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## ANALYTICAL EDITION

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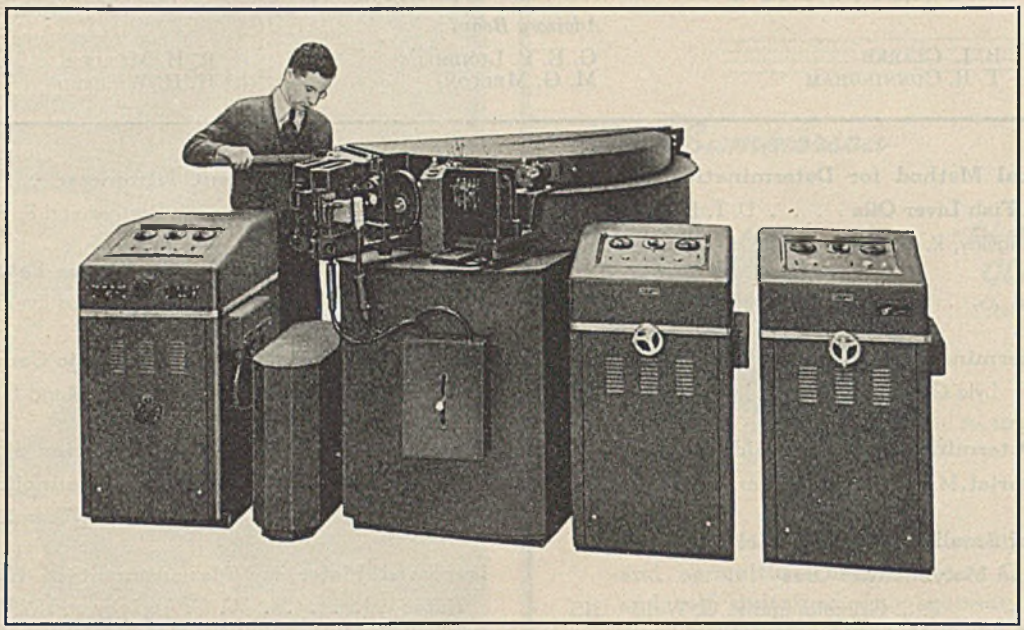

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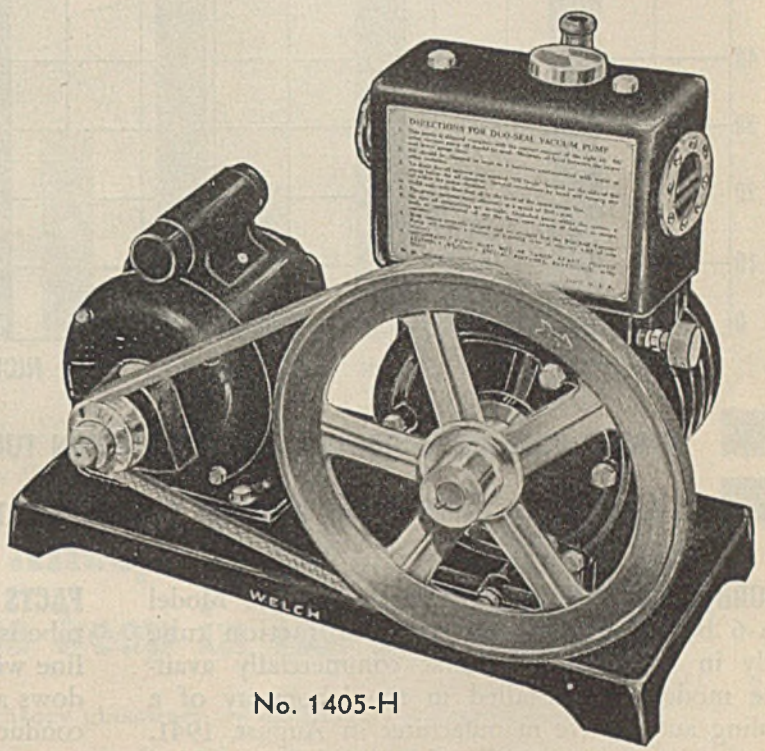


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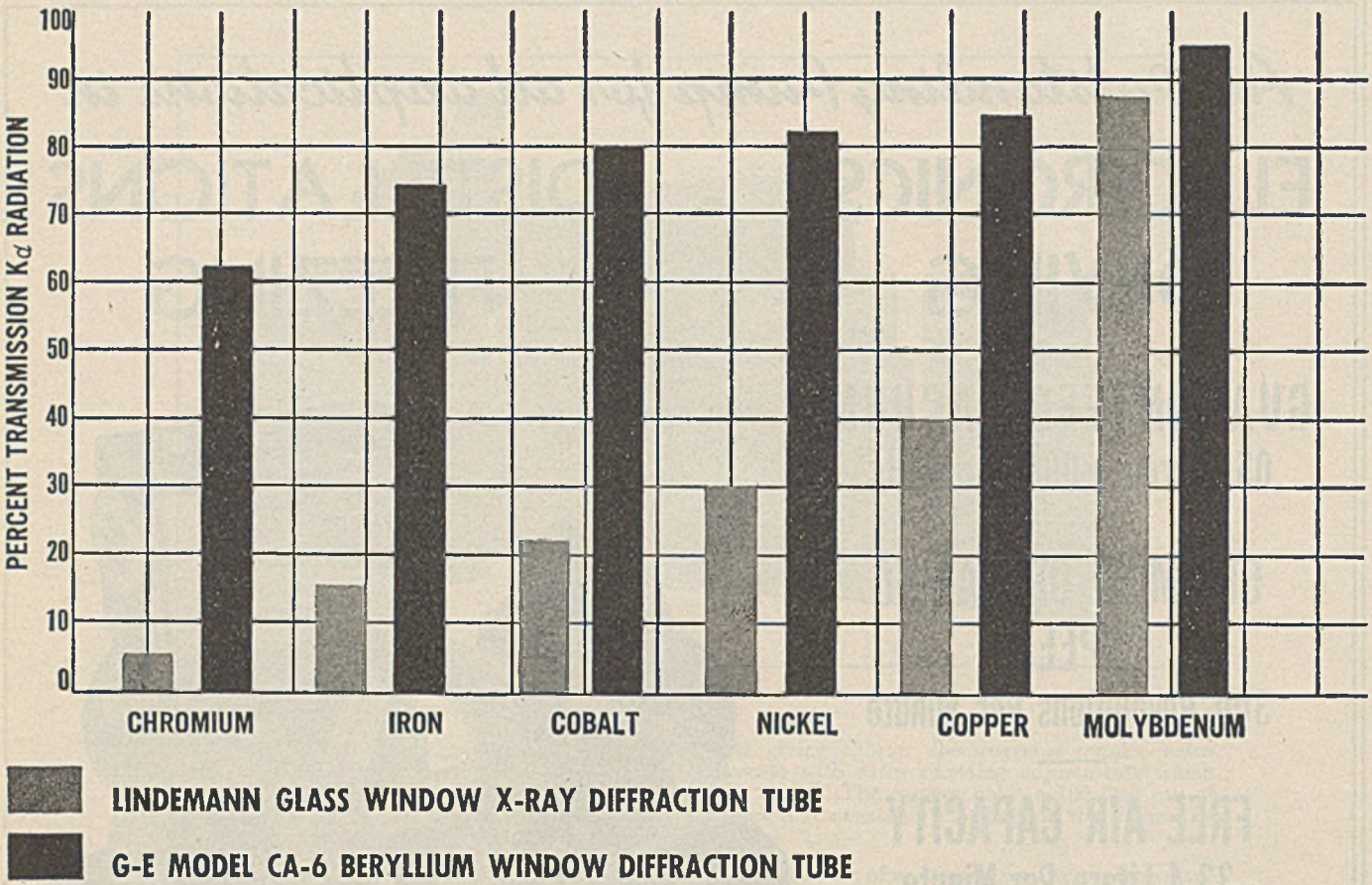


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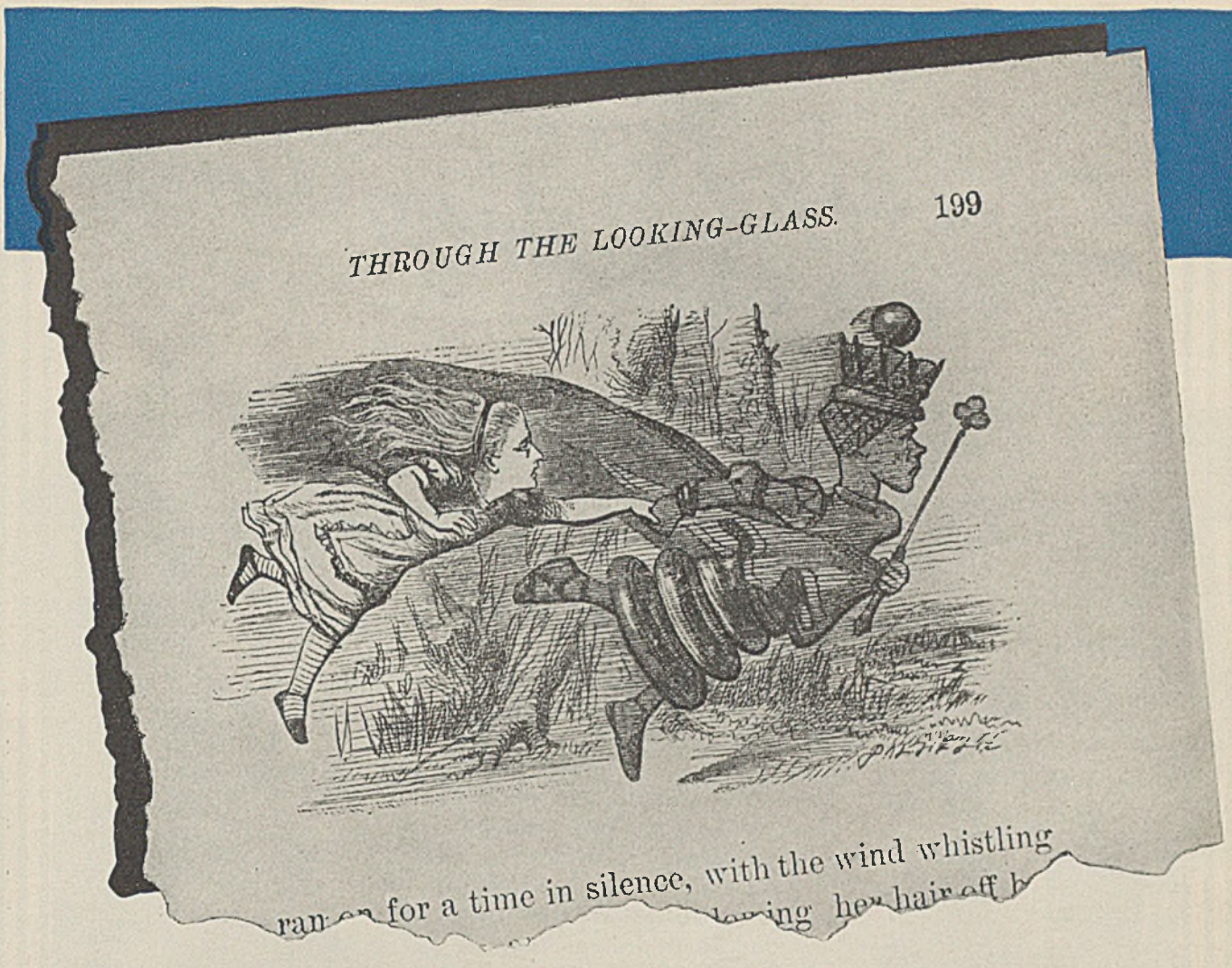


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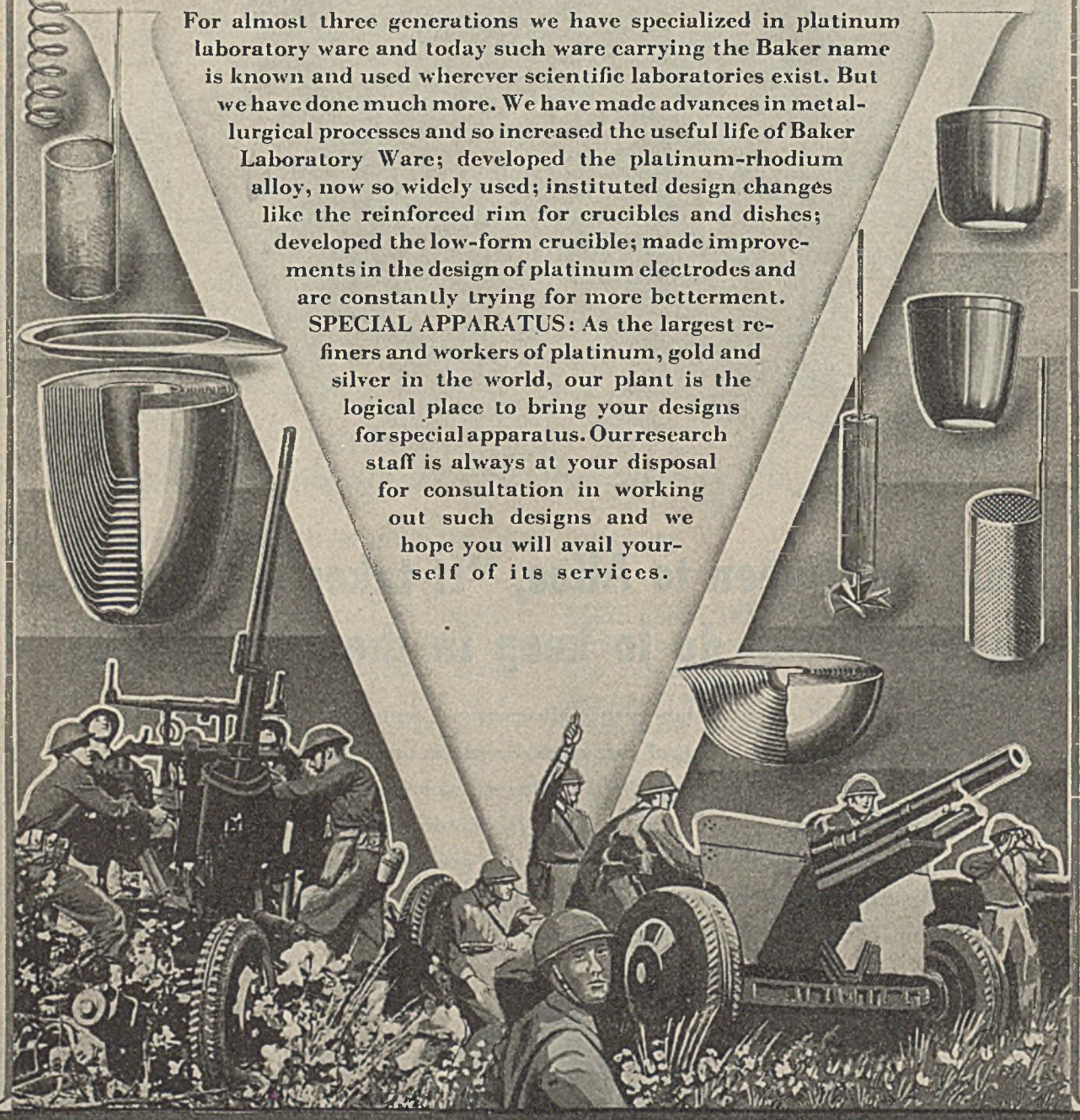

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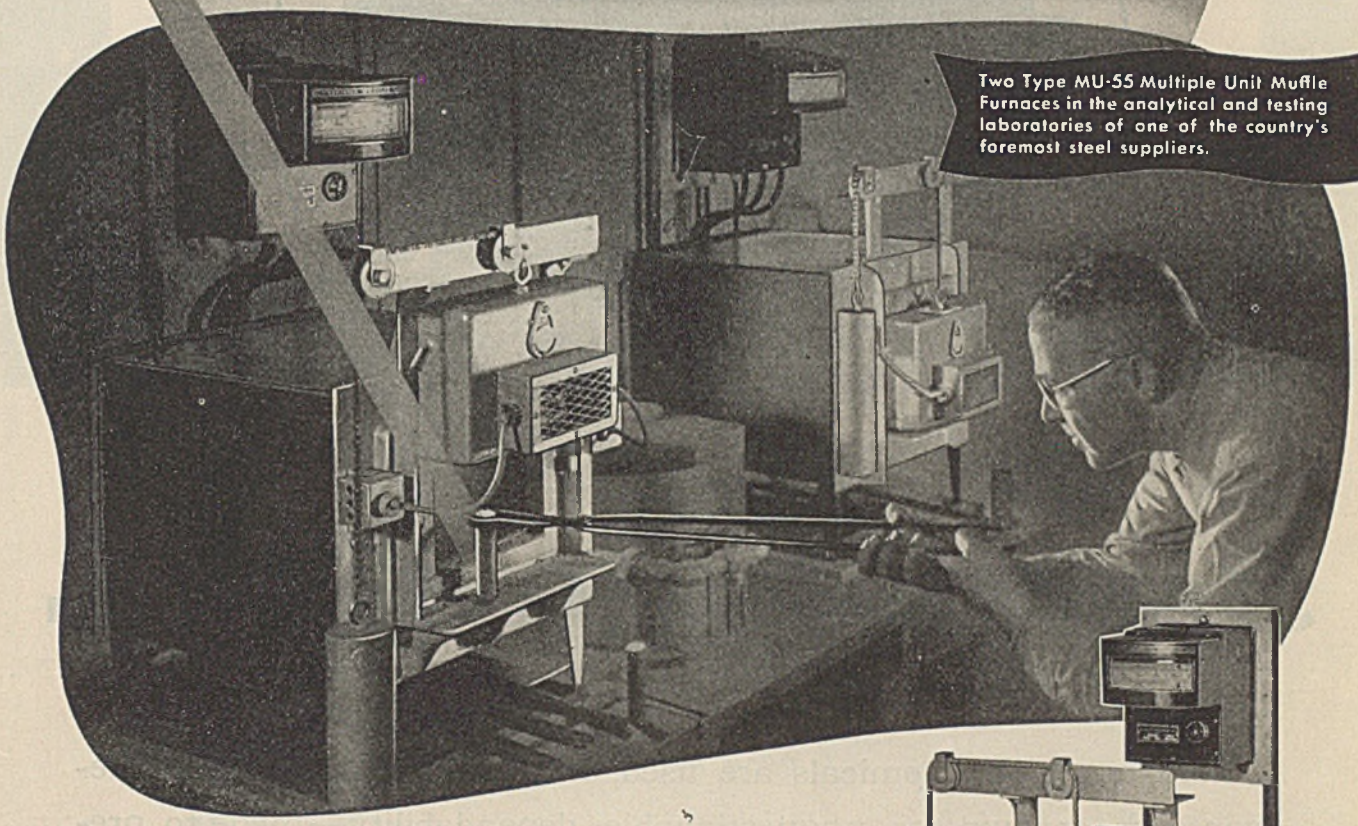

