indicators and permanganate as its own indicator. The possible accuracy was not greatly different in the three cases, but the blank values with permanganate as its own indicator were largest by a considerable amount, those with Setopaline C being least. The ease of titration was definitely greatest with the latter indicator. The permanganate titration was inferior to the excess ceric sulfate method, even when an internal indicator was used, because the accuracy could not be retained in the lower ranges of oxalate concentration.

## Direct Drop-Scale Permanganate Titrations

Direct titration of oxalate with permanganate on the drop scale could best be done with *o*-phenanthroline–ferrous sulfate as the indicator, since in this case it was stable enough to be added at the start of the titration while Setopaline C was not. The permanganate was so diluted on this scale that it did not interfere with the perception of the blue end point.

Varying amounts of 0.01 *N* sodium oxalate were titrated with 0.0213 *N* potassium permanganate. In order to avoid indicator blank, the *o*-phenanthroline–ferrous sulfate was titrated exactly to its end-point color and the necessary amount was then added directly to the oxalate solution to be titrated. In measuring the sample an amount of indicator proportional to the amount of oxalate was always taken. In performing calcium analyses, the indicator would have to be added separately, but there could be no indicator blank in either case because of the preliminary adjustment of the indicator to its end-point color. After addition of manganous sulfate solu-

tion as a catalyst, the titration was performed in the cold. Table VI shows the accuracy and precision of such titrations on the drop scale. Permanganate used in this manner is a satisfactory oxidant for drop-scale use, but was not found to be so favorable for this purpose as the excess ceric sulfate method previously studied (*4*).


### Summary

A comparative study of micro- and drop-scale titrations of oxalate and of calcium determination was made with excess ceric sulfate, ammonium hexanitrato cerate, ammonium hexaperchlorato cerate, and potassium permanganate solutions as reagents.

The necessary conditions for the titration were defined and the range, accuracy, and general utility studied for each titration.

The excess ceric sulfate method was found to have the widest range of applicability for both micro- and drop-scale titrations, and to be capable of the greatest accuracy.

Both ammonium hexaperchlorato cerate and potassium permanganate could be used for direct microtitrations of oxalate, but only the latter was useful for drop-scale work. Improvement in the use of permanganate was achieved by titrating the cold solution, using an internal indicator and manganous salt as a catalyst.


### Literature Cited


(1) Ellis, G. H., IND. ENG. CHEM., Anal. Ed., 10, 112 (1938).
(2) Kolthoff, I. M., and Furman, N. H., "Volumetric Analysis", New York, John Wiley & Sons, 1928.
(3) Larson, C., and Greenberg, D. M., *J. Biol. Chem.*, 123, 199 (1938).
(4) Lindner, R., and Kirk, P. L., *Mikrochemie*, 22, 291 (1937).
(5) Rappaport, F., and Rappaport, D., *Ibid.*, 15, 107 (1934).
(6) Smith, G. F., and Getz, C. A., IND. ENG. CHEM., Anal. Ed., 10, 304 (1938).


# Determination of Dietary Fluorine


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IN A STUDY of physiological action of ions of sea water the presence of fluorine (*14*) and the difficulties in making an artificial sea water were noted. Later it was found that after sea water was evaporated for easier transportation the fluorine would not redissolve. In a study of nutrition of rats, evaporated sea water was fed (*13*) and sea water was added to the drinking water. A study of the solubility of fluorapatite in saliva (*18*) led to the idea that fluorine is beneficial to the teeth. When fluorapatite was used as the sole source of calcium and phosphorus in rats' diet no evidence of fluorine poisoning was noted (*12*).

In a search for micromethods for fluorine analysis the assistance of A. G. Mulder (*16*) and W. D. Armstrong (*2*) was obtained. Since then Armstrong (*1*), Willard and Winter (*19*), Hoskins and Ferris (*8*), Churchill, Bridges, and Rowley (*3*), Jacob, Reynolds, and Marshall (*9*), McClure (*15*), Clifford (*4*), Dahle, Bonnar, and Wichmann (*6*), Reynolds and Hill (*17*), and others have contributed to the microanalysis of fluorine.

Notwithstanding the precise work on the fluorine content of teeth, bones, and phosphate rocks, the meager reports on dietary fluorine leave much to be desired. Cox, Matuschak, Dixon, Dodds, and Walker (*5*) state that "in agreement with Armstrong, Dahle, and McClure the value of analysis for fluorine on materials which must be ashed is questionable". The authors interpret this to mean that losses occur on ashing or that combustion products interfere with analysis. It therefore seems justifiable to publish the authors' more exacting technique, which has enabled them to find a diet (adequate for three generations of rats) so low in fluorine as to cause dental caries in 100 per cent of all rats. This diet has been made up in a number of batches which vary somewhat in fluorine content, but all contain about $^1/_3$ part per million of fluorine.

Whereas thorium tetrafluoride may be formed when thorium is the only cation in addition to hydrogen, in the presence of sodium ion it is possible to have $ThF_4.NaF$ which disturbs the stoichiometric titration of fluoride with thorium, and some failures are apparently caused by adding sodium hydroxide to distillates.

A spectrophotometric study was made of the thorium-alizarin-fluorine interaction, determining the pH with the glass electrode. The absorption curves (logarithm of reciprocal of transmittance) of alkaline alizarin (red) and of acid alizarin (yellow) are given in Figure 1. Since the Coleman spectrophotometer had a slit 300 Å. wide, the peaks of the absorption curves may not have been sharply determined.