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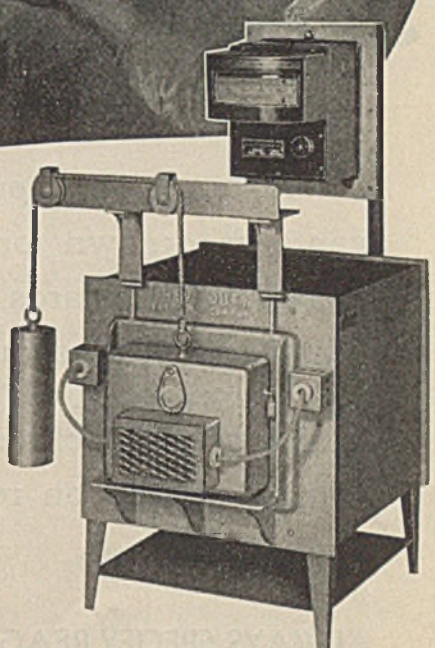

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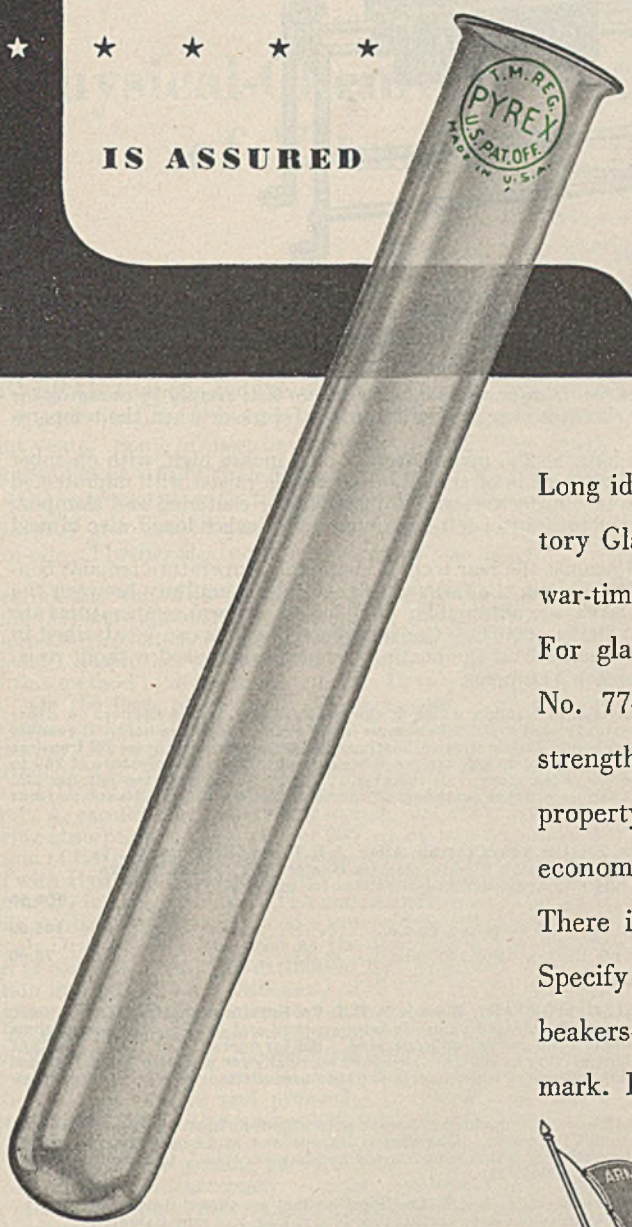


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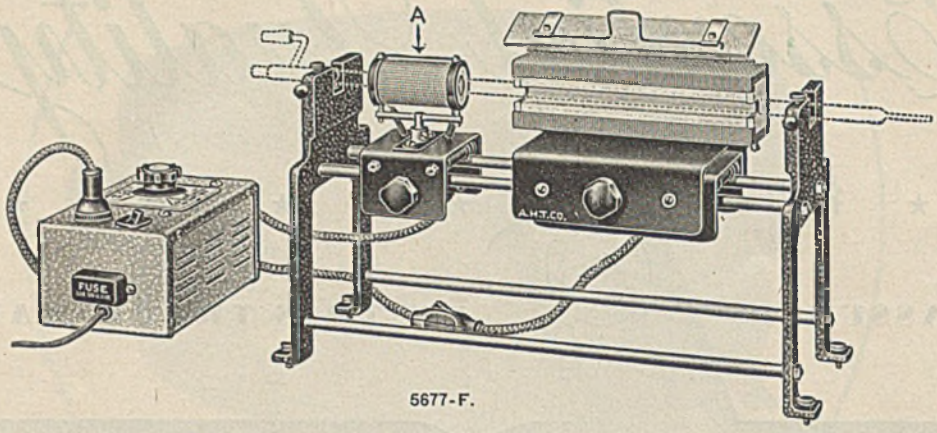


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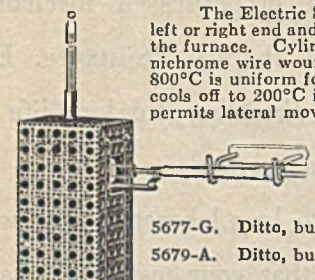

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INDUSTRIAL AND ENGINEERING CHEMISTRY

ANALYTICAL EDITION

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# Physical-Chemical Method for Determination of Vitamins D in Fish Liver Oils

D. T. EWING AND G. V. KINGSLEY
Michigan State College, East Lansing, Mich.
R. A. BROWN AND A. D. EMMETT
Parke, Davis and Company, Detroit, Mich.

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SEVERAL methods for the physical-chemical determination of vitamins D have been studied and reported on in recent years. Some of these have given good results on nearly pure samples of vitamins $D_2$ and $D_3$. In the case of fish liver oils ($D_3$), agreement with the bioassay values has been obtained only over limited concentration ranges for a small number of oils. Further study therefore seemed warranted.

Reerink and van Wijk (*16*) and later Töpelmann and Schuhknecht (*22*) were successful in determining the concentration of vitamin $D_2$ (irradiated ergosterol) by measuring the height of the ultraviolet absorption maximum at 265 mμ. Ol Khin (*14*) also used this method in determining vitamin $D_2$ in irradiation products. On the basis of the work of Brockmann (*1*, *2*) that the molecular extinctions of the two vitamin forms were the same, Marcussen (*11*) applied the measurement at this maximum to the determination of both vitamins $D_2$ and $D_3$. In the case of fish liver oil $D_3$, Marcussen first saponified the oil, then removed vitamin A, carotenoids, inactivated sterols, and other substances showing absorption in the region of 265 mμ by passing a heptane solution of the nonsaponifiable fraction through a Tswett column filled with Hydraffin $K_4$ as adsorbent. Thereby, he separated the vitamin $D_3$ in the percolate. The concentration of vitamins D in this solution was then obtained spectrographically. Calciferol (British Drug House) was used as the standard for converting the *E* (1 per cent, 1 cm.) (the extinction, $\log_{10} I_0/I$, for a 1 per cent solution in a cell of 1-cm. thickness) of the fish liver oil to units per gram of oil. Marcussen gave values for two tuna liver oils, one halibut liver oil, and one oil solution of an irradiated product.

Halden and Tzoni (*8*, *9*, *24*) obtained quantitative results with pure calciferol ($D_2$) based on a color reaction formed by heating a solution of the vitamin and pyrogallol in benzene, petroleum ether, or chloroform with a fresh solution of anhydrous aluminum chloride in absolute alcohol. The deep violet color formed was dissolved in absolute alcohol, giving a lilac-red solution suitable for colorimetric measurements. In the determinations of fish liver oils, it was found necessary to remove vitamin A. Cholesterol, ergosterol, and lumisterol did not interfere, but some of the irradiation products of ergosterol did give this color reaction.

Shear (*20*) suggested a colorimetric method based on the red color given by vitamins D in liver oils with a mixture of aniline and hydrochloric acid. Levine (*10*) reported that this color reaction was not specific for vitamins D.

Stoeltzner (*21*) proposed a colorimetric method based on the formation of color when phosphorus pentachloride was added to an oil solution of vitamins D. However, this reaction was found by Christensen (*4*) to be typical of other compounds present in natural oils.

Rutkovskiĭ (*19*) showed that the green color of the Tortelli-Jaffee reaction (*23*), formed when an acetic acid solution of vitamins D was mixed with a 2 per cent solution of bromine in chloroform, was also characteristic of the provitamins.

Robinson (*18*) reported a colorimetric method based on the yellow color formed when an alcohol solution of the vitamins D was boiled with sodium nitrite and acetic acid, then made slightly alkaline. This reaction was not given by cholesterol, ergosterol, dehydroergosterol, or lumisterol, but vitamin A gave a strong orange color and the nonsaponifiable fractions of olive oil and arachis oil also gave some color.

The antimony trichloride color reaction employed by Brockmann and Chen (*3*) has been studied by a number of investigators as the basis for a quantitative method for vitamins D. They used a cold saturated solution of antimony trichloride in dry, alcohol-free chloroform. Both vitamins $D_2$ and $D_3$ gave an orange-yellow colored solution with an absorption maximum at 500 mμ. Tachysterol gave a similar reaction, as did cholesterol, sitosterol, ergosterol, 7-dehydrocholesterol, lumisterol, suprasterol I, and suprasterol II. These all gave much weaker color, however, and did not interfere unless present in concentrations over thirty times that of the vitamins D. Vitamin A, with an absorption maximum at 620 mμ, interfered only when present up to six times the concentration of the vitamins D. This reagent thus seemed to serve as a dependable means for the colorimetric determination of vitamins D in the pure state, even when vitamin A and sterols were present in low concentrations.

On the other hand, Emmerie and van Eekelen (*5*) found that all vitamin A must be removed from fish liver oils before the antimony trichloride color reaction is used.

Wolff (*25*) used the Brockmann and Chen color reaction for the determination of vitamin $D_2$ in nine samples of irradiated ergosterol, taking pure calciferol as the reference standard. His results showed deviations of from 8 to 40 per cent from the bioassay values. He also investigated the determination of vitamins D in fish liver oils, removing the vitamin A and carotenoids by chromatographic adsorption on Montana earth from benzene solution. He found it necessary to remove most of the sterols by the use of digitonin, when the potency of the oil was low.

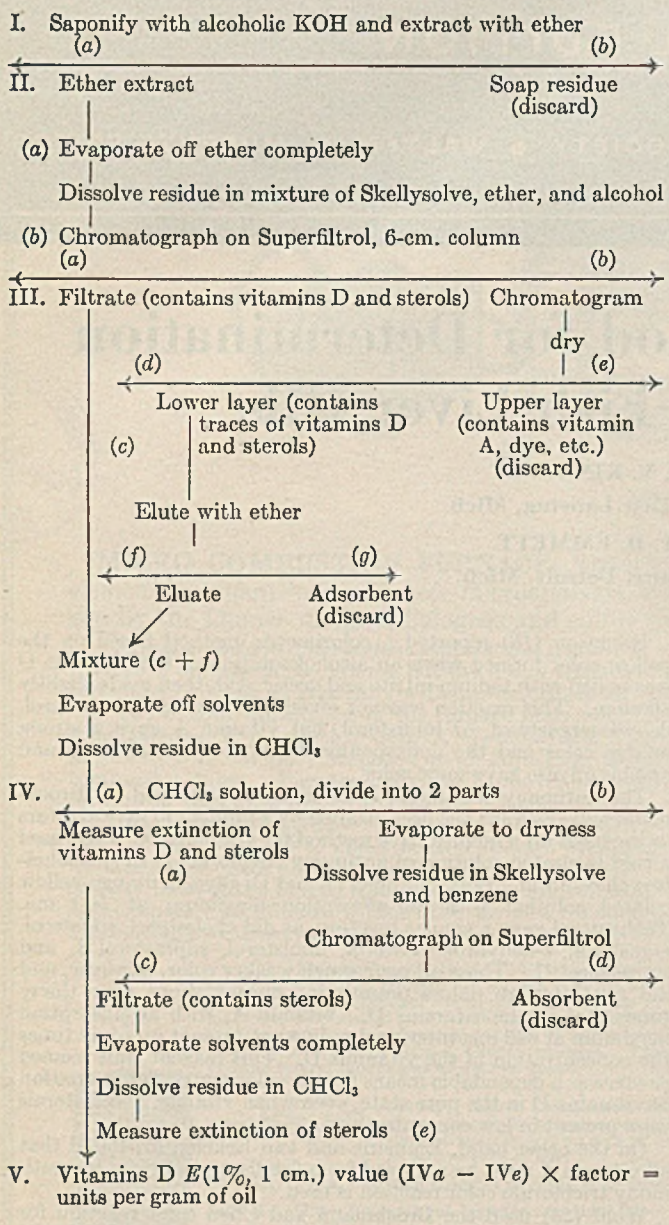
Ritsert (*17*) removed vitamin A by chromatographic adsorption on aluminum oxide, but reported that this colorimetric method was not applicable to fish liver oils nor to the mixed products obtained by irradiation of the provitamins.

Raoul and Meunier (*15*) modified the reagent of Brockmann and Chen by adding a small amount of acetic anhydride and sulfuric acid to the chloroform solution of antimony trichloride. They attempted to make use of the fading of the vitamin A color and of the concurrent slow formation of a cholesterol color to compute the vitamins D concentration by the rate of change in the absorption maxima. They suggested the use of digitonin to remove most of the sterols.

Nield, Russell, and Zimmerli (*13*) also modified the reagent of Brockmann and Chen by adding acetyl chloride and changing the concentration of the antimony trichloride. Thereby, they increased the sensitivity and permanency of the color.

Milas, Heggie, and Raynolds (*12*) determined the vitamin A potency of fish liver oils by measuring the 620 mμ maximum.

Schematic Outline of Vitamins D Determination




Cholesterol, with an absorption maximum increasing slowly with time, was estimated by measuring the 480 mμ maximum exactly 30 minutes after adding the antimony trichloride reagent to the fish oil sample. Corrections for vitamin A and cholesterol, based on the above measurements, were then applied. Milas *et al.* found better agreement, however, between the calculated potencies and bioassay values when the vitamin A, carotenoids, and possibly 7-dehydrocholesterol were first removed by treatment with maleic anhydride. The vitamins D concentration was then obtained by measurement of the 500 mμ maximum. Gudlet (*7*) also tried the use of maleic anhydride for removing vitamin A and freezing for the removal of sterols.

Ewing and Tomkins (*6*) separated vitamin A from the nonsaponifiable fraction of 16 fish liver oils by chromatographic adsorption on Superfiltrol from hexane-ether-alcohol solution. After removal of sterols with digitonin, the modified reagent of Nield, Russell, and Zimmerli (*13*) was used for the color reaction for vitamins D. Special attention was found necessary in the purification of the chloroform, including a final treatment with activated carbon.

This review indicates that certain interfering substances must be removed from the nonsaponifiable fraction before a quantitative determination of the vitamins D can be made. Then it seems possible to use some modification of the antimony trichloride color method after removal of the vitamin A. The authors chose for removal of the carotenoids, vitamin A, and pigments a modification of the chromatographic adsorption procedure used by Ewing and Tomkins (*6*). Further, while they depended upon freezing and treatment with digitonin for the removal of the sterols, the present authors base their procedure upon a two-step chromatographic treatment where the $E$(1 per cent, 1 cm.) is determined first for the combined vitamins D and sterols and second for the separated sterols. Then, by difference, the value for the vitamins D is obtained.

The schematic outline of the proposed method is given in stages I to V. The procedure is somewhat complicated; in fact, the minutest details should be followed exactly. To this end, definite instructions are given.

## Equipment and Reagents

1. The adsorption columns must be carefully and uniformly prepared to obtain reproducible results. The tubes for the chromatographic determinations are made by sealing a 6-cm. length of 7-mm. Pyrex tubing to the bottom of a 1.6 × 15 cm. (0.625 by 6-inch) Pyrex test tube. These tubes are cleaned before filling by soaking in sulfuric acid–dichromic acid solution, rinsing with distilled water and with alcohol, and finally drying in the flame of a Meker burner. The suction apparatus is designed so that a bank of 8 columns can be developed simultaneously, controlling the pressure with the aid of an open-tube mercury manometer attached to the suction flask. The adsorbent used is a finely divided grade of activated bentonite clay (Superfiltrol, obtained from The Filtrol Corp., 315 West 5th St., Los Angeles, Calif.).

The adsorption columns for the first chromatographic separation are prepared by placing a small wad of cotton in the bottom of one of the adsorption tubes, pressing this down firmly, and adding enough of the clay, so that when very firmly pressed down with a piston, using a cork on the end of a glass rod, under 6 cm. of suction, the height of the packed column will be 3 cm. A second and equal portion of the adsorbent is then added, and pressed down as before to give a hard, level surface. It is important that there should be no air pockets, as they cause irregularly shaped adsorption bands. To aid in overcoming this the piston-head cork used in packing the columns is but slightly smaller than the inside diameter of the Pyrex tube. This also helps avoid loosening of the adsorbent by suction when this cork piston is raised. Other columns for the chromatographic separation of vitamins D and sterols are prepared in the same manner, except that the height is only 1.5 cm. and the filling is done under a suction of 4 cm.

2. Alcoholic potassium hydroxide is prepared by dissolving 14 grams of C. P. potassium hydroxide pellets in 95 per cent ethyl alcohol to give 500 ml. This stock solution is protected from carbon dioxide and filtered through hardened filter paper as needed.

3. C. P. ethyl ether is used without further purification for extracting the saponified oils and for elution of adsorption columns.

4. The anhydrous ethyl ether for the chromatograph is purified by washing C. P. ether with 1 per cent ferrous sulfate solution to remove peroxides, then 10 times with distilled water to remove alcohol, drying with phosphorus pentoxide, filtering, and storing over sodium. This ether is distilled from metallic sodium as needed.

5. The Skellysolve is purified by shaking with concentrated sulfuric acid, washing twice with 10 per cent sodium carbonate solution, then with a mixture of 10 per cent sodium carbonate and 5 per cent potassium permanganate solution. It is washed 15 times with distilled water, the reagent being decanted into a dry flask and dried over sodium. The dried solvent is then distilled (68° to 70° C.) from sodium, the first 5 per cent and the last 10 per cent of the distillate being discarded.

6. The absolute ethyl alcohol is a high-grade commercial product.

7. C. P. chloroform is washed thoroughly with 7 approximately equal portions of water, dried over anhydrous potassium carbonate, decanted, and fractionated, discarding the first and

last 10 per cent of the distillate. This purified chloroform is relatively unstable and hence is prepared in small quantities, and protected from light. Before use, it is tested with silver nitrate solution for chlorides and with potassium iodide and starch solution for oxidizing agents. If these are absent, the chloroform is shaken with activated carbon and filtered just before using.

8. The antimony trichloride reagent is prepared by dissolving 18 grams of C. P. antimony trichloride in the purified chloroform, diluting to 100 ml., filtering, and then adding 2 ml. of redistilled acetyl chloride (*13*). The best results are obtained when not more than one week's supply of the purified chloroform and antimony trichloride reagent is prepared at one time.

9. C. P. thiophene-free benzene is dried over sodium, distilled, and shaken with Superfiltrol before use.

10. Measurements of the absorption maxima at 500 mμ are made on a Bausch & Lomb visual spectrophotometer equipped with a Martins polarizing unit and cells 1 or 2 cm. thick.

Table I. Determination of Conversion Factor[a]

| Date Assayed | $E^{1\%}_{1\,cm.}$, 500 mμ | Date Assayed | $E^{1\%}_{1\,cm.}$, 500 mμ | Date Assayed | $E^{1\%}_{1\,cm.}$, 500 mμ |
|---|---|---|---|---|---|
| 3/21 | 0.75 | 3/27 | 0.80 | 4/27 | 0.78 |
| | 0.80 | | 0.78 | | 0.78 |
| 3/23 | 0.76 | 3/31 | 0.77 | 5/20 | 0.76 |
| | 0.80 | | 0.76 | | 0.80 |
| 3/24 | 0.74 | 4/1 | 0.79 | 5/22 | 0.81 |
| | 0.75 | | 0.80 | | 0.79 |
| 3/25 | 0.80 | 4/13 | 0.74 | ... | ... |
| | 0.82 | | 0.75 | | ... |
| 3/26 | 0.77 | 4/14 | 0.77 | ... | ... |
| | 0.78 | | 0.79 | | ... |

Average of 26 determinations $E(1\%, 1\ cm.)$, 0.778
Maximum deviation from average, ±5 per cent
Conversion factor $= \frac{15{,}000}{0.778} = 19{,}300$

[a] Using as reference a fish liver oil mixture No. 47,761 having a U. S. P. biological assay value of 15,000 units per gram.

## Application of Method

Fish oil samples are weighed in duplicate into 125-ml. Erlenmeyer flasks. Samples containing 4000 to 100,000 U. S. P. units of vitamins D are most convenient. Usually, 0.5 to 2 grams are weighed for natural oils and 0.1 gram for concentrates. If the samples weigh 1 gram or less, 10 ml. of alcoholic potassium hydroxide are added, but for samples weighing more than 1 gram, 10 ml. of alcoholic potassium hydroxide per gram of sample are used. A short-stemmed funnel is then placed in the neck of the flask, to serve as a condenser. The sample is placed in a water bath and kept at 70° to 75° C. for 1 hour, or longer if saponification is not complete. Agitation of the flask at frequent intervals aids this reaction. (At this point the sample may be allowed to stand overnight.)

The sample is cooled to room temperature, 20 ml. of water per 10 ml. of alcoholic potassium hydroxide are added, and it is then extracted in a separatory funnel with four 25-ml. portions of ethyl ether. Gentle shaking in the separatory funnel can be used without danger of emulsification, provided more than the indicated quantity of water has not been added. If an emulsion does form, it can be readily broken by the addition of a few drops of alcohol. The ether layer must always be clear before separation of the two layers.

Finally, all the ether extracts are combined in the separatory funnel and 50 ml. of water are added. When both ether and water layers are clear, the water layer is withdrawn and discarded, leaving the ether layer in the funnel. This preliminary washing, which removes most of the soaps, is repeated twice with fresh 50-ml. portions of water. Agitation during these preliminary washings may result in the formation of a stable emulsion. If this occurs 2 ml. of alcohol are added to break the emulsion. Twenty-five milliliters of water are now added to the ether extract in a separatory funnel and shaken vigorously, after which 25 ml. of water are added and the water and ether layers are allowed to separate. The water layer must be clear before it is withdrawn. This is again repeated twice; at the end of the third washing the ether and the water layers should separate quickly to a sharp interface and appear very clear. The water layer at this point should be colorless and not alkaline to phenolphthalein. If not, the washing must be continued until these conditions are satisfied.

The washed ether extract is now withdrawn into a 125-ml. Erlenmeyer flask, passing through a filter paper containing anhydrous sodium sulfate. The separatory funnel is rinsed with 25 ml. of ether and the rinsings are poured on the filter to remove vitamins D from the sodium sulfate and filter paper. These should be colorless. The ether solution is then evaporated to dryness under reduced pressure, using a water bath at 50° C.

The dry residue from this evaporation is taken up in 5 ml. of a special mixture of solvents prepared from the above purified materials—50 parts of Skellysolve, 10 parts of anhydrous ether, and 1 part of absolute alcohol by volume. One drop of Sudan III solution (25 mg. per liter in the above mixed solvents) is added as a color marker, in the separation of vitamin A and pigments from vitamins D, following the suggestion of Brockmann (*1*) in his isolation of pure vitamin $D_3$, when he used Sudan III in a 1 to 4 benzene–petroleum ether mixture.

The 6-cm. adsorption column (prepared as previously described) is wetted with 10 ml. of the mixed solvent, and the sample is added, followed by 5 ml. of the solvent for rinsing the flask and 35 ml. of the same mixture for developing the adsorption bands. Each addition of solvent is made just before the top of the adsorption column becomes dry, otherwise it shrinks away from the glass walls and development of the column is not regular. A pressure differential of 6 cm. of mercury is maintained until 35 ml. of the developing solution have been added, when the suction is increased to 10 cm. Further increase of the suction results in crevices in the adsorption column and packing of the adsorbent to such a degree that the flow of the developing solution is nearly stopped.

When the last of the 35 ml. of solution has passed through the column, the adsorbent is dried by drawing air through the column for 5 to 10 minutes. The Pyrex tube is then taken from its suction flask and the top layer of the column is removed to a point 2 mm. below the red Sudan III band, using an L-bent spatula. This removes the vitamin A and pigments. The tube with the remainder of the column (carrying vitamins D and sterols) is replaced in its suction flask and eluted with 25 ml. of ether.

The combined filtrate and eluate are evaporated to dryness under reduced pressure and taken up in 10 ml. of purified chloroform. To 1 ml. of this solution are added 10 ml. of the antimony trichloride reagent. The flask is swirled for 30 seconds, the absorption cell filled, and the extinction determined on the Bausch & Lomb visual spectrophotometer exactly 3 minutes after starting to add the reagent to the vitamin sample. The sample at this point contains vitamins D and sterols.

Table II. Effect of Added Amounts of Sterols to Reference Oil 47,761

(Bioassay, 15,000 U. S. P. units per gram)

| Sample No. | Sterol Added | $E^{1\%}_{1\,cm.}$, 500 mμ, 3 Minutes S + D[a] | S | D | Physical-Chemical Method U. S. P. units/g. (Calcd.) |
|---|---|---|---|---|---|
| | *Gram* | | | | |
| Reference oil alone | ..... | .. | .. | 0.779 | 15,035 |
| 1 | 0.010 cholesterol | 0.98 | 0.20 | 0.78 | 15,050 |
| 2 | 0.025 cholesterol | 1.00 | 0.23 | 0.77 | 14,900 |
| 3 | 0.050 cholesterol | 1.01 | 0.25 | 0.76 | 14,700 |
| 4 | 0.100 cholesterol | 1.12 | 0.39 | 0.73 | 14,100 |
| 5 | 0.250 cholesterol | 1.36 | 0.64 | 0.72 | 13,900 |
| 6 | 0.500 cholesterol | 1.84 | 1.11 | 0.73 | 14,100 |
| 7 | 0.001 ergosterol | 1.02 | 0.21 | 0.81 | 15,600 |
| 8 | 0.005 ergosterol | 0.92 | 0.12 | 0.80 | 15,400 |

[a] S, sterols.
D, vitamins D.

To correct for the absorption at 500 mμ due to the sterols present, another 1-ml. aliquot of the chloroform solution of vitamins D plus sterols is evaporated to dryness and taken up in 5 ml. of 1 to 2 Skellysolve-benzene mixture. A tightly packed 1.5-cm. Superfiltrol column is wetted with 5 ml. of the mixed solvent and the sample added, followed by the addition of 5 ml. of solvent to rinse the flask and then 50 ml. to elute the sterols. A lavender-blue band carrying the vitamins D is fixed in the upper portion of the adsorption column. The sterols pass into the filtrate. The filtrate is evaporated to dryness under reduced pressure and the

TABLE III. COMPARATIVE VITAMIN D VALUES OF FISH LIVER OILS BY PHYSICAL-CHEMICAL AND BIOLOGICAL METHODS

| Sample No. | Physical-Chemical Method: Weight of sample, grams | $E^{1\%}_{1\text{ cm.}}$, 500 mμ | Calcd., U. S. P. units/g. | Biological Method, U. S. P. Units/G. | Difference, % |
|---|---|---|---|---|---|
| | **A. Low Vitamin D Fish Liver Oil Blend** | | | | |
| 74,912 | 2.00 | 0.14 | 2,700 | | |
| | | 0.15 | 2,890 | 3,000 | − 6.8 |
| | | Av. 0.145 | 2,795 | | |
| 62,321 | 2.00 | 0.27 | 5,210 | | |
| | | 0.25 | 4,830 | 4,750 | + 5.7 |
| | | Av. 0.260 | 5,020 | | |
| 56,211 | 1.00 | 0.26 | 5,020 | | |
| | | 0.27 | 5,210 | 4,750 | + 7.7 |
| | | Av. 0.265 | 5,115 | | |
| 50,261 | 2.00 | 0.33 | 6,370 | | |
| | | 0.31 | 5,980 | 6,000 | + 2.9 |
| | | Av. 0.320 | 6,175 | | |
| 78,992 | 1.00 | 0.286 | 5,520 | | |
| | | 0.242 | 4,670 | 4,500 | +13.2 |
| | | Av. 0.264 | 5,095 | | |
| 77,462 | 1.00 | 0.43 | 8,300 | | |
| | | 0.42 | 8,110 | 6,300 | +30.2 |
| | | Av. 0.425 | 8,205 | | |
| 66,831 | 1.00 | 0.34 | 6,550 | | |
| | | 0.36 | 6,850 | | |
| | | 0.36 | 6,950 | | |
| | | 0.35 | 6,750 | 6,600 | + 2.4 |
| | | Av. 0.352 | 6,775 | | |
| 60,771 | 2.00 | 0.38 | 7,330 | | |
| | | 0.36 | 6,950 | 6,600 | + 8.2 |
| | | Av. 0.370 | 7,140 | | |
| | **B. High Vitamin D Fish Liver Oil Blend** | | | | |
| 55,691 | 1.00 | 0.66 | 12,700 | | |
| | | 0.65 | 12,500 | 12,000 | + 5.0 |
| | | Av. 0.655 | 12,600 | | |
| 65,251 | 0.75 | 0.67 | 12,900 | | |
| | | 0.68 | 13,100 | | |
| | | 0.67 | 12,900 | | |
| | | 0.71 | 13,700 | 12,000 | + 9.6 |
| | | Av. 0.682 | 13,150 | | |
| 65,231 | 1.00 | 0.66 | 12,700 | | |
| | | 0.69 | 13,300 | 14,000 | − 7.1 |
| | | Av. 0.675 | 13,000 | | |
| 65,221 | 0.75 | 0.70 | 13,500 | | |
| | | 0.75 | 14,500 | | |
| | | 0.69 | 13,300 | | |
| | | 0.67 | 12,900 | 14,000 | − 3.2 |
| | | Av. 0.702 | 13,550 | | |
| 41,860 | 0.50 | 0.81 | 15,600 | | |
| | | 0.79 | 15,200 | 16,000 | − 3.8 |
| | | Av. 0.800 | 15,400 | | |
| 57,481 | 1.00 | 0.75 | 14,500 | | |
| | | 0.78 | 15,100 | 16,500 | −10.3 |
| | | Av. 0.765 | 14,800 | | |
| 40,090 | 0.50 | 0.97 | 18,700 | | |
| | | 0.98 | 18,900 | 20,000 | − 6.0 |
| | | Av. 0.975 | 18,800 | | |
| 55,951 | 0.50 | 1.49 | 28,800 | | |
| | | 1.56 | 30,100 | 30,000 | − 1.8 |
| | | Av. 1.525 | 29,450 | | |
| 60,761 | 0.25 | 1.80 | 34,700 | | |
| | | 1.89 | 36,500 | 35,000 | + 1.7 |
| | | Av. 1.845 | 35,600 | | |
| | **C. Tuna Liver Oils** | | | | |
| 68,881 | 1.00 | 0.60 | 11,600 | | |
| | | 0.60 | 11,600 | | |
| | | 0.62 | 12,000 | | |
| | | 0.59 | 11,400 | 12,000 | − 2.9 |
| | | Av. 0.602 | 11,650 | | |
| 76,902 | 1.00 | 0.64 | 12,400 | | |
| | | 0.67 | 12,900 | 12,000 | + 5.4 |
| | | Av. 0.655 | 12,650 | | |
| 62,071 | 0.50 | 0.68 | 13,100 | | |
| | | 0.66 | 12,700 | 16,500 | −21.8 |
| | | Av. 0.670 | 12,900 | | |
| 76,892 | 1.00 | 0.797 | 15,400 | | |
| | | 0.802 | 15,500 | 16,000 | − 3.4 |
| | | Av. 0.800 | 15,450 | | |
| 76,882 | 1.00 | 1.19 | 23,000 | | |
| | | 1.15 | 22,200 | 21,000 | + 7.6 |
| | | Av. 1.170 | 22,600 | | |
| 68,871 | 0.50 | 1.38 | 26,600 | | |
| | | 1.27 | 24,500 | 25,000 | + 2.2 |
| | | Av. 1.322 | 25,550 | | |
| 57,951 | 0.50 | 1.40 | 27,000 | | |
| | | 1.38 | 26,600 | 28,000 | − 4.3 |
| | | Av. 1.390 | 26,800 | | |
| | **D. Tuna Liver Oil Concentrates** | | | | |
| 70,202 | 0.25 | 3.13 | 60,400 | | |
| | | 3.22 | 62,100 | 65,000 | − 5.8 |
| | | Av. 3.175 | 61,250 | | |
| 63,201 | 0.10 | 4.29 | 82,800 | | |
| | | 4.39 | 84,700 | 80,000 | + 4.7 |
| | | Av. 4.340 | 83,750 | | |
| 70,712 | 0.10 | 3.44 | 66,400 | | |
| | | 3.56 | 68,700 | | |
| | | 3.50 | 67,600 | | |
| | | 3.50 | 67,600 | 80,000 | −15.6 |
| | | Av. 3.500 | 67,570 | | |
| 49,831 | 0.10 | 7.80 | 151,000 | | |
| | | 7.69 | 148,400 | 160,000 | − 6.4 |
| | | Av. 7.750 | 149,700 | | |
| 44,470 | 0.10 | 12.30 | 237,400 | | |
| | | 11.9 | 229,700 | 240,000 | − 2.7 |
| | | Av. 12.100 | 233,550 | | |
| 57,381 | 0.10 | 12.5 | 241,300 | | |
| | | 12.3 | 237,400 | 300,300 | −20.2 |
| | | Av. 12.4 | 239,350 | | |
| 61,751 | 0.10 | 15.2 | 293,400 | | |
| | | 14.9 | 288,000 | 325,000 | −11.9 |
| | | Av. 15.05 | 290,500 | | |
| | **E. Vitamins D Fish Liver Oil Distillates** | | | | |
| 55,031 | 1.00 | 0.96 | 18,500 | | |
| | | 0.93 | 17,900 | 18,500 | − 1.6 |
| | | Av. 0.945 | 18,200 | | |
| 44,170 | 0.25 | 0.84 | 16,200 | | |
| | | 0.76 | 14,700 | 15,000 | + 3.0 |
| | | Av. 0.800 | 15,450 | | |
| 56,961 | 1.00 | 1.11 | 21,400 | | |
| | | 1.09 | 21,000 | 20,000 | + 6.0 |
| | | Av. 1.100 | 21,200 | | |
| 44,090 | 0.50 | 1.12 | 21,600 | | |
| | | 1.13 | 21,800 | 22,000 | − 1.4 |
| | | Av. 1.125 | 21,700 | | |
| 43,040 | 0.25 | 1.68 | 32,400 | | |
| | | 1.69 | 32,600 | 30,000 | + 8.3 |
| | | Av. 1.685 | 32,500 | | |
| 44,080 | 0.25 | 1.56 | 30,100 | | |
| | | 1.52 | 29,300 | 31,000 | − 4.2 |
| | | Av. 1.540 | 29,700 | | |
| | **F. Miscellaneous Vitamins D Fish Liver Oils** | | | | |
| | Type | | | | |
| 85,682 | Cod | 4.10 | 0.0124 | 239 | |
| | | | 0.0165 | 318 | 250 +11.2 |
| | | | Av. 0.0144 | 278 | |
| 62,151 | Cod | 5.00 | 0.008 | 154 | |
| | | | 0.0102 | 197 | 287 −39.4 |
| | | | Av. 0.009 | 174 | |
| 62,331 | Cod | 4.10 | 0.0091 | 186 | |
| | | | 0.0096 | 186 | 300 −38.0 |
| | | | Av. 0.0094 | 186 | |
| 52,051 | Halibut | 0.40 | 0.066 | 1,270 | 1,200 + 5.8 |
| 62,171 | Halibut | 2.00 | 0.099 | 1,910 | |
| | | | 0.094 | 1,810 | 1,200 +55.0 |
| | | | Av. 0.097 | 1,860 | |
| 57,991 | Yellow Fin | 1.00 | 0.153 | 2,950 | |
| | | | 0.162 | 3,130 | 3,000 + 1.3 |
| | | | Av. 0.158 | 3,040 | |
| 80,352 | Swordfish | 1.00 | 0.37 | 7,140 | |
| | | | 0.34 | 6,560 | 5,000 +37.0 |
| | | | Av. 0.355 | 6,850 | |
| 80,362 | Swordfish | 1.00 | 0.48 | 9,260 | |
| | | | 0.43 | 8,300 | 10,000 −12.2 |
| | | | Av. 0.455 | 8,780 | |
| 57,971 | Albacore | 0.25 | 3.03 | 58,500 | |
| | | | 2.94 | 56,700 | 55,000 + 4.7 |
| | | | Av. 2.985 | 57,600 | |
| 58,011 | Bonita | 0.25 | 3.11 | 60,000 | |
| | | | 3.33 | 64,300 | 65,000 − 4.4 |
| | | | Av. 3.220 | 62,150 | |
| 75,442x[a] | Outside oil | 0.50 | 2.33 | 45,000 | |
| | | | 2.42 | 46,700 | |
| | | | 2.46 | 47,500 | |
| | | | 2.37 | 45,700 | 50,000 − 7.6 |
| | | | Av. 2.40 | 46,225 | |
| B2861[a] | Outside oil | 0.50 | 1.43 | 27,600 | |
| | | | 1.44 | 27,800 | 32,500 −14.1 |
| | | | Av. 1.44 | 27,700 | |
| P6846x[b] | Outside oil | 1.00 | 1.05 | 20,300 | |
| | | | 1.02 | 19,700 | 20,000 0.0 |
| | | | Av. 1.04 | 20,000 | |
| V3428[b] | Outside oil | 0.10 | 61.5 | 1,187,000 | |
| | | | 65.4 | 1,260,000 | 1,200,000 + 2.0 |
| | | | Av. 63.5 | 1,223,500 | |

[a] Sample and biological data supplied by S. H. Fox, Gelatin Products, Inc.
[b] Sample and biological data supplied by A. E. Briod, National Oil Products Co.

residue taken up in 1 ml. of chloroform. To this are added 10 ml. of the antimony trichloride reagent, using a 2-cm. absorption cell. The extinction is determined at 500 mμ exactly 3 minutes after mixing.

From these two extinction values, the $E$(1 per cent, 1 cm.) is calculated for vitamins D and sterols combined and for sterols alone. The difference between these two values gives the $E$ (1 per cent, 1 cm.) for the vitamins D in the original sample. This value, multiplied by the factor 19,300 referred to below, gives the potency in U. S. P. units per gram of oil.

Reference Oil. A typical vitamins D mixed fish liver oil, No. 47,761, was selected as the reference oil and was bioassayed several times by the official U. S. P. method, giving an average potency of 15,000 U. S. P. units per gram. This oil was also carefully assayed 26 times by the proposed chemical method over a period of 2 months. The extinctions are given in Table I.