The alizarin-thorium lake (dashed line, Figure 1) shows nearly the same absorption spectrum as the alizarin, but because the lake precipitates at pH greater than 4 except in extreme dilutions and low ionic strength, and the cell of the authors' Coleman spectrophotometer is only 19 mm. deep, the presence of a trace of the acid form of the alizarin could not be excluded. The peaks of the two absorption curves appear to be at the same wave length (5100 Å.). The difference in color is due to less absorption by the lake in the longer

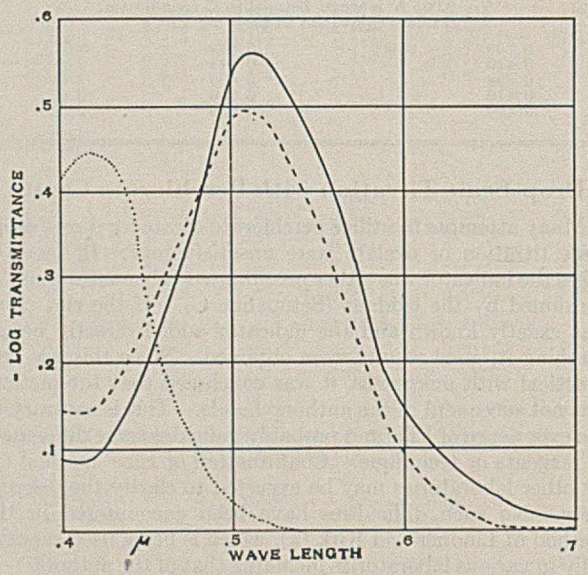



FIGURE 1. ABSORPTION CURVE OF ALKALINE AND ACID ALIZARIN AND ALIZARIN-THORIUM LAKE

1 cc. of sodium alizarin sulfonate in 25 cc. of 0.1 *N* hydrochloric acid (dotted line) and in 25 cc. of 0.01 *N* sodium hydroxide (solid line), and of thorium lake (dashed line)

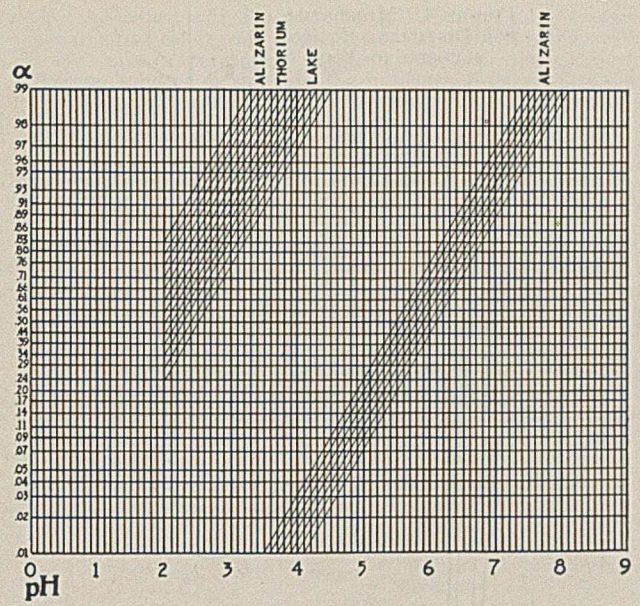



FIGURE 2. CHANGE IN DISSOCIATION OF ALIZARIN AND ALIZARIN-THORIUM LAKE WITH CHANGE IN pH

wave lengths. It therefore appears probable that the basis of the thorium procedure is the change in ionization constant of alizarin produced by thorium, and the following procedure is based on that hypothesis.

The results of pH-spectrometric measurements are plotted in Figure 2 on special graph paper (for sale at the Hahnemann Book Shop at 1 cent per sheet). Kolthoff (*10*) has shown that changes in ionic strength may change the logarithm of the reciprocal of the apparent ionization constant of alizarin as much as 0.06. The ionization, $\alpha$, of alizarin with change in pH is represented as a broad band in Figure 2. The breadth of the band may be due to changes in ionic strength and room temperature. The point is that under ordinary conditions, between pH 3 and 3.5, alizarin is never more than 1 per cent ionized (red), whereas the thorium lake is more than 50 per cent in the red form. The thorium-alizarin lake is not so simple as the alizarin, and is shown as a guide to titration. The band is not continued to pH values less than 2 because the pH of distillates is never less than 2. The determinations of $\alpha$ were made at 5400 Å. at which the undissociated dye shows practically no absorption, whereas the ionized dye and thorium lake show nearly maximum absorption (see Figure 1).

From the data in Figure 2 it follows that the thorium solution should be standardized at the exact pH, ionic strength, and temperature at which the fluoride is titrated. The pH should not be greater than 3.5, and since reduction in the pH causes reduction in the red color and consequently in the sensitivity of the method, it is not wise to have the pH less than 3.

The acidity of the distillate is kept as low as possible by an efficient trap and the addition of silver sulfate or perchlorate to the distilling flask. The distillate is brought to pH 3 by addition of minute amounts of perchloric acid or sodium hydroxide and matched with a blank at the same pH.

## Apparatus

Since open ashing leads to loss of chloride, and potassium chloride and fluoride have the same sublimation temperatures, there is danger of loss of fluoride on open ashing.

To avoid such losses, the closed combustion tube system shown in Figure 3 was adopted. This consists (from left to right) of a Pyrex absorber with three sintered Pyrex disks (from the Corning Glass Works, Corning, N. Y.), a platinum combustion tube, and an automatic stoker.

The furnace is made of two firebricks set with their broad faces parallel, 4 cm. apart, and covered with bright metal. They rest on a rectangular 3.785-liter (1-gallon) tin can laid on its narrow side with a slot 4 cm. wide cut lengthwise of the side turned uppermost. Convenient holes are cut in the front, back, and bottom of this can to operate three large Fisher burners. The can is blocked to prevent the tops of the burners from projecting more than about 1 mm. up between the bricks, when they will be overheated and strike back. The table top and rubber tubing are protected with high-temperature aluminum paint (from the Chemical Rubber Co., Cleveland, Ohio). The platinum combustion tube is 10 mm. in outside diameter at one end and 14 mm. in bore at the other. A 52-mesh platinum grid is pushed into the tube to prevent cinders from entering the absorber. The end of the absorber should be flared so that the small end of the combustion tube can be pushed into it about 1 cm.

A piece of 10-mm. stainless steel tubing about 30 cm. (1 foot) long [18–8 type 304 stainless steel tubing 0.788 cm. (0.315 inch) in inside diameter and 0.985 cm. (0.394 inch) in outside diameter, made by the Bishop & Co. Platinum Works, Malvern, Penna., which also makes the platinum parts] is used to introduce the sample into the wide end of the combustion tube. To make the connection, a platinum "stopper" is used, perforated with a 10-mm. hole for the stainless steel tube and a 3-mm. hole to admit oxygen. This stopper is cooled by a water jacket with 3-mm. inlet and outlet tubes.