During this period several new lots of adsorbent, solvents, and antimony trichloride were used. The average $E$(1 per cent, 1 cm.) value is 0.778 with a maximum deviation of only ±5 per cent. With this figure and the biological potency value of 15,000 units per gram, the standard conversion factor is calculated as 19,300 and is used throughout this report.

As a further check on the method, it was decided to determine whether cholesterol and ergosterol would interfere with the actual analysis of a fish liver oil. Accordingly, varying amounts of these substances were added to 1-gram samples of the reference oil (No. 47,761) and analyzed for vitamins D (Table II). These data show that adding even 0.5 gram of cholesterol above that already present in the natural fish liver oil caused an error of less than 7.3 per cent in the analysis. This amount of cholesterol is far higher than would be found in a fish liver oil. For 0.050 gram of added cholesterol the error due to the added cholesterol is only 2 per cent and this is well within the limit of reproducibility of the method as shown by Table I.

Further, the presence of the provitamin ergosterol (Table II, 7, 8), in concentrations of slightly higher order than the vitamin itself, caused only an insignificant increase in the calculated potency, indicating that no measurable amount of the vitamins D was formed during the analytical procedure.

## Experimental Results

The application of the method was carried out on the following types of fish liver oil (Table III):

| | | *Units per gram* |
|---|---|---|
| A | Low vitamin D oil blends | 3,000 to 7,000 |
| B | High vitamin D oil blends | 12,000 to 35,000 |
| C | Tuna liver oils | 12,000 to 28,000 |
| D | Tuna liver oil concentrates | 65,000 to 325,000 |
| E | Vitamins D liver oil distillates | 15,000 to 31,000 |
| F | Miscellaneous fish liver oils | 250 to 1,200,000 |

The results in Table III are self-explanatory, and show that the method is capable of reproducibility if the details of the procedure are followed exactly. The chemical determinations were made in duplicate or triplicate—that is, two or more samples were weighed out and carried through the entire procedure.

The over-all inspection of the results indicates a fairly close agreement between the physical-chemical method and the official U. S. P. procedure. The most outstanding variance lies in miscellaneous vitamins D fish liver oils, where it seems that with oils of a potency of 1200 or less U. S. P. units per gram, the errors are large. This may perhaps be attributed chiefly to taking large samples for test. This point is brought out clearly in the case of halibut oils 52,051 and 62,171. The outstanding differences may be due in part to a combination of errors, including those in the bioassay values which in themselves have a range of at least ±10 per cent. The average percentage difference for all 51 oils is 9.7, and if we exclude the 7 oils which show a range of 20 per cent or more, the average for the remaining 44 samples is 5.8 per cent.

The last four oils listed in Table III were submitted as unknowns, for a further check on the efficiency of the chemical method. The biological assays were reported as having been carried out by the official U. S. P. method. The results agree satisfactorily.

## Summary and Conclusions

A physical-chemical method for determining vitamins D in fish liver oils is based upon the separation of the vitamins D from vitamin A and other interfering substances by chromatograph adsorption, and then measurement of the extinction coefficient at 500 mμ of the reaction product in antimony trichloride.

Data are presented for the vitamins D potency of 51 liver oils from various types of salt-water fish. The values indicate that the proposed physical-chemical method, as outlined, gives results which are in fairly close agreement with those by the U. S. P. procedure for oils ranging from 5000 units per gram and up in potency. For weaker oils, the method is not so satisfactory. Both possible simplification and its application to irradiated products are being studied.

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# Pantothenic Acid

## Optical Rotation As a Measure of Stability

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STABILITY studies in this laboratory on synthetic and naturally occurring pantothenic acid have indicated a high degree of lability for the compound in presence of acid or alkali. The rate of destruction in preliminary studies appeared as a direct function of pH and of temperature. Furthermore, the presence of certain compounds in solution catalyzed the destruction of pantothenic acid even in the pH range of optimum stability.

A rapid chemical or physical method of analysis was desired to follow the rate of destruction of pantothenic acid and its salts under various controlled conditions. Since the nature of the pantothenate molecule made it unlikely that chemical derivatives of specific analytical value could be found, the author turned to physical methods of analysis. The optical rotatory power of the compound appeared as one possibility, but presented some difficulties, since the nature of the destruction was not entirely known and at least one of the known hydrolysis products was also optically active. Only the dextrorotatory form of pantothenic acid is biologically active and the study was limited to this form.

Evidence of the structure of pantothenic acid first arose from a knowledge of its normal degradation products, and this in turn provided clues to the various methods of synthesis.

Williams, Weinstock, Rohrmann, Truesdail, Mitchell, and Meyer (*6*) in 1939 indicated that pantothenic acid undergoes cleavage in acid or alkaline media to form β-alanine and an aliphatic dihydroxy acid. Woolley, Waisman, and Elvehjem (*7*) confirmed this finding by condensing the acid halide of the dihydroxy acid fragment of pantothenic acid with β-alanine to regenerate a compound having the full biological action of the original compound. Weinstock, Mitchell, Pratt, and Williams (*4*) isolated β-alanine as one of the cleavage products shortly thereafter, and Woolley, Waisman, and Elvehjem (*8*) made the further observation that the dihydroxy acid portion of the molecule readily forms a lactone. The dihydroxy acid portion of the molecule was later determined as α,γ-dihydroxy-β,β-dimethyl butyric acid by Williams and Major (*5*). Various synthetic methods have since appeared from many laboratories which depend on the condensation of the dihydroxy acid, or its lactone or salt, with β-alanine to form pantothenic acid.

### Theory

Simple hydrolytic cleavage (I) of the pantothenate ion to yield β-alanine and *d*(+)-α,γ-dihydroxy-β,β-dimethylbutyric acid [hereinafter designated as *d*(+)-dihydroxy acid] and lactonization (II) of the *d*(+)-dihydroxy acid to form *d*(−)-α-hydroxy-β,β-dimethylbutyrolactone [hereinafter designated as *d*(−)-lactone] are pictured as in the formula to the right.

A 1 per cent solution of calcium *d*(+)-pantothenate theoretically gives rise to a 0.545 per cent solution of the *d*(−)-lactone, assuming complete hydrolysis of pantothenate and complete lactonization of the *d*(+)-dihydroxy acid. The rate and completeness of lactonization were not known; however, it appeared likely that rotation values could be established for known systems of the *d*(−)-lactone–*d*(+)-dihydroxy acid equilibrium which would be of value in predicting the nature of unknown systems which also contained unchanged pantothenate.

Experiments were carried out to determine whether or not the above reactions could be reduced to a mathematical basis in which the measured rotation would serve as an index to the amount of unchanged pantothenate ion in solution at any time.

### Experimental

The specific rotation of several samples of synthetic calcium *d*(+)-pantothenate in 1 per cent aqueous solution ranged from +25.5° to +27°. The values for highly purified *d*(−)-α-hydroxy-β,β-dimethylbutyrolactone ranged from −50° to −51.5°. [Grüssner, Gätzi-Fichter, and Reichstein (*1*) reported the $[\alpha]_D^{17.5}$ of the *d*(−)-lactone to be −49° (*C* = 4.012 in water) and the $[\alpha]_D^{19}$ of the barium salt of the corresponding acid to be +5.5° (*C* = 2.8 in water).] All readings were taken at 25° to 26° C., using a sodium arc lamp, a 1-decimeter tube, and a rotary polariscope with Lippich polarimeter.

From the above-determined values a freshly made solution containing 2 parts of calcium *d*(+)-pantothenate to 1 part of *d*(−)-lactone would be expected to display zero rotation, since in these proportions the opposite rotatory powers of the two compounds almost exactly cancel out. This point was proved by experiment. A 0.545 per cent solution of the *d*(−)-lactone was found to have the calculated rotation, −27.5°. In 0.1 *N* sodium hydroxide the rotation shifted to +7 as the lactone ring opened to give the *d*(+)-dihydroxy acid. This change was quantitatively reversed by making the solution strongly acid. The reaction rate in either direction was greatly accelerated by heat.

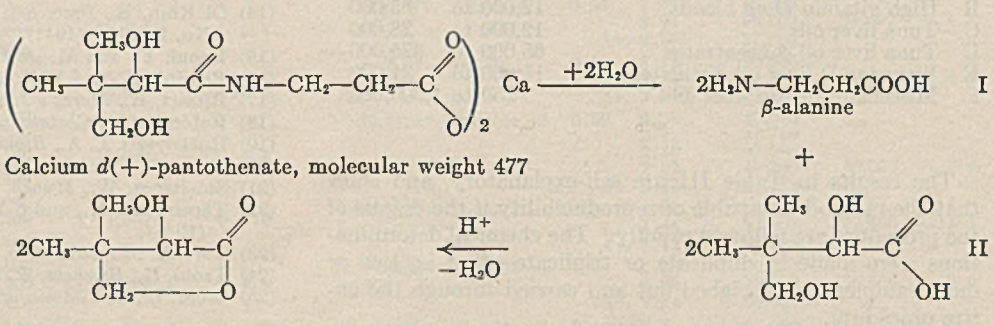
The changes in optical rotation of a 1 per cent solution of calcium *d*(+)-pantothenate as it undergoes hydrolysis and lactonization of the *d*(+)-dihydroxy acid are pictured in two steps as follows:

Ca *d*(+)-pantothenate → *d*(+)-dihydroxy acid $\xrightarrow{H^+}$ *d*(−)-lactone

$[\alpha]_D^{25} = +27°$ $[\alpha]_D^{25} = +7°$ $[\alpha]_D^{25} = -27.5°$

*C* = 1 per cent *C* = 0.545 per cent

Ideal curves representing the rotation through all stages of cleavage of calcium pantothenate, assuming zero (curve I)



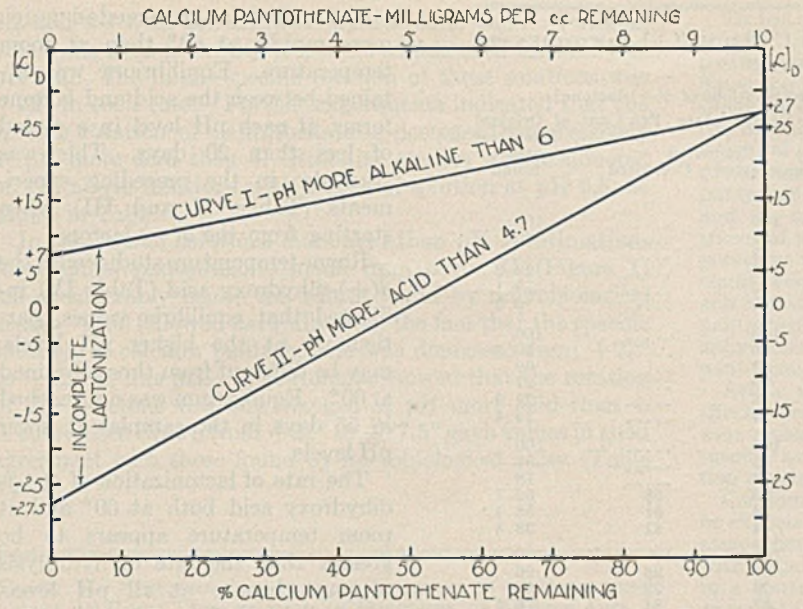



FIGURE 1. SPECIFIC ROTATION OF 1 PER CENT CALCIUM *d* (+)-PANTOTHENATE
Representing zero to complete hydrolysis at different pH levels

and complete (curve II) lactonization of the *d*(+)-dihydroxy acid, are shown in Figure 1.

The validity of curve I was tested by subjecting 1 per cent solutions of calcium pantothenate to treatment with 0.01 *N* sodium hydroxide at various temperatures and taking the rotation at intervals. In all instances the specific rotation finally shifted from +27° to +7°. The reaction required several weeks at room temperature, but only about 24 hours at 100° C.

In order to test the validity of curve II, the following experiment was conducted, in which unbuffered solutions of calcium *d*(+)-pantothenate were made to acid pH to favor lactonization of the *d*(+)-dihydroxy acid.

CALCIUM PANTOTHENATE UNBUFFERED. One per cent solutions of calcium *d*(+)-pantothenate were made to pH 4.0, 4.6, and 5.2 with hydrochloric acid and placed in well-capped bottles in a 60° oven. The rate of destruction was followed at 5-day intervals by measurement of the rotation and by microbiological assay by the method of Strong, Feeney, and Earle (*2*). The general good agreement between the values estimated from curve I and the microbiological assay values is shown in Table I.

TABLE I. ESTIMATION OF RATE OF DESTRUCTION OF CALCIUM *d*(+)-PANTOTHENATE AT 60° C. IN UNBUFFERED SOLUTION BY MEASURE OF OPTICAL ROTATION

| Sample | Treatment | | pH | $[\alpha]^{25}_D$ ($C$ = 1%) | Calcium Pantothenate, Per Cent of Original: Estimated | Microbial assay |
|---|---|---|---|---|---|---|
| | ° C. | Days | | | | |
| A | Original | | 4.0 | +27 | 100 | 100 |
| | 60 | 5 | 4.3 | +11 | 71 | 73 |
| | 60 | 10 | 4.6 | + 7 | 63.5 | 66 |
| | 60 | 15 | 4.8 | + 3 | 56 | 55.5 |
| | 60 | 20 | 5.0 | − 1 | 48 | 45.6 |
| B | Original | | 4.6 | +27 | 100 | 100 |
| | 60 | 5 | 4.8 | +18.5 | 85 | 84 |
| | 60 | 10 | 5.1 | +17 | 82 | 81 |
| | 60 | 15 | 5.3 | +14 | 76.5 | 81 |
| | 60 | 20 | 5.5 | +12 | 71 | 68 |
| C | Original | | 5.2 | +27 | 100 | 100 |
| | 60 | 15 | 5.9 | +27 | 100 | 100 |
| | 60 | 20 | 5.9 | +27 | 100 | 95 |

The gradual rise in the pH of all solutions can be ascribed to the liberation of β-alanine, which has an isoelectric point close to pH 6, and to the gradual lactonization of the *d*(+)-dihydroxy acid, which exerts a pH effect about 3.5 as the free acid. The rate of pH change decreased as the more stable range was approached. This paralleled the lessened rate of destruction.

EFFECT OF pH ON *d*(−)-α-HYDROXY-β,β-DIMETHYLBUTYROLACTONE. The above experiment indicated that lactone formation was complete below pH 5.0, as might be expected. In order to determine the effect of pH on the extent of lactonization, the following experiments were conducted:

A 1 per cent solution of the *d*(−)-lactone was subjected to 60° for 10 days. The original pH was 4.6 and had changed to 3.6 at the end of the period; however, the original specific rotation of −51° did not change significantly throughout the period.

The necessity of buffering solutions for further study was indicated, and equal aliquots of a 1 per cent solution of the *d*(−)-lactone were made to pH 7.4, 6.6, 6.0, and 4.8 with mixtures of 3 per cent solutions of monosodium and disodium phosphate. Original rotations were taken immediately. The solutions were placed at 60° and samples were removed for rotation measurements in 2, 5, and 7 days (Table II). Equilibrium between the lactone and acid forms was apparently reached in about 2 days and was not changed significantly thereafter.

TABLE II. EFFECT OF pH ON SPECIFIC ROTATION OF *d*(−)-α-HYDROXY-β,β-DIMETHYLBUTYROLACTONE AT 60° C.
($C$ = 0.545 per cent)

| | pH | $[\alpha]^{25}_D$ | pH | $[\alpha]^{25}_D$ | pH | $[\alpha]^{25}_D$ | pH | $[\alpha]^{25}_D$ |
|---|---|---|---|---|---|---|---|---|
| Original | 7.6 | −28 | 6.6 | −27 | 6.0 | −27 | 4.8 | −27 |
| 60° C., 2 days | 7.2 | + 7 | 6.4 | − 1 | 5.8 | − 9 | 4.6 | −26 |
| 60° C., 5 days | 7.2 | + 6 | 6.4 | + 1 | 5.8 | − 8 | 4.6 | −27 |
| 60° C., 7 days | 7.2 | + 6 | 6.4 | + 2 | 5.8 | − 7 | 4.6 | −25 |
| 60° C., 14 days | 7.2 | + 6 | 6.4 | + 2 | 5.8 | − 6 | 4.5 | −25 |

In view of the above finding, it appeared possible to calculate corrections from the standard decomposition curve of pantothenic acid to account for incomplete lactone formation at any constant pH. The following experiment was run to test this possibility:

CALCIUM PANTOTHENATE BUFFERED, CORRECTION FOR INCOMPLETE LACTONIZATION. A 1 per cent solution of calcium *d*(+)-pantothenate and a 0.545 per cent solution of the *d*(−)-lactone were made up and each solution was divided into four equal parts. The equal parts of the two solutions were then paired off and the members of each pair were made to comparable pH, using small amounts of monosodium and disodium phosphate, sodium hydroxide, and concentrated phosphoric acid as required. Calcium phosphate precipitated in the calcium pantothenate solutions, but did not interfere with the collection of data. All solutions were kept at 60° and aliquots were taken for rotation and assay at 5-day intervals. Small pH changes occurred throughout the experiment, but the pH in each case was maintained in a narrow range. The pH ranges for the paired solutions are shown in Table III.

The *d*(−)-lactone was stable at pH 3.9 to 4.7. In the range pH 4.9 to 5.2, the hydrolysis of the lactone occurred very slowly and to only a slight extent. At pH 5.6 to 5.9 the

TABLE III. ESTIMATION OF RATE OF DESTRUCTION OF CALCIUM *d*(+)-PANTOTHENATE AT 60° C. IN BUFFERED SOLUTION

[By measure of optical rotation and correction for paired solutions of *d*(−)-lactone]

| Group Pair | Sample | $[\alpha]_D^{25}$ Lactone ($C$ = 0.545%) | $[\alpha]_D^{25}$ Ca Pantothenate ($C$ = 1%) | Ca Pantothenate, Per Cent of Original: Estimated Uncorrected | Estimated Corrected | Microbial assay, found |
|---|---|---|---|---|---|---|
| | *Days* | | | | | |
| I | Original | −27.5 | +27 | 100 | ..... | 100 |
| pH 3.9–4.2 | 1 | −27 | +22 | 91 | ..... | 90 |
| | 5 | −27.5 | +12 | 73 | ..... | ... |
| | 15 | −27.5 | −16 | 21 | ..... | 23.3 |
| | 19 | −27.5 | −20.5 | 13 | ..... | 13.1 |
| | 25 | −27.5 | −24 | 6 | ..... | 6.3 |
| II | Original | −27.5 | +26 | 100 | ..... | 100 |
| pH 4.4–4.7 | 1 | −27 | +16 | 80 | ..... | 80.5 |
| | 5 | −27 | + 7 | 63 | ..... | 69 |
| | 15 | −27 | −11 | 29 | ..... | 27 |
| | 19 | −27.5 | −14 | 25 | ..... | 21.9 |
| | 25 | −27.5 | −19 | 14 | ..... | 14.4 |
| III | Original | −27.5 | +26 | 100 | ..... | 100 |
| pH 4.9–5.2 | 1 | −27.5 | +21 | 89 | ..... | 89 |
| | 5 | −27.5 | +13 | 74 | ..... | 78 |
| | 15 | −25 | + 3 | 55 | 53 | 52.7 |
| | 19 | −25 | + 1.5 | 53 | 51 | 58.4 |
| | 25 | −23 | − 3 | 44 | 41 | 38.3 |
| IV | Original | −27.5 | +27 | 100 | 100 | 100 |
| pH 5.6–5.9 | 1 | −23 | +25 | 97 | 96 | 96 |
| | 5 | −22 | +23 | 93 | 92 | 95 |
| | 15 | −16.5 | +19.5 | 87 | 84 | 76.6 |
| | 19 | −16 | +10 | 69 | 61.5 | 63.6 |
| | 25 | −15 | +11 | 70 | 62.5 | 61.8 |

hydrolysis of the lactone was more rapid in the initial stages but continued throughout the entire period. No correction appeared to be necessary in the calcium pantothenate estimations in groups 1 and 2, and curve I (Figure 1) was applied with fair success, as seen in Table III. Corrections for incomplete lactone formation were made, as indicated, for groups 3 and 4. The corrections were made by varying the standard curve (Figure 1) to correspond with the specific rotation value obtained for the *d*(−)-lactone at the particular pH and time in question. These corrections are rapidly obtained for paired solutions by extending a line on the standard curve from the +27° point for 1 per cent calcium pantothenate to the *d*(−)-lactone value in question and reading the residual calcium pantothenate of the paired pantothenate solution directly from its rotation value. All the correction curves fall naturally between curves I and II (Figure 1). As seen in Table III, the corrected estimates are in somewhat better agreement with the microbiological assay values than the uncorrected estimates.

EFFECT OF pH ON LACTONIZATION OF *d*(+)-α,γ-DIHYDROXY-β,β-DIMETHYLBUTYRIC ACID. In the foregoing experiments the equilibria values obtained at various pH values starting from the *d*(−)-lactone were thought to be equivalent to those which would be obtained starting from the corresponding *d*(+)-dihydroxy acid. That this assumption is essentially correct appears to be borne out by the results of the experiments; however, a large difference may exist in the rate of closing and opening of the lactone ring, and the effect of temperature on the equilibrium between the two forms obtained at any constant pH was not known. The following experiments were run to study the effect of pH and temperature on the rate and completeness of lactonization of the *d*(+)-dihydroxy acid.

A 0.545 per cent solution of the *d*(−)-lactone in 0.01 *N* sodium hydroxide was converted quantitatively to the *d*(+)-dihydroxy acid by mild heating. The solution was divided into 5 equal parts which were made to varying pH values by adding appropriate mixtures of dry phosphate buffer plus hydrochloric acid as needed (Table IV). Half of each buffered solution was placed at 60° and the other half maintained at room temperature. Optical rotation measurements were made at intervals, as shown in Table IV.

Lactonization proceeded much more rapidly at 60° than at room temperature. Equilibrium was attained between the acid and lactone forms at each pH level in a period of less than 20 days. This was true also in the preceding experiments (Tables II and III) when starting from the *d*(−)-lactone.

Room-temperature studies with the *d*(+)-dihydroxy acid (Table IV) indicated that equilibria values, particularly at the higher pH levels, may be different from those obtained at 60°. Equilibrium was not reached in 55 days in the samples at lower pH levels.

The rate of lactonization of *d*(+)-dihydroxy acid both at 60° and at room temperature appears to be greater than the rate of hydrolysis of pantothenate at all pH levels where the pH favors lactonization. Thus there should be no large excess of *d*(+)-dihydroxy acid arising from hydrolysis of pantothenate in solution at any time at pH levels which favor complete lactonization. At pH levels where lactonization is partial—i. e., pH 4.7 to 6—correction has been made most satisfactorily by use of paired solutions of the *d*(−)-lactone, as previously described. Use of the *d*(+)-dihydroxy acid has not proved so satisfactory for paired solutions at 60°.

As can be seen by comparing Tables III and IV, equilibria values at equal pH levels at 60° are about the same whether starting from the *d*(−)-lactone or the *d*(+)-dihydroxy acid. Thus, when equilibrium is reached, the correction will be about equal starting from either compound. Further study is needed to establish the validity of the method of correction as herein applied, particularly in regard to the beginning stages of pantothenate destruction.

TABLE IV. EFFECT OF pH ON LACTONIZATION OF *d*(+)-α,γ-DIHYDROXY-β,β-DIMETHYLBUTYRIC ACID AT ROOM TEMPERATURE AND AT 60°

[$C$ = 0.545%, as *d*(−)-α-hydroxy-β,β-dimethylbutyrolactone]

| Time of Sampling | pH 3.9–4.2 | pH 4.4–4.7 | pH 4.9–5.2 | pH 5.3–5.6 | pH 6.0–7.0 |
|---|---|---|---|---|---|
| *Days* | | | | | |
| | | | $[\alpha]_D^{25}$ | | |
| | | | Samples Maintained at 60° | | |
| Original | + 7 | + 7 | + 7 | + 7 | + 7 |
| 1 | − 6 | + 1 | + 3 | + 3 | + 5 |
| 4 | −13 | − 7 | − 4 | 0 | + 4 |
| 8 | −22 | −17 | −11 | − 6 | + 4 |
| 12 | −26 | −25 | −15 | −12 | + 4 |
| 20 | −28 | −27 | −23 | −17 | + 4 |
| 35 | −28 | −27 | −23 | −17 | + 4 |
| | | | Paired Room Temperature Samples | | |
| Original | + 7 | + 7 | + 7 | + 7 | + 7 |
| 1 | + 2 | + 6 | + 6 | + 7 | + 7 |
| 4 | 0 | + 4 | + 7 | + 7 | + 7 |
| 8 | − 3 | + 1 | + 5 | + 7 | + 7 |
| 12 | − 8 | 0 | + 3 | + 5 | + 7 |
| 20 | −11 | − 3 | 0 | + 3 | + 7 |
| 35 | −17 | − 7 | − 2 | + 3 | + 7 |
| 55 | −24 | −11 | − 5 | + 3 | + 7 |

EFFECT OF EXCESS ACID ON ROTATION AND RATE OF HYDROLYSIS OF CALCIUM *d*(+)-PANTOTHENATE. One per cent solutions of calcium *d*(+)-pantothenate, containing 0.5 per cent of chlorobutanol as a preservative, were made to pH

2.2 and 1.3 with concentrated hydrochloric acid. Twice as much was used to acidify to pH 1.3 as was used to acidify to pH 2.2. The initial specific rotation of these solutions was +22° in each case. Further experiments indicated that the specific rotation of pantothenate is decreased progressively at pH more acid than 4. Stability studies were conducted on the above solutions with a control solution at pH 6.3, as shown in Table V.

In the case of solutions more acid than pH 4, estimations for residual pantothenate made from curve II (Figure 1) fell considerably below the values found by microbiological assay. This followed naturally from the fact that the specific rotation of calcium pantothenate was depressed from +27° to +22° at this pH. Experiments showed that the rotation of *d*(−)-lactone was not changed at pH more acid than 4. A curve then drawn from +22° to −27.5° gave values in good agreement with those found by microbiological assay (Table V).

TABLE V. SPECIFIC ROTATION OF CALCIUM *d*(+)-PANTOTHENATE AT ROOM TEMPERATURE IN PRESENCE OF EXCESS ACID

| Sample | pH | $[\alpha]^{25}_{D}$ ($C$ = 1%) | Estimated | Microbial Assay, Found |
|---|---|---|---|---|
| *Days* | | | % | % |
| Original | 1.3 | +22 | 100 | ..... |
| 1 | 1.3 | +20.5 | 97 | 95 |
| 8 | 1.4 | + 6 | 67.5 | 67.2 |
| 20 | 1.5 | − 3 | 49 | 48.9 |
| 40 | 1.7 | −12 | 30.5 | 27.3 |
| Original | 2.2 | +22 | 100 | ..... |
| 1 | 2.0 | +20 | 96 | 93.7 |
| 8 | 2.1 | +16 | 88 | 86 |
| 20 | 2.26 | +14 | 83 | 79.4 |
| 40 | 2.3 | + 8 | 71 | 68 |
| Original | 6.3 | +27 | 100 | ..... |
| 1 | 6.2 | +27 | 100 | ..... |
| 8 | 6.2 | +27 | 100 | 97.5 |
| 20 | 6.5 | +27 | 100 | 99.8 |
| 40 | 6.9 | +28 | 100 | 98.2 |

## Application of Method

Under optimum conditions the presence of thiamine, riboflavin, nicotinamide, pyridoxine, or various combinations did not obviate application of the method for rapid study of stability of pantothenate. In the pH range in which it is most stable—i. e., more acid than 6.6—riboflavin has no appreciable optical rotatory power. None of the other compounds mentioned is optically active.

These studies clearly demonstrate the incompatibility of thiamine and pantothenate in aqueous mixtures. Pantothenate is most stable in a pH range of about 5.5 to 7. Hydrolysis of the molecule occurs at an increasing rate as the pH moves away from this range on either the acid or alkaline side. Thiamine becomes increasingly less stable at pH more alkaline than 4, as shown by a large number of independent experiments conducted in this laboratory during the last four years.

In presence of air, pyridoxine is oxidized to a colored compound of sufficient intensity to obviate rotation measurements. When oxygen was excluded pyridoxine was found to be compatible with pantothenate at neutral pH.

Nicotinamide in concentrations from 2 to 15 per cent had a stabilizing effect for pantothenate at pH 7 to 9, but not at pH 4 to 5 where it appeared to have a small labilizing effect. The effect of thiamine and riboflavin on pantothenate was negligible.

Since hydrolysis appeared as the main mechanism of destruction of pantothenate, it seemed likely that the molecule would resist dry heating up to the point of decomposition.

To test this hypothesis, dry calcium pantothenate was heated at 120° for 40 hours. Rotation measurements on the heat-treated pantothenate indicated about 12 per cent destruction. Since the presence of traces of moisture might account for considerable destruction of pantothenate, a similar experiment was run in which the calcium pantothenate was mixed with an equal weight of a dehydrating agent—i. e., silica gel or calcium sulfate—preliminary to heating. In this experiment no destruction of pantothenate occurred, as measured by both the rotation method and the microbiological assay method. Further evidence that traces of water may react to destroy pantothenate at high temperatures was obtained by mixing calcium pantothenate with an equal weight of disodium hydrogen phosphate dodecahydrate and heat-treating the mixture as above. About one third of the pantothenate was destroyed by this treatment. In presence of anhydrous disodium phosphate only 2 to 4 per cent of the original pantothenate was lost.

Acidic substances appeared to exert a catalytic destructive effect toward pantothenate even when only traces of moisture were present. Thus, addition of small amounts of benzoic acid, succinic acid, or nicotinamide hydrochloride caused rapid destruction of admixed calcium pantothenate at 120°.

Calcium pantothenate is moderately hygroscopic and might be expected to be somewhat less stable in a humid than in a dry atmosphere. Calcium pantothenate samples alone and admixed with equal weights of silica gel and calcium sulfate were placed in a container together with a vessel from which water could evaporate freely and the whole was subjected to 60° for 2 weeks. No destruction of pantothenate in presence of either desiccant took place as shown by rotation measurement. Destruction of pantothenate in absence of a desiccant was slight but detectable and was estimated at about 5 per cent of the original.

## Discussion

The suggested method for estimation of pantothenate in solutions of known chemical content deserves further study both to establish the validity of the method and to increase its precision. The microbiological assay method of Strong, Feeney, and Earle (*2*) is thought to be accurate to within ±5 per cent when no interfering substances are present. In most instances in this study, values for pantothenate estimated by rotation were within ±5 per cent of those found by the microbiological method. The rotation method is thought to be precise for simple mixtures at acid pH where lactone formation parallels the rate of destruction, and is applicable, though inherently less accurate, at neutral or alkaline pH where no lactone formation occurs. At intermediate pH values where correction for incomplete lactone formation is needed—i. e., pH 4.5 to 6—the accuracy of the method is largely dependent on the accuracy of the correction.

The pH of optimal stability of pantothenate is in the range of pH 5.5 to 7, but may vary somewhat, depending on the presence of materials which may catalyze hydrolysis. The presence of phosphate buffer appeared to catalyze hydrolysis of pantothenate (compare Tables I and III). This observation was confirmed by further studies in which the microbiological assay was used exclusively. The presence of electrolytes in general appeared to have slight catalytic effect toward destruction of pantothenate. Stability was not so satisfactory in presence of other β-complex vitamins at neutral or acid pH as in their absence at similar pH. Pantothenate in water alone appeared to be stable for 20 days at 60° at pH 5.5 to 7.

Susceptibility of pantothenate to hydrolytic cleavage in the semidry form where only small amounts of moisture are concerned was demonstrated in various ways. The rate of destruction, just as in solution, was dependent upon effective pH. Methods of food drying and processing can be expected to have a large effect on the amount of pantothenate destroyed. Rapid and complete drying and storage in the cold should yield by far the best results. Destruction of pantothenate in natural grain rations for the chick has been reported (*3*) to occur in 100 hours at 100° or more completely in 30 hours at 120°. The amount of water derived from the

feed mixture is apparently sufficient under these conditions to allow cleavage of the pantothenate contained therein. This appears as the most likely explanation, since pantothenate was found completely stable under even more drastic conditions when kept entirely free from moisture.

Unfortunately, the natural stability of many of the vitamins—i. e., thiamine, pyridoxine, and ascorbic acid—is poor at the pH of optimal stability of pantothenate. This fact deserves careful consideration in the case of pharmaceutical preparations and foods where loss of pantothenate is to be avoided.

## Summary

Pantothenate destruction under ordinary conditions can be traced to hydrolysis of the molecule. A method is described for following the destruction of calcium $d(+)$-pantothenate by rapid polarimetric analysis.

The rate of pantothenate destruction is a function of pH and temperature and is affected also by presence of other substances both in aqueous solution and in dry mixtures. Optimum stability of pantothenate lies in the approximate range, pH 5.5 to 7. The rate of destruction increases as the pH moves away from this range. Only traces of water are needed to cause significant destruction of pantothenate when other conditions favor hydrolysis.

Special significance is attached to the apparent incompatibility of pantothenate with certain other vitamins, notably thiamine.

## Acknowledgment

Acknowledgment is gratefully made to Eleanor Willerton, who conducted the microbiological assays. Thanks are expressed to Edmond E. Moore and Marjorie B. Moore for helpful discussion of many of the problems involved and for a supply of $d(-)$-$\alpha$-hydroxy-$\beta,\beta$-dimethylbutyrolactone, and to Carl Nielsen and E. H. Volwiler for helpful advice and support of this project.

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# Colorimetric Determination of Cobalt with *o*-Nitrosoresorcinol

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YOE and Barton (*2*) reported a study of the reaction of $\beta$-nitroso-$\alpha$-naphthol with cobalt, including spectrophotometric data, and found the reaction applicable to the colorimetric determination of small amounts of cobalt. The main disadvantage in using this reagent is due to the insolubility of the cobalt complex. The colored suspension tends to precipitate on standing, resulting in a limited stability.

The authors observed that a similar organic compound, *o*-nitrosoresorcinol, also reacts with cobalt and may be advantageously employed as a colorimetric reagent for this element. *o*-Nitrosoresorcinol is slightly less sensitive for cobalt than is $\beta$-nitroso-$\alpha$-naphthol, but solutions of the cobalt complex of *o*-nitrosoresorcinol are stable for several weeks.

Cronheim (*1*) used *o*-nitrosophenol for the colorimetric determination of cobalt, extracting the cobalt complex with petroleum ether and measuring the intensity of the colored ether fraction. The authors were unable to extract the cobalt complex of *o*-nitrosoresorcinol with any immiscible solvent.

Spectrophotometric data for solutions of *o*-nitrosoresorcinol, the cobalt complex, and the complexes of other metals are presented in this paper. A colorimetric method for the determination of cobalt in the presence of nickel is given.

## Apparatus and Materials

Transmittancy measurements were made with a Beckman spectrophotometer, Model D, using a solution thickness of 1 cm. and distilled water as a standard.

The visual observations were performed with 50-ml. (220-mm.) Nessler tubes.

pH measurements were made with the glass electrode.

*o*-NITROSORESORCINOL (Eastman No. 2088). An aqueous 0.05 per cent solution of the sodium salt was employed. This reagent solution is stable for several weeks.

COBALT. A stock solution containing 1 mg. of cobalt per ml. was prepared from C. P. cobalt nitrate hexahydrate. Solutions containing 20 or 100 p. p. m. of cobalt, prepared by dilution of the stock solution, were used in the experiments.

NICKEL. C. P. nickel nitrate hexahydrate was purified by precipitating, as potassium cobaltinitrite, any cobalt present and filtering. The nickel was precipitated as the hydroxide, filtered, washed thoroughly, and dissolved in hydrochloric acid. The nickel content was determined gravimetrically with dimethylglyoxime.

BUFFER. A buffer solution having a pH of 6.0 was prepared by adding 363 ml. of 0.5 *M* sodium hydroxide to 500 ml. of 0.4 *M* potassium biphthalate and diluting to 1 liter with water. The pH of the buffer is practically unchanged when diluted from 25 to 100 ml.

All other reagents used were of the highest purity obtainable.

## Experimental

The usual procedure followed in this work was to transfer the desired quantity of cobalt to a 100-ml. volumetric flask, add 25 ml. of buffer and 5 ml. of the reagent, and dilute to the mark with water. After thorough mixing, color comparisons were made in Nessler tubes and transmittancy measurements made on another portion of the solution.

Using Nessler tubes, 1 part of cobalt in 20,000,000 parts of solution may be determined at cobalt concentrations of 0 to 0.08 mg. per 100 ml.; 1 in 10,000,000 at 0.08 to 0.2 mg.; 1 in 5,000,000 at 0.2 to 0.25 mg. These are also the approximate increments to be employed in making up a standard series. The spectrophotometer is slightly more sensitive; 1 part of cobalt in 50,000,000 of solution may be detected.

Transmittancy curves for solutions of the reagent and of the cobalt complex are given in Figure 1. The concentration of the reagent must be kept relatively low to prevent a decrease

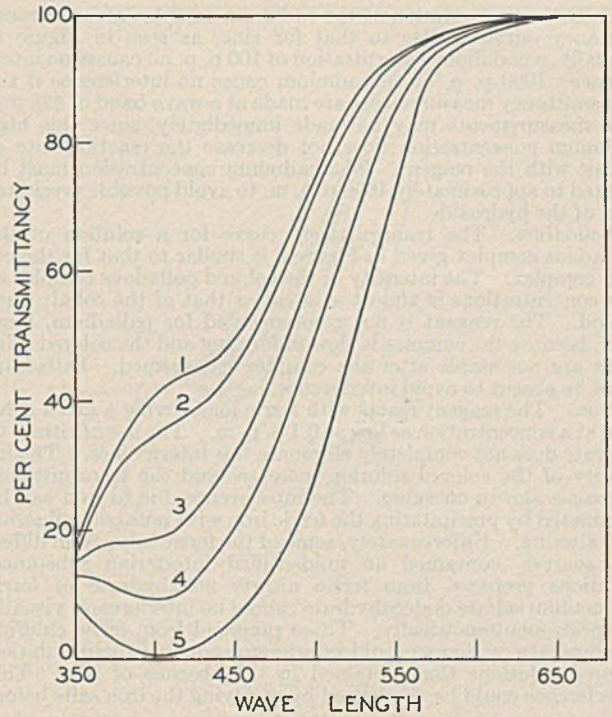


FIGURE 1. EFFECT OF COBALT CONCENTRATION

1. 25 p. p. m. of reagent, no Co
2. 0.1 p. p. m. of Co
3. 0.5 p. p. m. of Co
4. 1 p. p. m. of Co
5. 2.5 p. p. m. of Co

in the sensitivity for cobalt, because the reagent is highly colored and absorbs strongly in the same spectral region as the cobalt complex. The reaction rate, however, is prohibitively slow at very low reagent concentrations. The concentration of reagent recommended, 2.5 mg. per 100 ml., results in immediate color formation and also permits the determination of small amounts of cobalt.