A 0.625-cm. (0.25-inch) rod of stainless steel 35 cm. (14 inches) long is threaded with an adjustable 0.625-cm. (0.25-inch) die, 20 threads to the inch. The threads are cut in only halfway, leaving a "square" shoulder. A thimble-shaped cap of platinum 0.1-mm. thick is fitted loosely over one end of the rod.

Into one end of the stainless steel tube are driven 2.5 cm. (1 inch) of 0.625-cm. (0.25-inch) copper tubing 0.104-cm. (0.042-inch) wall (such as is used for the gas line in autos). The threaded rod is inserted through this end. A setscrew is inserted through the stainless steel and copper and into one of the threads in the rod. The other end of the rod is attached by means of a short piece of rubber tubing to the axle of a Telechron motor (B-2, 1 r. p. m., 60 cycles) that slides horizontally on a knitting needle.

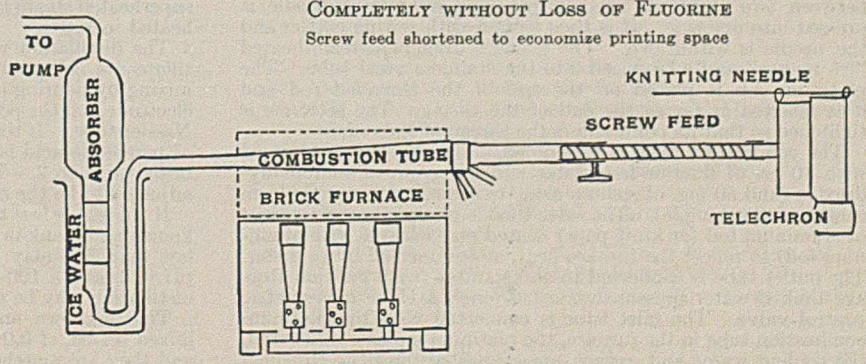
A Hoke micrometric control valve and Corning flowmeter are used to pass 200 cc. of oxygen per minute through the water-cooled platinum stopper.

The 50-cc. Pyrex microstill (made by the Precision Thermometer and Instrument Co., 1434 Brandywine St., Philadelphia, Penna.) is shown in Figure 4. The trap, which avoids the necessity of double distillation, is sufficiently close-fitting so that the water seal is not blown out during distillation. The filling tube has a Y at the top and the filling is done through the vertical arm. The lateral arm is used to pass in superheated steam at 150° C.

Steam is generated by boiling alkaline distilled water in a Pyrex flask with a Nichrome wire wrapped around its neck.

FIGURE 3. COMBUSTION TRAIN FOR BURNING FOOD SAMPLES COMPLETELY WITHOUT LOSS OF FLUORINE

Screw feed shortened to economize printing space



Two garden hose coupling clamps are attached to the two ends of the neck of the flask; 3.6 meters (12 feet) of 30-gage Nichrome wire and 3.6 meters (12 feet) of coarse sewing thread are attached to one clamp and wound together around the neck and the other ends are attached to the other clamp. A thick suspension of fireclay in water is poured over the winding and allowed to dry. Asbestos paper is wrapped around the fireclay and held with wire. The two clamps are connected with the two ends of a 110-volt electric circuit in series with a 200-watt lamp and switch. The flask is fitted with a 2-hole rubber stopper; in one hole is inserted a thermometer with its bulb in the neck of the flask and in the other a short piece of glass tubing to connect with the distilling flask. The temperature of 150° in the issuing steam is controlled by turning the switch on and off.

The vertical form of the distilling flask permits its being immersed in a Pyrex beaker filled with white mineral oil such as is used for constipation, and avoids the necessity of a thermometer inside the flask. The beaker also contains a thermometer and is heated by gas flame to 150° C.

The titrations are made in tall 100-cc. Nessler tubes with Lochte and Hoover (*11*) microburets (0.2 cc. graduated in 1000ths, from Williams, Brown and Earle, Philadelphia, Penna.). Titrations and pH determinations are made in a double-walled cellophane chamber about 210 cm. (7 feet) cube kept at a constant temperature of 25° C. by an electric thermostat, heater, and fan. The cellophane chamber is inside a steam-heated room. A small electric refrigerator unit is used to cool the chamber when the room is a few degrees too warm.

## Reagents

Fluorine-Free Water. Distilled water is made alkaline with sodium hydroxide and redistilled in a Pyrex still.

Fluorine-Free Perchloric Acid. Ten cubic centimeters of Baker's analyzed 70 per cent perchloric are distilled with steam at 150° C. in the microstill and 100 cc. of distillate are tested for fluorine. If as much as 1 microgram of fluorine distills over, the residual acid is used as reagent; otherwise this purification is omitted.

Thorium Nitrate Tetrahydrate, 7.26 grams dissolved in fluorine-free water to make 1 liter. This is intended to correspond to 1000 micrograms of fluorine per cc. Some of this is diluted 10 times and some 100 times with fluorine-free water.

Sodium Fluoride (Baker's analyzed) is dried at 150° C. and cooled in a desiccator and 2.2104 grams are weighed and dissolved in fluorine-free water to make 1 liter. Some of this is diluted 10 times and some 100 times with fluorine-free water. These solutions are kept in paraffin-lined bottles, made by filling cylindrical glass-stoppered bottles one-fifth full of melted 60° paraffin and rolling them on the table until cooled. These solutions are labeled 1000, 100, and 10 micrograms of fluorine per cc. (A possible error of a very few per cent in the purity of the standard is within the limit of error in the determination of dietary fluorine.)

Powdered Quartz is heated with sulfuric acid and washed with fluorine-free water.

Titrations are made in tall 100-cc. Nessler tubes against a white table top with a 20-watt fluorescent lamp 15 cm. (6 inches) above it, shielded from the eyes of the operator. The table top must be of uniform whiteness, the bottoms of the Nessler tubes clean, and the eye trained by practice.