The maximum amount of cobalt determinable is limited by the relatively small amount of reagent employed. No increase in intensity occurs if the quantity of cobalt present exceeds 0.26 mg. with 2.5 mg. of reagent. These quantities correspond to a molar ratio of reagent to cobalt of slightly greater than 3 to 1. This indicates that the compound formed is an inner complex of nitrosoresorcinol and the cobaltic ion, similar to that obtained with $\beta$-nitroso-$\alpha$-naphthol.

The maximum difference between the transmittancy of solutions of the reagent and of the cobalt complex occurs at a wave band of 420 to 430 m$\mu$ at low cobalt concentrations. Most of the measurements were made in this region. A wave length of 450 m$\mu$ may be advantageously used for cobalt concentrations above 0.15 mg. per 100 ml. The validity of Beer's law at 430 and 450 m$\mu$ is shown in Figure 2.

EFFECT OF pH. The color of the reagent varies markedly with the pH of the solution. Below pH 2.5 it has a pale greenish-yellow color which increases in intensity and becomes orange as the pH increases from 2.5 to 5.6. From 5.6 to 6.5 little change in the intensity occurs, but it increases slightly at 7.0. A further increase in pH results in a slight decrease in intensity. The transmittancy curves of solutions of the reagent at various pH's are given in Figure 3. No break in the curve at a wave length of 440 m$\mu$ is observable at a pH of 4.0.

The pH also influences the intensity of the red color of the cobalt complex. No visible reaction occurs below a pH of 2.0. The intensity of the color increases from 2.0 to 5.6, is practically constant from 5.6 to 6.3, and decreases slightly with increasing pH above 6.5. A pH of 6.0 was employed in all experiments, although the method is applicable at a pH range of 5.6 to 6.5. Figure 4 shows the transmittancy curves of solutions of the cobalt complex at various pH's.

To afford increased buffer capacity, the concentration of the buffer may be increased without any effect on the intensity of the color. A biphthalate-sodium hydroxide buffer was employed in preference to a phosphate or citrate buffer because the presence of phosphate or citrate causes a decrease in the intensity of the color of the cobalt complex.

The rate of reaction is dependent on the pH of the solution, being slower at a pH of less than 5.6 than above this value. The reaction occurs practically instantaneously at a pH of 6.0 and above.

STABILITY. Solutions of the cobalt complex are stable for at least a month. The transmittancy, at 450 m$\mu$, of a solution containing 0.15 mg. of cobalt was 0.128 when prepared and remained unchanged for a month. The transmittancy of solutions of the reagent changes slightly on long aging—for example, the transmittancy at 450 m$\mu$ increased from 0.488 to 0.510 after a month. The transmittancy increases slightly at wave lengths less than 500 m$\mu$ but decreases at longer wave lengths on aging. Visually, solutions of the reagent fade slightly on long aging in the buffer or aqueous medium.

INTERFERENCE. In these studies the usual procedure was followed but the ion in question was added prior to the addition of the reagent. Observations were made in the absence of cobalt and at a cobalt concentration of 1 p. p. m.

The presence of 5 grams of sodium nitrate, potassium chloride, ammonium nitrate, sodium chloride, or ammonium

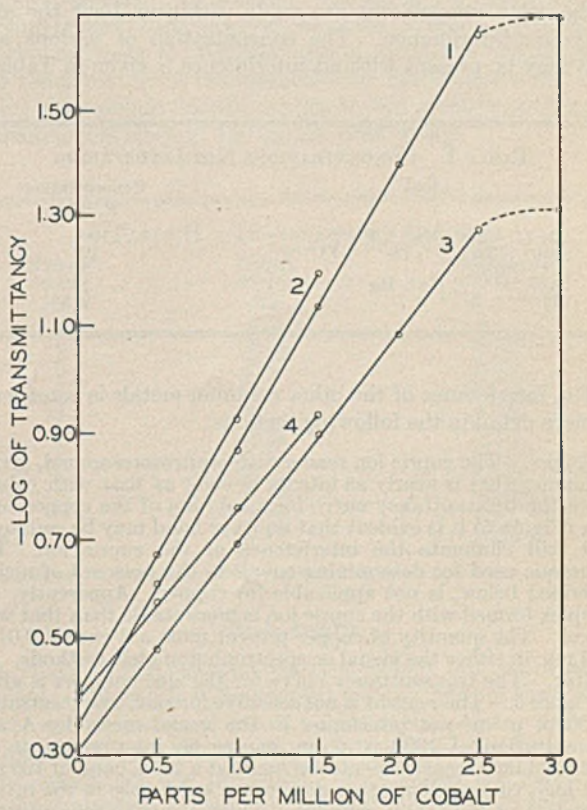


FIGURE 2. TEST OF BEER'S LAW WITH COBALT-NITROSO-RESORCINOL SOLUTION

1. 430 m$\mu$
2. 430 m$\mu$, 50 p. p. m. of Ni
3. 450 m$\mu$
4. 450 m$\mu$, 50 p. p. m. of Ni

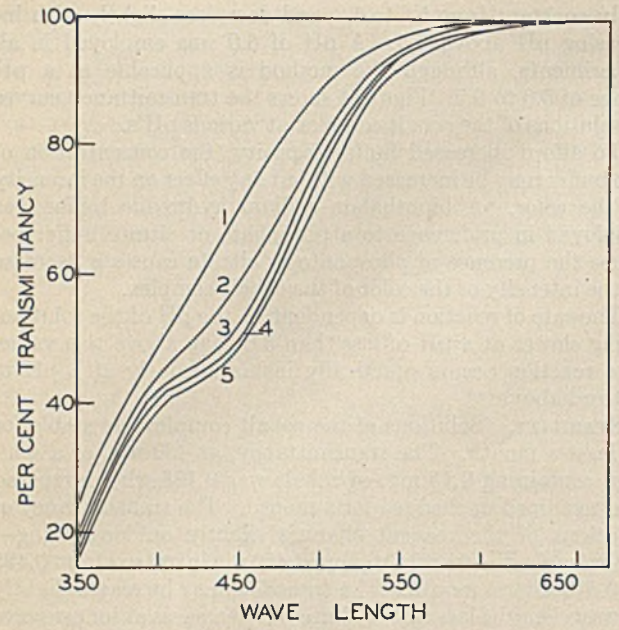


FIGURE 3. EFFECT OF pH ON NITROSORESORCINOL SOLUTIONS

1. pH = 4.0
2. pH = 4.6
3. pH = 5.2
4. pH = 5.6–6.3
5. pH = 7.0 (25 p. p. m. of reagent)

chloride had no effect on the transmittancy of the solutions at 430 mμ. Visually, the presence of more than 1 gram of ammonium chloride produced a green tint. All the other salts cause a similar off shade of color on standing. However, if the color comparisons are made within an hour the salts cause no interference. The concentration of various ions that may be present without interference is given in Table I.

TABLE I. CONCENTRATIONS NOT INTERFERING

| Ions | Concentration *P. p. m.* |
|---|---|
| $Ba^{++}$, $Ca^{++}$, $Mg^{++}$, $Pb^{++}$ | 100 |
| $Mn^{++}$, $Th^{++++}$, $Yb^{+++}$, $Yt^{+++}$ | 10 |
| $Cr^{+++}$ | 5 |
| $Ag^{+}$, $Al^{+++}$, $Hg^{+}$, $Hg^{++}$ | 1 |
| $Ti^{++++}$, $Zr^{++++}$ | 0.5 |

The interference of the other common metals is considered in more detail in the following sections.

*Copper.* The cupric ion reacts with *o*-nitrosoresorcinol, giving a complex that is nearly as intense in color as that with cobalt. From the transmittancy curve for a solution of the copper complex (Figure 5) it is evident that no wave band may be employed that will eliminate the interference of the cupric ion. The technique used for determining cobalt in the presence of nickel, described below, is not applicable for copper. Apparently, the complex formed with the cupric ion is more stable than that with nickel. The quantity of copper present must not exceed 0.01 to 0.02 mg. in either the visual or spectrophotometric methods.

*Zinc.* The transmittancy curve for the zinc complex is given in Figure 5. The reagent is not sensitive for zinc, a concentration of 20 p. p. m. not interfering in the visual method. A zinc concentration of 1000 p. p. m. causes no interference, if the transmittancy measurements are made at a wave band of 420 mμ. The high zinc concentration does cause a decrease in the rate of formation of the cobalt complex. Thus, at a zinc concentration of 1000 p. p. m. it is necessary to allow the solutions to stand 3 to 4 hours before making the measurements if the cobalt concentration is 1 p. p. m. At lower cobalt or zinc concentrations a shorter period of time is required. No evidence of the precipitation of zinc hydroxide was observed, even at a zinc concentration of 5000 p. p. m.

*Cadmium.* A solution of the cadmium complex gives a transmittancy curve similar to that for zinc, as seen in Figure 5. Visually, a cadmium concentration of 100 p. p. m. causes no interference; 1000 p. p. m. of cadmium cause no interference if the transmittancy measurements are made at a wave band of 425 mμ. The measurements may be made immediately, since this high cadmium concentration does not decrease the reaction rate of cobalt with the reagent. The cadmium concentration must be limited to approximately 1000 p. p. m. to avoid possible precipitation of the hydroxide.

*Palladium.* The transmittancy curve for a solution of the palladous complex given in Figure 5 is similar to that for the cobalt complex. The intensity of the colored palladous complex at low concentrations is almost as great as that of the cobalt compound. The reagent is not recommended for palladium, however, because the complex is slow in forming and the colored solutions are not stable after the complex has formed. Palladium must be absent to avoid interference.

*Iron.* The reagent reacts with ferric ions, giving a green solution at a concentration as low as 0.1 p. p. m. The use of citrate or tartrate does not completely eliminate this interference. The intensity of the colored solution increases and the transmittancy decreases slowly on aging. The interference due to iron can be eliminated by precipitating the ferric iron with potassium fluoride and filtering. Unfortunately, some of the ferric salts, from different sources, contained an unidentified interfering substance. Solutions prepared from ferric nitrate nonahydrate or ferric ammonium sulfate dodecahydrate caused no interference, visually or spectrophotometrically. Those prepared from ferric chloride hexahydrate or iron wire did interfere, producing slightly darker colored solutions than obtained in the absence of iron. This interference could be eliminated by purifying the iron salts before use.

The reagent is not recommended for cobalt if iron is present, because it is impossible to know whether or not the interfering substance accompanies the iron. The results obtained, however, do show that a clean-cut separation of cobalt from iron is possible by precipitating the iron as the complex fluoride—for example, no cobalt is lost when the iron is precipitated from a solution containing 500 mg. of iron and 0.05 mg. of cobalt, a ratio of 10,000 to 1.

## Determination of Cobalt in the Presence of Nickel

Nickel also forms a colored complex with *o*-nitrosoresorcinol under the conditions used for the determination of cobalt. The transmittancy curve for the nickel complex (Figure 5)

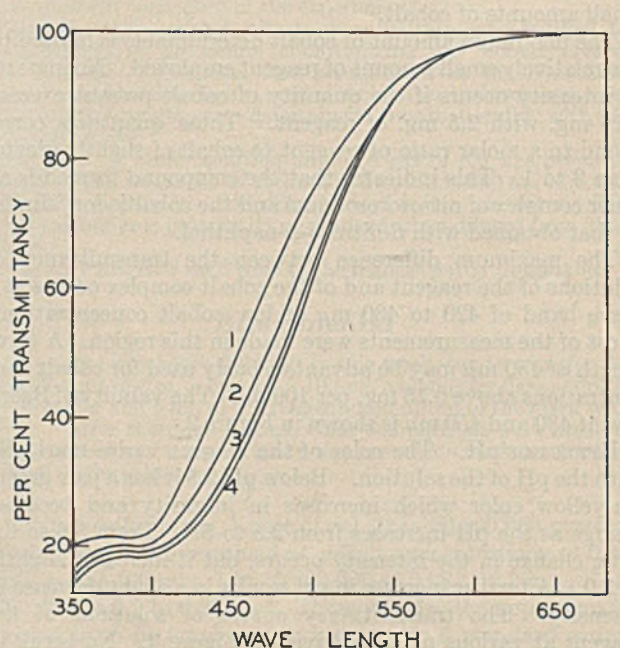


FIGURE 4. EFFECT OF pH ON COBALT-NITROSORESORCINOL SOLUTIONS

1. pH = 4.0
2. pH = 4.6
3. pH = 5.2 and 7.0
4. pH = 6.0 (0.5 p. p. m. of Co)

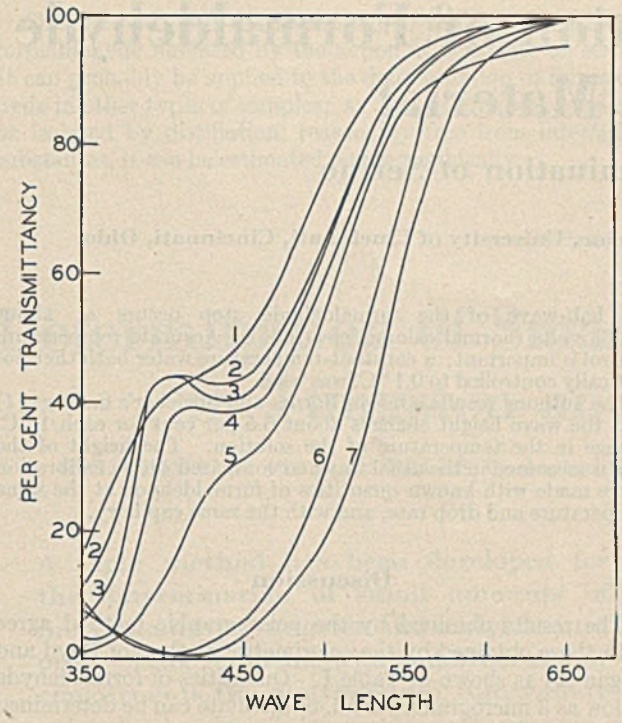


FIGURE 5. TRANSMITTANCY CURVES FOR SOLUTIONS OF METAL COMPLEXES OF NITROSORESORCINOL

1. 25 p. p. m. of reagent
2. 2000 p. p. m. of Cd
3. 2000 p. p. m. of Zn
4. 50 p. p. m. of Ni
5. 5 p. p. m. of Cu
6. 3 p. p. m. of Co
7. 10 p. p. m. of Pd

shows that at no wave band may the interference of nickel be effectively eliminated. The nickel concentration would have to be limited to 0.5 p. p. m., if this interference could not be eliminated by some method.

Using a constant cobalt concentration, the intensity of the color increases with increasing nickel concentration up to approximately 10 p. p. m. The intensity is less immediately after adding the reagent at higher nickel concentrations but increases on standing until the intensity is the same as at 10 p. p. m. This is understandable, if it is remembered that the total intensity of the color is the sum of that of the cobalt and nickel complexes and that the intensity of the cobalt complex is greater than that of the nickel at the same concentration. At the lower nickel concentrations, the cobalt complex forms immediately and any reagent in excess of that required for the cobalt forms the nickel complex simultaneously. At the higher nickel concentrations, however, the formation of the nickel complex is favored, but since the cobalt complex is more stable than the nickel complex a slow shift from the nickel to the cobalt complex occurs, resulting in the increase in the color intensity on standing.

The transmittancy of solutions of the nickel complex is practically independent of the nickel concentration, if the latter is above 10 p. p. m. This indicates that the concentration of nickel does not have to be controlled carefully, providing the concentration is above 10 p. p. m. The reaction rate, between the cobalt and reagent, however, decreases with increasing nickel concentration, thereby setting a practical limit to the amount of nickel that may be present.

An increase in the concentration of the reagent increases the reaction rate but results in a decrease in the sensitivity. This difficulty is avoided if the volume of the solution is kept below 50 ml. during the aging period and diluted to 100 ml. before making the measurements. The aging is carried out at approximately 100° C., because the reaction rate also increases with increasing temperature. Results of experimentation are given in the following procedure:

PROCEDURE FOR DETERMINING COBALT IN THE PRESENCE OF NICKEL. Transfer the test solution to a 100-ml. Erlenmeyer flask, and add 25 ml. of buffer and 5 ml. of reagent. Heat on a steam or water bath for a period of time depending upon the cobalt and nickel concentration present. Cool, transfer quantitatively to a 100-ml. volumetric flask, and dilute to the mark with water.

Using 5 mg. of nickel 2 hours of heating are required for a final cobalt concentration of 1 p. p. m.; 3 hours for 1.5 p. p. m. With 10 mg. of nickel 4 hours are required for 1 p. p. m. of cobalt; 6 hours for 1.5 p. p. m. For 15 mg. of nickel 6 hours are required for 1 p. p. m.; 10 hours for 1.5 p. p. m. of cobalt. A longer time is required if the volume of the solution is greater than 40 ml. during the heating period. It is impractical for the nickel concentration to exceed 150 p. p. m. and the cobalt concentration 1.5 p. p. m.

The use of a spectrophotometer is recommended. With this instrument it is possible to detect 0.002 mg. of cobalt in the presence of 10 mg. of nickel—i. e., 0.02 per cent of cobalt.

Some transmittancy values for mixtures of cobalt and nickel are given in Table II.

Additional results are shown in Figure 2 for various cobalt concentrations in the presence of 5 mg. of nickel. Beer's law is valid, although the lines have origins different from those obtained in the absence of nickel.

The visual method is applicable if appropriate amounts of nickel are present in the standards and the standards are heated for the proper periods of time. Visually, the method is slightly less sensitive for cobalt in the presence of nickel, owing to the increased intensity of the colored solutions.

The effect of potassium fluoride on the method was studied briefly in connection with the possible determination of cobalt in solutions also containing nickel and iron. The presence of potassium fluoride decreases the color intensity of the nickel complex, especially on heating. The aging can be carried out at room temperature, however, since the presence of potassium fluoride increases the reaction rate of the cobalt and reagent. The visual method is not applicable to mixtures of cobalt and nickel if potassium fluoride is present, but spectrophotometric measurements made at 450 mμ are satisfactory.

TABLE II. TRANSMITTANCY VALUES

| Nickel Added *Mg.* | Cobalt Added *Mg.* | Transmittancy at 430 mμ |
|---|---|---|
| 0 | 0.05 | 0.246 |
| 0 | 0.10 | 0.130 |
| 0 | 0.15 | 0.071 |
| 1 | 0 | 0.403 |
| 5 | 0 | 0.401 |
| 10 | 0 | 0.402 |
| 1 | 0.05 | 0.218 |
| 5 | 0.05 | 0.213 |
| 10 | 0.05 | 0.213 |
| 1 | 0.10 | 0.121 |
| 5 | 0.10 | 0.120 |
| 10 | 0.10 | 0.122 |
| 1 | 0.15 | 0.063 |
| 5 | 0.15 | 0.068 |

## Summary

A study of the reaction between cobalt and *o*-nitrosoresorcinol has shown that the reagent may be used for the colorimetric determination of cobalt. Spectrophotometric data for solutions of the reagent, for the cobalt complex, and for other metal complexes are presented. Procedures are given for the determination of cobalt alone and for cobalt in mixtures of nickel and cobalt.

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(2) Yoe, J. H., and Barton, C. J., *Ibid.*, 12, 405 (1940).