## Procedure

Sufficient dry foodstuff to contain 1 microgram or more of fluorine is ground, weighed, and introduced into lengths of 0.938-cm. (0.375-inch) cello-dialyzer tubing (from the Fisher Scientific Co., Pittsburgh, Penna.). The casing is tied with thread at each end, the excess is cut off, it is somewhat flattened between two sheets of clean paper, and a knitting needle is pressed into one side. It is then wound with sewing cotton and the needle is withdrawn. The casing is dried in a steam-heated "ice maker" and introduced into the stainless steel tube. The platinum cap is placed on the end of the threaded rod and it is inserted as far as the end of the casing. The setscrew is tightened so that its point enters the thread of the rod.

The combustion train is assembled. The absorber is charged with 10 cc. of fluorine-free water and 0.2 gram of sodium hydroxide (and 50 mg. of sodium azide to destroy nitrous acid from ashing of the sample). The inlet tube is passed through a piece of aluminum foil (or kraft paper coated on each side with aluminum foil) to reflect the furnace heat, and immersed in ice water. The outlet tube is connected to an aspirator (with vacuum storage tank if water pressure varies) through a Hoke micrometric control valve. The inlet tube is connected with the platinum combustion tube in the furnace, the platinum stopper is inserted, the cooling water and oxygen are connected, and the threaded rod is connected by means of a short piece of rubber tubing with the Telechron motor sliding on a knitting needle. The burners are lighted and a piece of asbestos board is used to cover the middle portion of the furnace top. About 200 cc. of oxygen per minute are admitted and suction is applied to maintain atmospheric pressure inside the combustion tube, counting the bubbles in the absorber or attaching a delicate manometer by means of a T-tube to the oxygen inlet. There should be no noticeable back pressure in the passage of the oxygen through the 3-mm. opening. When the combustion tube reaches bright luminosity the Telechron motor is started. In case the sample fills more than one length of casing, these lengths may be fed into the stoker one at a time by loosening the setscrew and removing the threaded rod.

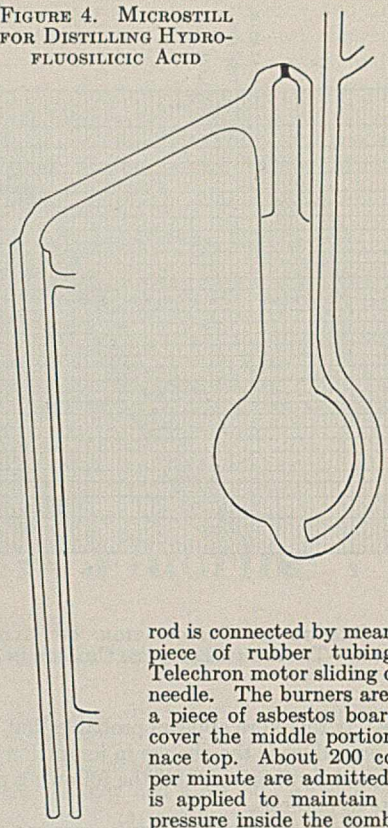


FIGURE 4. MICROSTILL FOR DISTILLING HYDROFLUOSILICIC ACID

When the casing is all fed into the combustion tube the platinum cap is allowed to drop off the rod, and the rod is withdrawn after loosening the setscrew. The heating is continued 5 minutes to burn the tar off the cap. The cooling water is reduced until it boils and the heating continued 5 minutes more. The rubber tubes are disconnected from the platinum stopper and it is allowed to heat for 5 minutes while air is drawn through to burn the tar from its inner face. The gas and suction are turned off and the contents of the absorber and the ash and washings of the platinum tube with 3 cc. of 0.1 *N* perchloric acid are transferred to the microstill with 0.1 gram of powdered quartz and enough silver sulfate or perchlorate to precipitate all the chloride.

Heat is applied to the oil bath to heat it to 150° C., while compressed air is led slowly through the filling tube to distill off most of the water. If the sample is reduced to 2 cc. before the oil bath reaches 150°, the still is raised and reinserted when the temperature is correct.

The condensing water must be running and a 100-cc. Nessler tube used as a receiver when 10 cc. of perchloric acid are introduced through the filling tube, and the orifice closed after the superheated steam is introduced from the superheater (previously heated to 150°).

The distillation with steam is continued until 100 cc. of distillate are collected. A blank distillation is also made. After mixing by pouring in a beaker the pH is determined with a glass electrode and the portion used to determine pH is returned to the Nessler tube. If the pH is greater than 3, the calculated amount of perchloric acid is added to the unknown and to the blank to bring them to 3. The pH of these solutions is then verified or adjusted to be the same with the glass electrode.

If the pH is less than 3, sodium hydroxide is added to the unknown and blank in a similar manner. (If the pH is only slightly less than 3 it may be diluted with fluorine-free water until the pH is 3, and a 100-cc. aliquot taken for analysis.) The rate of distillation may be regulated so that the pH is about 3.

The unknown and blank should be colorless; with each is mixed 0.1 cc. of 0.04 per cent aqueous sodium alizarin sulfonate and they are matched for color. The microburets are filled and

their tips wiped with San Fay tissue. Thorium solution corresponding to 1 microgram of fluorine is run into the blank, which should have a perceptibly pinker color than the unknown. Thorium solution is run into the unknown from a microburet until it matches the blank. The contents are throughly mixed by pouring in and out of a beaker; about 20 seconds are required to develop full color. The titer is recorded and an equal total amount of thorium added to the blank (remembering that some was added at the start), which should be perceptibly pinker. If no difference appears the fluorine in the unknown must be recorded as zero, but this has not occurred in the authors' work except in distillates from purified reagents.

If the blank is pinker than the unknown, fluoride solution is added until the colors match again, when the fluorine content of the unknown is equal to that added to the blank. A number of unknowns may be titrated, provided they have the same pH as the blank. After thorium equivalent to 1 microgram of fluorine is added to the blank, each unknown is titrated with thorium to match it in color. Beginning with the unknown to which the smallest amount of thorium was added, the blank is back-titrated to each unknown in turn.