# Polarographic Determination of Formaldehyde in Biological Material

## Application to the Determination of Serine

M. JOHN BOYD AND KARL BAMBACH, College of Medicine, University of Cincinnati, Cincinnati, Ohio

FORMALDEHYDE has been determined quantitatively by the polarograph according to Jahoda (*3*), Winkel and Proske (*5*), and Barnes and Speicher (*1*). Recently Boyd and Logan (*2*) have developed a colorimetric method for the determination of formaldehyde as a step in the procedure for estimating serine. Their method consists essentially of quantitatively separating by distillation the formaldehyde resulting from the action of periodate on protein hydrolyzates containing serine, and condensing this formaldehyde with chromotropic acid (Eegrewe's reagent) to form a colored compound.

TABLE I. DETERMINATION OF FORMALDEHYDE BY POLAROGRAPHIC AND COLORIMETRIC METHODS

| Sample | Polarographic Method | | Colorimetric Method |
|---|---|---|---|
| | In 3 ml. of distillate | Per ml. | Per ml. |
| | *Micrograms* | | |
| 1 mg. of serine | 8 | 2.7 | 2.7 |
| 1 mg. of serine | 8.7 | 2.9 | 2.8 |
| 5 mg. of serine | 41 | 13.7 | 14.1 |
| 5 mg. of serine + 100 mg. of threonine | 42 | 14 | 14.1 |
| 5 mg. of serine + 100 mg. of threonine | 42 | 14 | 14.2 |
| 50 mg. of serine | 382 | 127 | 128.8 |
| 50 mg. of serine | 385 | 128 | 131.6 |
| Gelatin hydrolyzate | 155 | 52 | 49.5 |
| Gelatin hydrolyzate | 155 | 52 | 48.7 |
| Salmin hydrolyzate | 20.5 | 6.8 | 6.5 |
| Salmin hydrolyzate | 21 | 7 | 6.7 |
| Salmin hydrolyzate | 22 | 7.3 | 6.5 |
| Cascin hydrolyzate | 21 | 7.0 | 7.0 |
| Cascin hydrolyzate | 22 | 7.3 | 7.0 |
| Horse hemoglobin (crystallized) hydrolyzate | 19 | 6.3 | 6.1 |
| Horse hemoglobin (crystallized) hydrolyzate | 19.5 | 6.5 | 6.4 |
| Dog hemoglobin (crystallized) hydrolyzate | 19 | 6.3 | 6.1 |

In order to ascertain whether the polarograph can be used to determine the formaldehyde found in the distillate in the above procedure, the authors compared polarographic analyses against those run colorimetrically. The polarographic procedure proved suitable for this type of determination, since no substances which interfered with the production of smooth current-voltage curves came over in the distillation.

### Procedure

The formaldehyde is separated from the protein hydrolyzate and the periodate reaction mixture by distillation, as described by Boyd and Logan (*2*). Three milliliters of the distillate are mixed with 1 ml. of a solution of electrolytes (0.4 *N* potassium chloride, 0.2 *N* potassium hydroxide), and nitrogen is bubbled through the mixture for 3 minutes. This does not result in loss of formaldehyde, since repeated runs on the same solution showed that gassing for as long as 15 minutes did not change the height of the step. The polarogram of the solution is then taken from −1.4 to −1.8 volts with the Leeds & Northrup Electro-chemograph; the half-wave of the formaldehyde step occurs at about −1.63 volts (normal calomel electrode). Accurate temperature control is important; a constant-temperature water bath thermostatically controlled to 0.1° C. was used.

The authors' results confirm Barnes and Speicher's findings (*1*) that the wave height changes about 6.5 per cent for each 1° C. change in the temperature of the solution. The height of the step is measured in the usual way and compared with a calibration curve made with known quantities of formaldehyde at the same temperature and drop rate, and with the same capillary.

### Discussion

The results obtained by the polarographic method agree with those obtained by the colorimetric method of Boyd and Logan (*2*) as shown in Table I. Quantities of formaldehyde as low as 3 micrograms per ml. of distillate can be determined by the polarographic procedure.

According to Barnes and Speicher, when potassium hydroxide is the only supporting electrolyte, the full height of the formaldehyde step is not given in concentrations of potassium hydroxide below 0.5 per cent, and so those workers used 1.25 per cent solutions in their determinations. In the authors' experience, satisfactory curves with the full height of the formaldehyde wave are obtained with 0.05 *N* potassium hydroxide in 0.1 *N* potassium chloride, so that it is not necessary to employ stronger alkali, thereby decreasing the possibility of condensation of the formaldehyde.

Acetaldehyde (which arises from the action of periodate on threonine, *4*) does not interfere in the analysis unless it is present in extremely large amounts, since it is not reduced until a potential of −1.8 volts is reached (Figure 1). This method cannot be used for the determination of small amounts of acetaldehyde unless the potassium ion in the supporting electrolyte is replaced by lithium, because the potassium wave interferes with that of acetaldehyde. However, good results can be obtained with acetaldehyde in lithium hydroxide. Other aldehydes, higher in the aliphatic series, are reduced at still higher voltages.

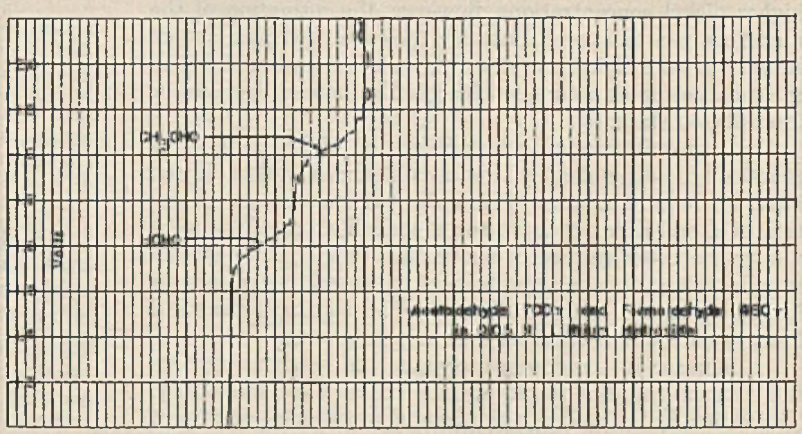

FIGURE 1. POLAROGRAM OBTAINED WITH LEEDS & NORTHRUP ELECTRO-CHEMOGRAPH

This method need not be limited to the determination of formaldehyde liberated by the action of periodate on serine. It can probably be applied to the determination of formaldehyde in other types of samples; as long as the compound can be isolated by distillation, reasonably free from interfering substances, it can be estimated polarographically.

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# Determination of Small Amounts of Molybdenum in Tungsten and Molybdenum Ores

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**A rapid method has been developed for the determination of small amounts of molybdenum in tungsten and molybdenum ores. After removing iron and other major constituents the molybdenum thiocyanate color is developed in water-acetone solutions, using ammonium citrate to eliminate the interference of tungsten. Comparison is made by titrating a blank with a standard molybdenum solution. Aliquots are adjusted to deal with amounts of molybdenum ranging from 0.01 to 1.30 mg.**

MOST of the calcium tungstate ores from contact-metamorphic deposits in the western states contain at least a trace of molybdenum, and in many such ores as much as 0.5 per cent of molybdenum may be present. As concentrates from such ores are subject to a penalty, the determination of molybdenum is important.

In its search for new supplies of the different strategic metals the U. S. Geological Survey has examined various samples of possible molybdenum content that were on the border line between a little and no molybdenum. For such work a quick but accurate method was needed. One method developed for this purpose is based on fluorescence (*1*); the other depends on chemical analysis and colorimetric estimation of the molybdenum.

The amber color developed by molybdenum, thiocyanate, and a reducing agent has long been used as a sensitive test for molybdenum, the colored complex usually being concentrated by extraction with organic solvents such as ether, butyl acetate, or cyclohexanol, before colorimetric estimation. Ether, the most common solvent employed, has several objections, as noted by Sandell (*5*). The authors have found that even a few milligrams of tungsten or vanadium cause high results with the ether extraction alone, unless tartrate or citrate is present. Hurd and Allen (*3*) studied the conditions for maximum stability of color, using various solvents, and stated that "butyl acetate, although an excellent solvent for the thiocyanate, should be used with care. The color produced by a given concentration of molybdenum appears to depend to no slight extent upon the manner in which the solvent has been saturated with the reagents." They reported that cyclohexanol is difficult to handle, separates slowly from aqueous solution, and may develop a turbidity.

The writers dispense with extractions altogether, but employ an acetone-water solution, as acetone appears to have a stabilizing action and prevents fluctuations of the color with time. The interference of tungsten is eliminated by the use of ammonium citrate.

The essential features of the procedure are that the molybdenum thiocyanate color is developed in a water-acetone solution and the comparison is made by titrating a blank containing the necessary reagents with a standard molybdenum solution.

## Reagents

STANNOUS CHLORIDE (10 PER CENT). Dissolve 20 grams of stannous chloride dihydrate in 100 ml. of concentrated hydrochloric acid and 100 ml. of water.

AMMONIUM THIOCYANATE (10 PER CENT). Dissolve 20 grams in 200 ml. of water.

AMMONIUM CITRATE, reagent grade.

ACETONE, reagent grade.

STANDARD MOLYBDENUM SOLUTION. 1 ml. = 0.1 mg. of molybdenum trioxide. Dissolve 0.1 gram of pure molybdenum trioxide in a little sodium hydroxide, neutralize to litmus with hydrochloric acid, and make up to 1 liter in a volumetric flask. As the standard degenerates with time, it is necessary to make up a fresh solution every month.

## Solution of Sample

With substances like scheelite that contain up to 40 per cent of tungstic oxide and possibly as much as 1 per cent of molybdenum oxide, fuse in platinum 0.5 gram of the sample with 2.5 grams of sodium carbonate and about 0.05 gram of sodium nitrate (*2*). Begin the heating at a low temperature and gradually increase the heat until a homogeneous melt is obtained. Extract the cooled melt with water containing a drop of alcohol to reduce manganate, add a little paper pulp, filter, and wash with warm 1 per cent sodium carbonate solution. Make just acid with hydrochloric acid. If any chromate is present, reduce it with a little sulfurous acid. Evaporate to about 25 ml. on the steam bath to remove carbon dioxide. At this point silica may separate out. Without removing silica neutralize with 10 per cent sodium hydroxide and add 10 ml. in excess, add a little paper pulp, and digest on the bath about 10 minutes. Filter hot and wash with dilute sodium hydroxide. Add phenolphthalein to the filtrate, make it barely acid with hydrochloric acid, and transfer it to a 100-ml. volumetric flask. The solution is now ready for the estimation of molybdenum.

With molybdenite ores, wulfenite ores, and ores high in silica, in which tungsten is low, an acid attack of the sample is preferable. The usual practice (*6*) involves treatment with nitric and sulfuric acids and eventual extraction of the molybdenum in a sulfuric acid solution. On a 0.5-gram sample, 2 ml. of sulfuric acid are sufficient.

## Colorimetric Estimation of Molybdenum

Take a 15-ml. aliquot of the neutral molybdenum solution in a 100-ml. beaker; if a larger or smaller aliquot is taken, adjust volume to 15 ml. Add to the solution at room temperature 1.5

grams of ammonium citrate and stir until it is dissolved. Now add 5 ml. of 10 per cent ammonium thiocyanate and 25 ml. of acetone, cool, add dropwise by pipet exactly 7 ml. of stannous chloride solution, and stir. Accurate measurement is important, as the acidity of the solution is controlled by the quantity of stannous chloride added. This develops the amber color of the molybdenum thiocyanate. To a blank in another 100-ml. beaker add 15 ml. of water, 1.5 grams of ammonium citrate, 5 ml. of ammonium thiocyanate, and 25 ml. of acetone. Add by pipet exactly 7 ml. of stannous chloride solution. Titrate this blank to a color match by adding standard molybdenum solution from a buret.

Occasionally the unknown will precipitate silica on the addition of stannous chloride, especially if the addition is too rapid. If this occurs, filter off the silica at this point before matching the color.

## Results with Known Mixtures

In the experimental work accurately measured quantities of solutions of known titer, containing the sodium salts of the elements in question, were transferred to 100-ml. beakers. The solutions were adjusted to 15 ml. and made just acid before the addition of the reagents used for the development of the color. Estimations were made by titrating blanks with standard molybdenum solution.

Table I lists the results obtained in the presence of tungsten. The color is stable in the presence of tungsten for at least 3 hours.

Table I. Estimation of Molybdenum in the Presence of Tungsten

| No. | Taken $WO_3$ | Taken $MoO_3$ | $MoO_3$ Found |
|---|---|---|---|
| | *Gram* | *Mg.* | *Mg.* |
| 1 | None | 0.06 | 0.06 |
| 2 | None | 0.20 | 0.19 |
| 3 | None | 0.50 | 0.51 |
| 4 | None | 0.80 | 0.82 |
| 5 | 0.20 | 0.03 | 0.03 |
| 6 | 0.20 | 0.06 | 0.06 |
| 7 | 0.20 | 0.10 | 0.11 |
| 8 | 0.20 | 0.40 | 0.39 |
| 9 | 0.20 | 0.70 | 0.72 |
| 10 | 0.20 | 1.00 | 1.02 |
| 11 | 0.20 | 1.50 | 1.43 |
| 12 | 0.40 | 0.03 | 0.03 |
| 13 | 0.40 | 0.40 | 0.39 |
| 14 | 0.40 | 0.70 | 0.69 |

## Interference

Iron causes a serious positive error and must be removed completely. The error is not so much a function of the quantity of iron present as of the time elapsed before the sample is matched. Table II illustrates this interference of iron when the solution is matched immediately. Table III shows the interference as a function of time.

Iron as well as many other interfering bases is, however, removed at the start by the carbonate fusion. Small or moderate amounts of iron are further removed with sodium hydroxide as described in the procedure. Excess ammonium hydroxide may be substituted for sodium hydroxide but the digestion is best conducted in a stoppered Erlenmeyer flask. In four experiments, 0.05, 0.30, 1.00, and 50.0 mg. of iron in the presence of 0.3 gram of tungstic oxide were completely removed by this method. Phosphate causes no interference when tungsten is absent. With tungsten present the color fades and turns blue-green with time. If matched within 10 minutes, 20 mg. of phosphorus pentoxide in the final aliquot with 0.3 gram of tungstic oxide show negligible interference. With 0.1 gram of tungstic oxide, 80 mg. of phosphorus pentoxide cause little trouble during this 10-minute interval. The interference of phosphate may be minimized by using more ammonium citrate.

Large amounts of vanadium interfere because of the blue-green color of vanadium in reduced solutions. Amounts of vanadium up to 2 mg. of vanadium trioxide in the final aliquot, however, do not appreciably affect the molybdenum color. Arsenic, antimony, sodium, and ammonium salts, calcium, aluminum, and silica do not interfere. Nitrates tend to cause a fading of color with time. Preferably the final solution should contain less than 0.3 gram of nitrate.

Table II. Interference of Iron

(Sample matched immediately)

| No. | Taken $WO_3$ | Taken Fe as $FeCl_3$ | Taken $MoO_3$ | $MoO_3$ Found | Error |
|---|---|---|---|---|---|
| | *Gram* | *Gram* | *Mg.* | *Mg.* | *Mg.* |
| 1 | 0.3 | 0.02 | 0.03 | 0.08 | +0.05 |
| 2 | 0.3 | 0.07 | 0.03 | 0.09 | +0.06 |
| 3 | 0.3 | 0.02 | 0.10 | 0.19 | +0.09 |
| 4 | 0.3 | 0.07 | 0.10 | 0.20 | +0.10 |
| 5 | 0.3 | 0.02 | 0.30 | 0.52 | +0.22 |
| 6 | 0.3 | 0.03 | 0.40 | 0.65 | +0.25 |
| 7 | 0.3 | 0.05 | 0.40 | 0.65 | +0.25 |
| 8 | 0.1 | 0.05 | 0.40 | 0.63 | +0.23 |
| 9 | 0.1 | 0.10 | 0.40 | 0.64 | +0.24 |
| 10 | 0.3 | 0.0001 | 0.50 | 0.55 | +0.05 |
| 11 | 0.3 | 0.0002 | 0.50 | 0.60 | +0.10 |
| 12 | 0.3 | 0.0010 | 0.50 | 0.75 | +0.25 |

Table III. Interference of Iron with Time

| No. | Taken $WO_3$ | Taken Fe | Taken $MoO_3$ | $MoO_3$ Found: Matched immediately | $MoO_3$ Found: Matched after 15 minutes |
|---|---|---|---|---|---|
| | *Gram* | *Gram* | *Mg.* | *Mg.* | *Mg.* |
| 1 | 0.3 | 0.07 | 0.30 | 0.55 | 0.65 |
| 2 | 0.3 | 0.07 | 0.40 | 0.68 | 0.89 |
| 3 | 0.3 | 0.07 | 0.50 | 0.77 | 1.10 |

## Determination of Molybdenum in Synthetic Scheelite

K. J. Murata of this laboratory prepared pure synthetic members of the scheelite-powellite mineral series by the method of Michel (4), and these were analyzed for molybdenum. The ground samples were attacked by 10 ml. of hydrochloric acid and 2 drops of nitric acid, evaporated to dryness, and taken up with 1.5 grams of ammonium citrate and 4 drops of ammonium hydroxide. The color was then developed and compared.

The results, as indicated in Table IV, showed good agreement with the synthetics.

Table IV. Determination of Molybdenum in Synthetic Scheelite

| Preparation No. | $MoO_3$ in Synthetic Scheelite | $MoO_3$ Found |
|---|---|---|
| | % | % |
| 4 | 0.07 | 0.07 |
| 26 | 0.22 | 0.23 |
| 28 | 0.25 | 0.24 |
| 6 | 0.29 | 0.31 |
| 27 | 0.36 | 0.34 |
| 29 | 0.36 | 0.34 |
| 7 | 0.51 | 0.50 |
| 31 | 0.72 | 0.68 |
| 5 | 0.79 | 0.74 |

## Acknowledgment

The authors wish to thank K. J. Murata for kindly supplying samples of synthetic scheelite of known molybdenum content.

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# Quantitative Estimation of Acetyl in Carbohydrate Acetates

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METHODS for the quantitative determination of carbohydrate acetyl have been developed principally along two separate lines: (1) for the simple sugar acetates which require the use of mild conditions to prevent errors which might arise from the destruction of the labile carbohydrate molecules; (2) for cellulose acetates which, because of the heterogeneous reaction involved, require somewhat more drastic conditions or considerably extended reaction time. Separate, essentially noninterchangeable methods were, therefore, evolved for the two classes of carbohydrate derivatives. Adaptations of the aforementioned methods have been applied to starch acetates, but none appears to have received general acceptance.

This paper describes a quantitative method of acetyl analysis which is applicable not only to the polysaccharide acetates of starch and cellulose but, on slight modification, to the simple sugar acetates as well. The method employs the sodium methoxide catalyst of Zemplén (*7*) to transesterify carbohydrate acetyl in methanol with the production of methyl acetate which is then quantitatively removed and determined by a modification of the well-known Freudenberg (*2*) procedure. Starch and cellulose do not yield volatile acidic decomposition products on contact with alkaline catalyst during the separation of the methyl acetate by distillation. The lower sugars, being labile, are decomposed on boiling in the presence of the catalyst. Therefore, in these cases, the transesterification is accomplished at room temperature, and the catalyst is neutralized by the addition of a methanol solution of sulfuric acid before proceeding with the distillation.