Table I. Recovery of Fluorine Added to Rats' Bones and Teeth

| | F | Recovery |
|---|---|---|
| | *Micrograms* | % |
| Femur, 117.7 mg. | 3.0 | ... |
| Femur, 117.7 mg. + 10γ F | 13.4 | 104 |
| Teeth, 162.6 mg. | 3.5 | ... |
| Teeth, 162.6 mg. + 10γ F | 14.0 | 105 |

## Results

A comparison of the authors' microstill with those devoid of traps shows that negligible amounts of perchloric acid pass their trap on distilling with steam at 150° C. With slow distillation the pH of the distillate was 4.6 (after standing), whereas that of water redistilled in Pyrex from sodium hydroxide without trap was 5.5, but after standing (and absorbing carbon dioxide from the air) was 5.05. This indicates that the normality of the acid in the distillate was about 0.00014. In addition to the 0.00014 *N* perchloric, the acids that might come over in analyses are halogen and nitrogen acids and sulfuric acid. The former (except the fluorine) are held back by silver. The substitution of silver sulfate for silver perchlorate did not noticeably increase the acidity of the distillate, but increasing the rate of distillation does increase the acidity.

The recovery of fluoride added to rats' bones and teeth is shown in Table I.

Table II. Fluorine in Teeth and Bones of Rats on Diet Analyzed in Table III

| Rats | Fluorine Found Enamel | Fluorine Found Dentine | Rats | Bone | Fluorine Found |
|---|---|---|---|---|---|
| | % | % | | | % |
| 1 and 2 | 0.004 | 0.010 | 4 | Left femur | 0.007 |
| 3 and 4 | 0.010 | 0.010 | 4 | Right femur | 0.008 |
| 5 and 6 | 0.004 | 0.007 | 5 | Right femur | 0.010 |
| | | | 6 | Right femur | 0.010 |
| | | | 7 | Right femur | 0.010 |
| | | | 8 | Right femur | 0.004 |

Table III. Fluorine in Rats' Diet and Recovery of Added Fluorine

(Diet, 5 grams)

| Fluorine Added | Fluorine Found | Recovery |
|---|---|---|
| *Micrograms* | | % |
| 0 | 1.5 | ... |
| 10 | 12 | 104 |
| 0 | 1.5 | ... |
| 10 | 11.3 | 98 |
| 0 | 1.9 | ... |
| 5 | 7 | 101 |
| 0 | 1.8 | ... |
| 2.5 | 4.5 | 105 |
| 0 | 1.8 | ... |
| 1 | 3 | 107 |

The only analyses of fluorine in rats' dentine and tooth enamel found in the literature were made by Armstrong (7); the fluorine in the diets was unknown, although one diet was known to contain more fluorine and produced "mottled" teeth. Armstrong found (average) 0.0082 per cent of fluorine in "normal" enamel and 0.0109 per cent in the dentine. He found (average) 0.196 per cent in "mottled" enamel, and 0.257 per cent in the dentine.

The authors have analyzed the fluorine in the bones and teeth of rats receiving from weaning about 0.33 microgram of fluorine per gram of diet as shown in Tables II and III.

## Summary

In determining fluorine in the presence of large amounts of organic matter, a closed system of ashing prevents the loss of fluorine by volatilization.

An improved still allows separation of fluosilicic acid in a single distillation. The perchloric acid carried over may be 0.00015 *N* and does not interfere with the titration.

The interference of sodium ion may be due to the formation of $ThF_4.NaF$, precipitation of the lake, and effect of change in ionic strength on alizarin.

At pH greater than 3.5 there may be error due to free alizarin in red form. At pH less than 3.5 the thorium lake does not show its full color; therefore the thorium must be standardized with fluoride at exactly the same pH at which the titration is made. The back-titration on a blank distillate after adjusting pH with the glass electrode allows this to be accomplished, as well as correcting for fluorine in reagents.

From 2.5 to 10 micrograms of fluorine added to the sample may be recovered with an error of 5 per cent. The greater percentage inaccuracy with smaller quantities may be due to fluorine in the reagents and to the uncertainty of the end point. It appears that the color of the thorium lake is not entirely suppressed by adding the equivalent of fluoride. The method is considered valuable in determining 1 microgram of fluorine in foodstuffs (error, 7 per cent).

## Literature Cited

(1) Armstrong, W. D., *J. Am. Chem. Soc.*, 55, 1741 (1933); Ind. Eng. Chem., Anal. Ed., 5, 315 (1933); 8, 384 (1936).
(2) Armstrong, W. D., "Studies in Fluorine Analysis", Ph.D. thesis, University of Minnesota, 1932.
(3) Churchill, H. V., Bridges, R. W., and Rowley, R. J., Ind. Eng. Chem., Anal. Ed., 9, 222 (1937).
(4) Clifford, P. A., *J. Assoc. Official Agr. Chem.*, 23, 303 (1940).
(5) Cox, G. J., Matuschak, M. C., Dixon, S. F., Dodds, M. L., and Walker, W. E., *J. Dental Research*, 18, 486 (1939).
(6) Dahle, D., Bonnar, R. U., and Wichmann, H. J., *J. Assoc. Official Agr. Chem.*, 21, 459 (1938).
(7) Hodge, H. C., Luce-Clausen, E. M., and Brown, E. F., *J. Nutrition*, 17, 333 (1939).
(8) Hoskins, W. M., and Ferris, C. A., Ind. Eng. Chem., Anal. Ed., 8, 6 (1936).
(9) Jacob, K. D., Reynolds, D. S., and Marshall, H. L., *Am. Inst. Mining Met. Engr. Tech. Pub.* 695 (1936); gives reference to other papers.
(10) Kolthoff, I. M., *J. Phys. Chem.*, 32, 1820 (1938).
(11) Lochte, H. L., and Hoover, A., Ind. Eng. Chem., Anal. Ed., 5, 335 (1933).
(12) McClendon, J. F., *Rock Products*, 26, 27 (1923).
(13) McClendon, J. F., *Science*, 55, 358 (1922).
(14) McClendon, J. F., Carnegie Institution of Washington, *Year Book* 15, 208 (1916).
(15) McClure, F. J., Ind. Eng. Chem., Anal. Ed., 11, 171 (1939).
(16) Mulder, A. G., "Physiological Chemistry of Fluorine", Ph.D. thesis, University of Minnesota, 1926.
(17) Reynolds, D. S., and Hill, W. L., Ind. Eng. Chem., Anal. Ed., 11, 21 (1939).
(18) Robb, E. F., Medes, G., McClendon, J. F., Graham, M., and Murphy, I. J., *J. Dental Research*, 3, 50 (1921).
(19) Willard, H. H., and Winter, D. B., Ind. Eng. Chem., Anal. Ed., 5, 7 (1933).

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# MODERN LABORATORIES

## Laboratories of the Best Foods, Inc.

H. W. VAHLTEICH, Best Foods, Inc., Bayonne, N. J.

THE new research and development laboratory of the Best Foods, Inc., is a $2^1/_2$-story rectangular brick building about 50 by 80 feet. It was designed by Lockwood Greene Engineers, Inc., in cooperation with the laboratory staff, which is also responsible for the special features and equipment layouts. Its purpose was to consolidate previously scattered laboratory operations, to supplant outworn and outmoded facilities with up-to-date equipment, and to provide room for expansion. Extensions on one or more of three sides may be made with a minimum of inconvenience.

In a number of practical aspects the design is unique for a rectangular building—for example, the main entrance is at one corner. This corner is built in the form of a semicircular tower, a feature which permits the following practical applications: Four stories are provided in the tower; a low basement level for a darkroom, a ground-level entrance foyer, and offices on each of the two upper floors; a pleasant circular effect in the offices and foyer as a relief from the usual straight-sided rooms; shorter halls inside the building with resulting economy of working space; and a complementary inside circular rotunda, facing which are a library, a model kitchen, and an experimental bakery. The object is to facilitate tours of consumer visitors who can, without being wearied by complicated laboratory equipment, gain a general impression of the organization's care in providing for technical control of its products. Large curved plate-glass windows are set in the glass-block walls of the rotunda lobby to permit an unobstructed view of technicians at work.

Another unique design feature is the safety balcony, extending along the north and west sides of the building, which provides means of egress from all rooms.

Headroom on the top floor is 12 feet, on the first floor 13 feet, and in the basement 14 feet; however, a 20 × 20 foot bay in one corner of the basement is 4 feet below floor level, providing 18 feet of headroom for tall pilot-plant equipment, and in one corner of this bay steel reinforcing in the concrete is omitted from a 5 × 5 foot ceiling area to permit easy removal should an apparatus column up to 31 feet be desired. Tile walls are used throughout all laboratories to avoid expensive repainting, and plywood forms for all laboratory ceilings gave a ceiling sufficiently smooth to paint on directly without the use of plaster. Hauserman steel partitions carrying glass above 3 feet are used to section off working space and simultaneously permit easy inspection of working spaces from a distance. Handrails and doors are of similar metal construction.

Floors are concrete with a 0.75-inch dressing of steel-gray cement, with the exception of terrazzo in the first-floor passageways, kitchen, bakery, and entrance lobby.

Lighting, which has received special attention, in all rooms is of the high-intensity indirect type, a circular hanging fixture with plastic shields and reflecting globes having been used. Glass brick has been used freely for both interior and exterior walls, and this, together with large windows, affords well-lighted rooms and laboratories. Throughout, steel sash and casement-type windows have been used, while wire glass instead of plate glass in many instances provides an additional safety measure. Offices, library, and corridors have acoustical ceilings. Under the roof is a 2-inch layer of cork, which, together with the high headroom, minimizes discomfort due to heat in summer.

To prevent water from entering electrical outlets when rooms are flushed in cleaning, all sockets have been placed 2 feet above the floor. The electrical system is 4-wire polarized, making available at each outlet 120- and 208-volt, 1- and 3-phase current. Every room has a master panel which controls individual outlets on benches and work tables. Laboratory benches are also provided with 110-volt direct current.

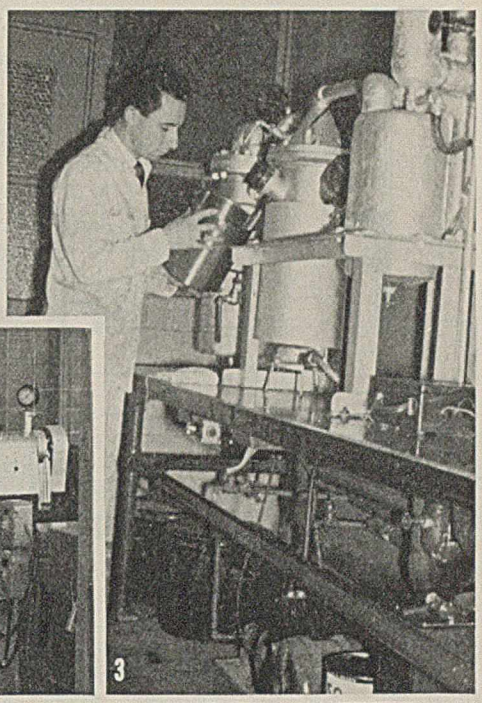
Ceilings purposely have been made high to aid ventilation, and to remove piping from the line of vision. Pipes in the walls are readily accessible, being housed behind removable metal partitions.

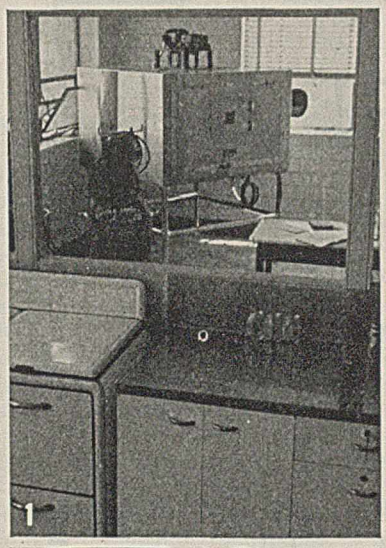
Instead of a sprinkler system, the A. D. T. system of fire detection has been installed. Primarily, this method of detection relies upon the pressure increase of air, within a fine copper tube, as the temperature within a room increases. Sudden increases will cause an alarm to sound, but slower changes due to normal conditions have no effect, as provision is made for the escape of gradually heated air.

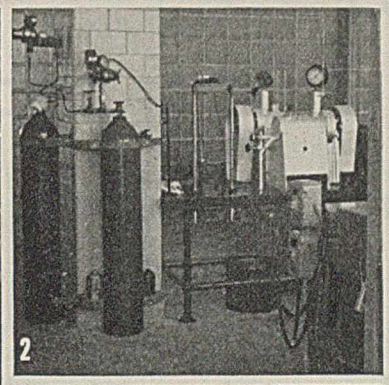
The laboratory furniture and equipment, like the building, were designed to meet a variety of special requirements. The furniture is steel of the double-wall type, lead-coated and finished in laboratory green. Alberene stone tops are used throughout. Drawers are on roller bearings and the ends of all benches include recessed sinks and waste cans. Interiors of all furniture have been lacquered in white to facilitate finding things, a feature found practical from previous trials.

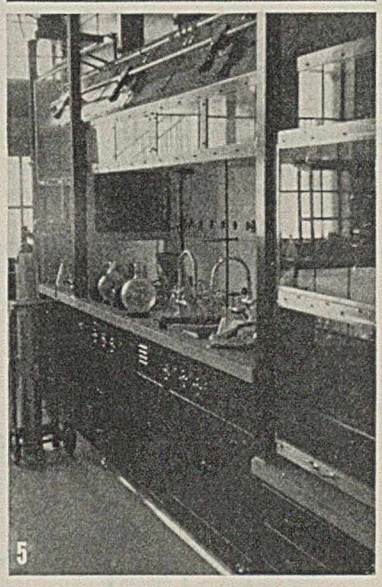
In providing for about 70 lineal feet of hood space it was also necessary to provide for properly distributing and heating the incoming air when the hoods are in operation. This has been done by means of Trane Torridor units, synchronized to operate automatically when the hood fans are turned on, and thermostatically controlled to provide uniform room temperature in cool weather. Both features are achieved by means of a Johnson system of control. The hoods have the usual dual control system, providing equal ease of operation from within and from the front of the hood when the glass doors are down. The large hoods are in three sections separated

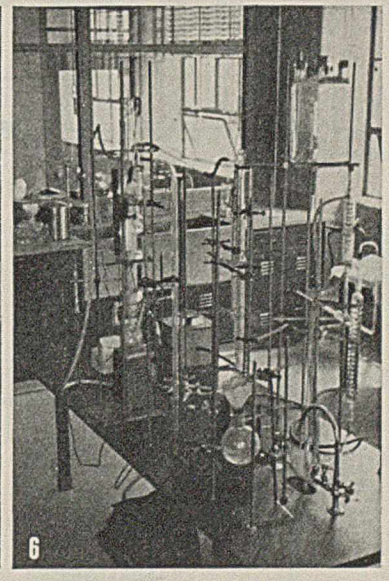
1. View in Bakery Control Laboratory
2. Equipment Setup for Hydrogenation of Vegetable Oils
3. Vacuum Deodorizer Setup for Vegetable Oils
4. Part of the Laboratory Kitchen
5. Organic Research Laboratory, Showing Hood
6. Low Bench in Organic Research Laboratory

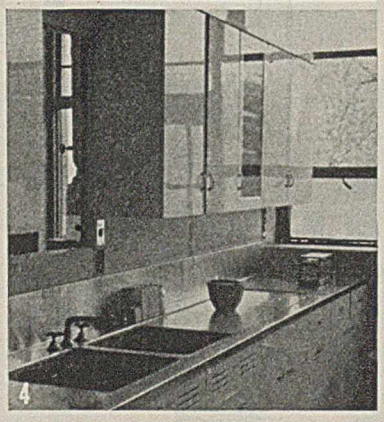












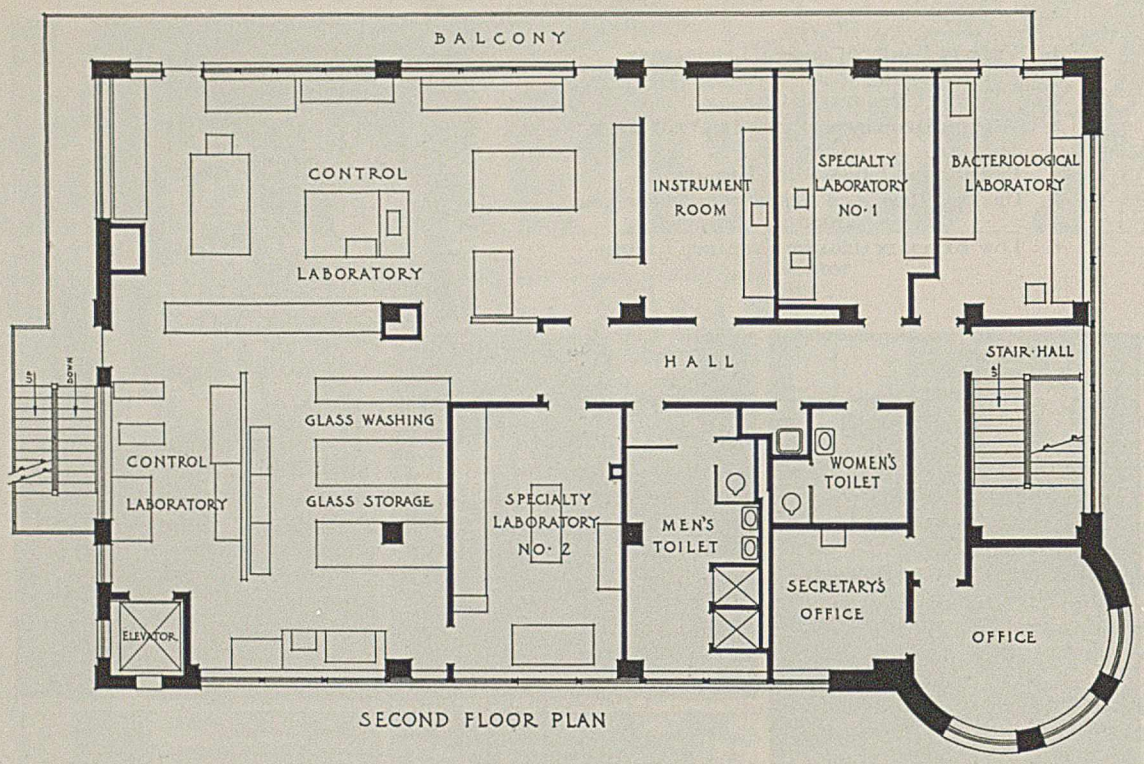
by transoms, and may be operated as one hood or in three sections as desired. One end section of each hood is provided with two flush hot plates, one steam and the other electrically heated. A large hood in the organic research laboratory is provided with one low section to facilitate the handling of tall apparatus. All hoods are provided with steam-heated cabinets and Transite-lined cabinets for corrosive liquid containers. All hood ducts lead to the roof, are of Transite lined with pitch, and the Transite sections are removable.

All laboratories are provided with at least one safety shower, as well as with modern equipment for fighting small fires. One laboratory with hood space has been set aside for analytical work requiring inflammable solvents. Here there are no electrical switches and no gas whatever is provided; all heating must be done with steam. All electrical equipment in this room is of the explosion-proof type (lights, unit heater and hood motors, mercoid control, etc.). The usual doorknob is replaced by a special device as an additional reminder that smoking is not permissible.

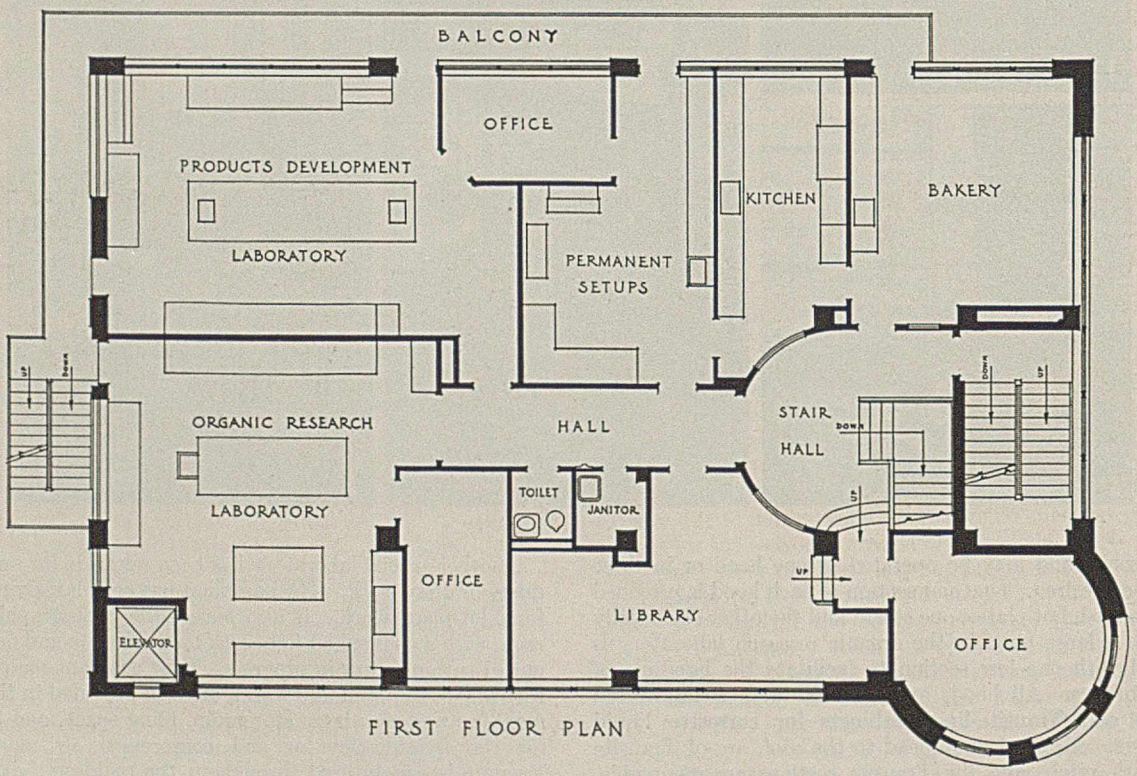
Another special laboratory has been set aside for unusually dusty or dirty work. The basement has been largely reserved for pilot-plant work. It also houses the photographic darkroom with a convenient light lock for passing in and out without disturbing work in progress. Four constant-temperature rooms, two cold and two warm, are also provided in the basement, as well as a large storeroom, filing space, and housing for the freight elevator and compressed air machinery. Vacuum is widely used throughout the building, but is supplied by individual electrically driven pumps.

There are also an experimental bakery, a model home kitchen, a permanent "setups" room for small-scale hydrogenation equipment, a small-scale edible oil deodorization system and oil-refining equipment, a bacteriological laboratory, etc.

The apparatus-washing facilities are largely concentrated adjacent to the analytical laboratory on the second floor, where they are most needed, but special washing facilities are also provided in the organic research laboratory. Dis-




SECOND FLOOR PLAN




FIRST FLOOR PLAN

tilled water is produced adjacent to the analytical laboratory and piped to the first floor. One end of the analytical laboratory is walled off to provide a room for housing instruments. Here the chemist may work in relative quiet and is also provided with a means for darkening the room when using such instruments as the polariscope.

Services to laboratory benches (including fume hoods) include hot and cold water, live steam, gas, electricity, and air. Stainless-steel steam baths are provided in several locations on the benches and hoods. A Schwartz sectional system in the analytical laboratory houses hundreds of samples of all sizes and shapes out of sight and yet readily available. The organic research laboratory is provided with a special low bench of modern design. The library, one side of which forms the curved glass-block wall of the rotunda, has bookcases built by a laboratory furniture company like conventional laboratory glassware cabinets, but grained in a walnut finish and provided with plate-glass doors having narrow stainless-steel trim. A special telephone system is provided to ease the service load on the switchboard.