The transesterification is rapid, requiring only 15 minutes at room temperature, for acetates of the simple carbohydrates. Even in the case of polysaccharide acetates, which remain insoluble, the reaction is completed in about 25 minutes. In certain instances with dense or somewhat horny acetates the reaction proceeds slowly. In these cases it is expedient first to swell the acetate by heating in methanol for 0.5 to 1 hour before proceeding with the analysis.

The apparatus employed for the analysis is a modification of the Freudenberg (*2*) apparatus as shown in Figure 1.

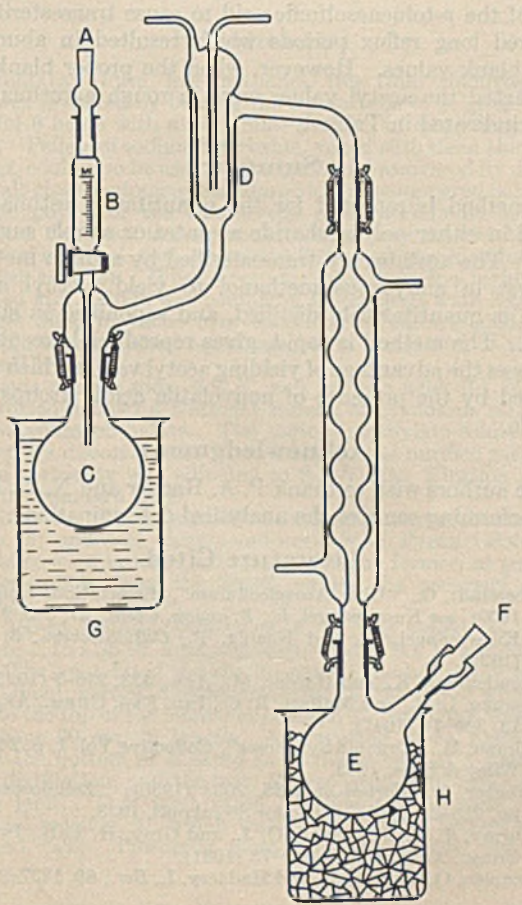


FIGURE 1. APPARATUS

## Analytical Procedure

REAGENTS. Anhydrous methanol was prepared by drying methanol with magnesium methoxide (*4*) and distilling through a fractionating column.

Sodium methoxide was prepared by dissolving clean sodium in anhydrous methanol. The sodium was cut under xylene to expose fresh surfaces and weighed. It was then rapidly passed through three separate portions of anhydrous methanol contained in beakers and finally dissolved in anhydrous methanol in a volumetric flask and made up to volume. Approximately 0.2 *N* solutions were employed.

An approximately 0.2 *N* solution of sulfuric acid in methanol was prepared by adding a calculated amount of concentrated sulfuric acid to anhydrous methanol and allowing the mixture to stand several days to permit formation of monomethyl sulfate. The solution was then standardized and was found to maintain its normality for a considerable time.

PROCEDURE FOR POLYSACCHARIDE ACETATES. A 0.2- to 0.3-gram sample of finely divided polysaccharide acetate, previously dried in vacuo over phosphorus pentoxide, is placed in 20 ml. of anhydrous methanol in the reaction flask (*C*, Figure 1), containing a boiling tube to prevent bumping. Approximately 5 to 10 ml. of 0.2 *N* sodium methoxide (3 to 8 milliequivalents per gram of sample) in anhydrous methanol are added through the dropping funnel, *B*, which is protected by a small drying tube, *A*. The temperature of the water bath, *G*, is raised until slow distillation into the receiving flask, *E*, occurs, the rate of distillation being approximately 35 drops per minute. During the distillation, the receiving flask is cooled by immersion in crushed ice. The receiving flask may be protected by a small tube, *F*, filled with soda lime. When the liquid in the distillation flask has been concentrated to a small volume (approximately 5 ml.) a fresh 20-ml. portion of anhydrous methanol is admitted through the dropping funnel and the solution is again distilled to a small volume. Two 10-ml. portions of anhydrous methanol are then added successively and the solution is concentrated to a small volume after the addition of each portion. During the concentration the water level in the water bath is maintained just below the level of liquid in the distillation flask in order to prevent baking of material on the flask walls.

After the final concentration, the distillation flask is removed or the stopcock on dropping tube *B* is opened, and 25 ml. of 0.2 *N* sodium hydroxide are pipetted into the receiving flask by way of the side neck and the neck closed by a glass stopper. The bath, *H*, of crushed ice is removed and a hot water bath substituted. The contents of the receiving flask are refluxed 15 minutes, the flask is cooled, 75 ml. of boiled distilled water are added, and the excess sodium hydroxide is titrated with 0.1 *N* hydrochloric acid to a phenolphthalein end point. The base consumed in the saponification, minus that consumed in a separate blank determination, is equivalent to the acetyl content of the carbohydrate acetate. Blank determinations are made by refluxing 50 ml. of

methanol 15 minutes with 25 ml. of 0.2 *N* sodium hydroxide and titrating as described above.

It is not necessary that the catalyst remain in the reaction mixture throughout the distillation. After the first concentration of the liquid in the distillation flask the catalyst may be neutralized or the solution slightly acidified by addition of sulfuric acid dissolved in methanol, and the remainder of the distillation carried out as described. This procedure causes no change in the observed acetyl value. Samples not in a state of fine subdivision are refluxed with methanol for 0.5 to 1 hour before introducing the catalyst and proceeding with the analysis. For the reflux, water is circulated through condenser *D* but at all other times this condenser remains empty.

Procedure for Simple Sugar Acetates. A 0.20- to 0.25-gram sample of sugar acetate is added to the reaction flask and dissolved in 20 ml. of anhydrous methanol by gentle heating. When complete solution has occurred the mixture is cooled to room temperature, and 5 to 10 ml. of 0.2 *N* sodium methoxide in anhydrous methanol are added and well mixed with the solution. After 15 minutes, slightly more of a solution of sulfuric acid in methanol is added than is needed to neutralize the catalyst. The methyl acetate is then quantitatively distilled and determined as described in the procedure for polysaccharide acetates. The sample may be left in contact with the catalyst for periods longer than 15 minutes without affecting the results. The equilibrium effected by the catalyst apparently produces methyl acetate to the almost complete exclusion of carbohydrate acetate.

## Discussion

Since the presence of sodium hydroxide in the solution would tend to prevent quantitative transesterification and distillation of the methyl acetate, it is important that water be carefully excluded from the reaction flask and that dry samples and anhydrous reagents be employed.

The amount of catalyst employed need not be precisely controlled. A several-fold variation in the ratio of catalyst to sample may be used without detriment to the analytical results. In general, 3 to 8 milliequivalents of catalyst per gram of sample were employed. Barium methoxide gives good results with the simple sugar acetates, but is not so effective with the polysaccharide acetates as is sodium methoxide.

Methanol is used in preference to ethanol because of the more favorable distillation of methyl acetate from a solution in methanol as compared to ethyl acetate from ethanol.

Results are reproducible to about ±0.1 per cent acetyl—for example, the value reported in Table I for cornstarch acetate I represents the mean value of 18 determinations all values of which were within ±0.2 per cent of the mean value and 15 results were within ±0.1 per cent. The accuracy of the results is good in the case of the lower sugar acetates. Since no method exists for definitely establishing the acetyl content of starch and cellulose acetates, the accuracy of the results in the case of these polysaccharides cannot be precisely determined. For this reason it was of interest to compare the values obtained for the polysaccharide acetates with values obtained by the use of a previously widely used procedure. The methods in the literature, applying principally to the determination of acetyl or combined acetic acid in cellulose acetates, have been comprehensively reviewed and criticized by several workers (*3*, *5*, *6*). The method recommended (*3*, *6*) as giving the most reliable results is a modification of the method of Eberstadt (*1*), which consists in saponification of the acetate in standard alkali and subsequent titration of the excess alkali with acid.

Values obtained from quadruplicate determinations by this method were compared with values resulting from the sodium methoxide procedure. In general, the results obtained by the two methods were in good agreement (Table I). In several cases, however, the modified Eberstadt method gave acetyl percentages 0.2 to 0.4 higher than the values obtained by the sodium methoxide procedure. In these instances, a blank run on the unacetylated starches showed that the samples possessed an acidity equivalent to 0.2 to 0.4 per cent acetyl. Acidic groups may result from the presence of fats, phosphoric acid residues, or carboxyl groups produced by partial oxidation of the carbohydrate. Correction for these acidities brought the results into agreement with those of the sodium methoxide procedure. Acetyl values which were thus corrected are indicated in Table I. Since cornstarch acetate III was a commercial product and none of the unacetylated starch was available, correction of the acetyl value for acidic contaminants could not readily be made. This acetate was impure, as indicated by its brown color. In contrast to the Eberstadt method, the sodium methoxide procedure is not affected by the presence of nonvolatile acidic contaminants such as fats and phosphoric acid residues or by the existence of free carboxyl groups resulting from oxidation of the polysaccharide.

Table I. Analysis of Carbohydrate Acetates [a]

| Substance | Per Cent Acetyl: By sodium methoxide | By Eberstadt method | Theoretical |
|---|---|---|---|
| Cornstarch acetate I | 44.36 | 44.48 [b] | 44.78 [c] |
| Cornstarch acetate II | 44.17 | 44.25 [d] | ... |
| Cornstarch acetate III [e] | 36.94 | 37.64 | ... |
| White potato starch acetate | 44.15 | 44.20 [d] | ... |
| Waxy cornstarch acetate | 43.90 | 44.04 [b] | ... |
| Wheat starch acetate | 44.31 | 44.10 [d] | ... |
| Cellulose acetate I [e] | 40.51 | 40.21 | ... |
| Cellulose acetate II [e] | 44.20 | 43.97 | ... |
| Glucose penta-acetate | 55.23 | ... | 55.13 |
| Mannose penta-acetate | 55.19 | ... | 55.13 |
| Maltose octa-acetate | 50.79 | ... | 50.74 |
| Gentiobiose octa-acetate | 50.77 | ... | 50.74 |

[a] All values are average of at least triplicate determinations.
[b] No acidity found present in unacetylated starch.
[c] Calculated for $[C_6H_7O_2(CH_3CO)_3]$.
[d] Corrected for acidity in unacetylated starch.
[e] Commercial preparations.

Attempts to adapt the Freudenberg procedure (*2*) to polysaccharide acetates were not entirely successful. The slowness of the *p*-toluenesulfonic acid to cause transesterification required long reflux periods which resulted in abnormally large blank values. However, when the proper blanks were subtracted the acetyl values were in rough agreement with those indicated in Table I.

## Summary

A method is reported for the quantitative estimation of acetyl in either polysaccharide acetates or simple sugar acetates. The acetates are transesterified by sodium methoxide catalyst in anhydrous methanol to yield methyl acetate, which is quantitatively distilled, and saponified in standard alkali. The method is rapid, gives reproducible results, and possesses the advantage of yielding acetyl values which are not affected by the presence of nonvolatile acidic groups in the sample.

## Acknowledgment

The authors wish to thank P. A. Haeder and N. H. Koenig for performing some of the analytical determinations.

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# Estimation of Volatile Acyl Groups in Cellulose Esters

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GENUNG and Mallatt (*4*) not only reviewed the literature of this important estimation but also submitted the principal method, that of Ost, to a critical experimental study. In this method, the cellulose ester is completely dissolved in cold, aqueous sulfuric acid (1 to 1), the solution containing the liberated organic acid, or acids, is distilled with steam, and the acidity of the distillate is found by titration.

During the past five years (*1*), several operators in this laboratory have obtained reliable and concordant analyses for the volatile acyl content of various cellulose esters by the following procedure, which is based on the work of Zemplén (*9*), Perkin (*7*), Freudenberg and Harder (*2*), and particularly Phillips (*8*). The acyl groups are recovered as methyl esters by a transesterification in methanol, with sodium methylate followed by excess *p*-toluenesulfonic acid as catalysts. Since the lower methyl esters are more volatile in methanol than the free acids are in steam, a short distillation, instead of the rather tedious one characteristic of the Ost process, transfers the esters to the distillate. This distillate is trapped in an excess of standard aqueous alkali. Saponification, removal of excess methanol by distillation, and titration of the residual aqueous solution complete the estimation.

## Experimental

MATERIALS. Pure, dry methanol, free from aldehydes and acids, was prepared by heating the commercial C. P. grade under reflux for 6 hours with a few grams per liter of clean magnesium ribbon. Pellets of sodium hydroxide, mixed with clean aluminum turnings, could also be used. The methanol recovered by distillation in all-glass equipment was stored in glass-stoppered bottles.

If the purity of the commercial *p*-toluenesulfonic acid hydrate was doubted, the aqueous solution was filtered through absorbent charcoal and steam-distilled until the distillate was no longer acid (*8*). When crystallization from the highly concentrated still residue was complete, the crystals were recovered and were dried in vacuo over concentrated sulfuric acid. The stock solution contained equal weights of water and of the sulfonic acid hydrate.

Aqueous caustic soda, 0.2 *N*, and sulfuric acid, 0.1 *N*, were standardized against a National Bureau of Standards sample of acid potassium phthalate. The sodium methylate solution was prepared by dissolving the clean metal in the purified methanol and the normality was adjusted to 0.1 *N* after titrating an aliquot with standard acid.

APPARATUS. The apparatus was a modification of that used by Phillips (*8*) and was constructed entirely of Pyrex. Soft glass and rubber were avoided lest alkali from the former, or traces of hydrogen sulfide from the latter, reduce the accuracy of the estimation.

The 250-cc. round-bottomed flask, *A*, was sealed, as shown in Figure 1, to a 35-cc. dropping funnel and a No. 11 ground-glass joint. The joint fitted the similar one on the bottom of the 40-cm. (16-inch), vertical Liebig condenser, *B*, the top of which was sealed to the top of the similar condenser, *E*, through a horizontal tube about 20 cm. in length. An inverted 25-cc. Erlenmeyer flask at the bottom of *E* acted as a trap in case of a suck-back during distillation, and the exit, *D*, was sealed through a No. 20 ground joint. This joint fitted the one on the neck of the ellipsoidal receiver, *C*, which was blown from a 500-cc. round-bottomed flask. Powdered glass was fused inside the lower part of this receiver to promote uniform boiling during distillations. When the receiver was attached to the exit tube, *D*, in the position shown, the end of the tube remained above the level of the liquid in *C*. Rotation of the latter through 180° about the glass joint caused the end of the exit tube to become immersed.

The equipment was not particularly fragile when kept clamped to its vertical iron stand and accidental breakages were very few.

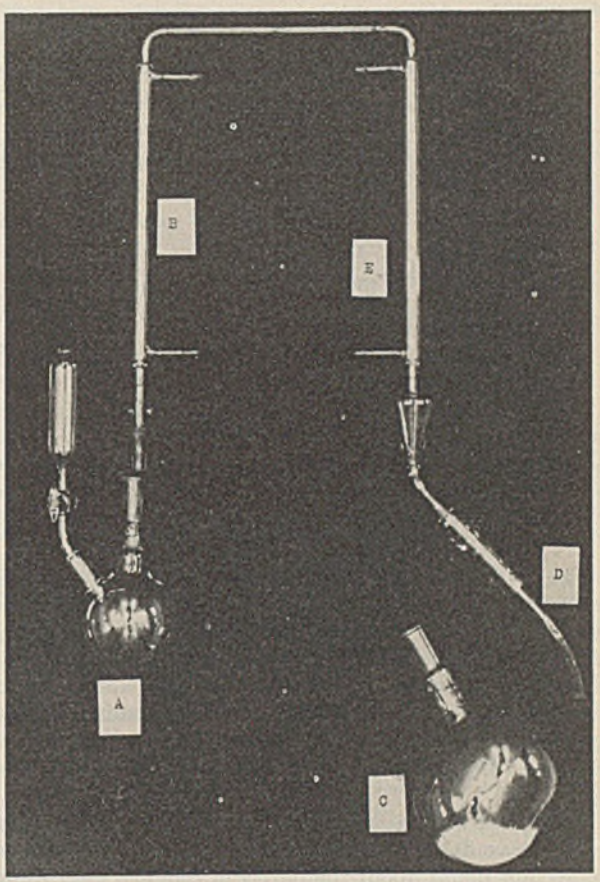


FIGURE 1. APPARATUS

ANALYSIS. After the cellulose ester, which had to be finely divided, had been thoroughly dried at 60° C. over phosphorus pentoxide in vacuo, a weighed sample, 0.3 to 0.4 gram, was put into flask *A*, together with 50 cc. of methanol, 10 cc. of the sodium methylate solution, and two or three glass beads. The flask was held in position on condenser *B* by means of rubber bands and the contents were kept in gentle ebullition for 3 hours by heat from a small flame. Cooling water was meanwhile circulated in condenser *B*. A cellulose acetate with which this preliminary transesterification was omitted analyzed 24.6 instead of 39.2 per cent acetyl.

After 3 hours, flask *C*, containing an accurately measured volume, 25 cc., of the caustic soda solution, was very loosely attached to *D*, so that the exit tube dipped below the surface of the liquid. The lower part of the flask was immersed in an ice bath and cold water was circulated in condenser *E*. Condenser *B*

[1] Present address, Du Pont Rayon Company, Buffalo, N. Y.
[2] Present address, Tennessee Eastman Company, Kingsport, Tenn.

was emptied. A large excess, 3 cc., of the *p*-toluenesulfonic acid solution was added through the dropping funnel attached to *A* and the mixture of methanol and methyl ester was slowly distilled into *C*. When the still residue had been reduced to about 15 cc., an additional 80 cc. of methanol was added dropwise from the funnel at such a rate that the volume in the still remained at 20 to 25 cc. The stopcock on the dropping funnel was then left open and receiver *C* was firmly attached by rubber bands to *D*, so that the exit tube remained above the surface of the liquid. After the ice bath was replaced by a small flame under an asbestos sheet, the methyl esters in *C* were saponified by 1 hour of gentle ebullition. A low result was obtained with ethyl acetate when this time was reduced by one half.

Condenser *E* was then allowed to empty, cooling water was circulated in *B*, and the asbestos sheet over the flame was removed. These changes caused the excess methanol to distill from *C* and thereby made it possible to obtain a sharp end point, instead of a difficult one, when the residual aqueous liquor was titrated with the standard acid in presence of phenolphthalein. The sulfuric acid and the caustic soda were frequently standardized against each other and not more than 0.2 cc. of the alkali was utilized in blank experiments with the reagents. The difference between these blanks and the milliequivalents of alkali remaining in an actual experiment was accepted as equivalent to the amount of volatile acyl contained in the cellulose ester sample.

Table I. Acyl Analyses of Cellulose Mixed Esters[a]

| Present Data | | Eastman Kodak Company Data | | | |
|---|---|---|---|---|---|
| Apparent acetyl % | Mean % | Apparent acetyl % | Acetyl % | Propionyl % | Butyryl % |
| 40.48 | 40.35 | 40.2 | 29.3 | 13.9 | 0.6 |
| 40.21 | | | | | |
| 42.87 | 42.76 | 42.6 | 30.2 | 16.5 | .. |
| 42.65 | | | | | |
| 30.86 | 30.74 | 30.7 | 19.8 | 14.6 | .. |
| 30.62 | | | | | |
| 40.37 | 40.28 | 40.1 | 29.5 | .. | 17.5 |
| 40.18 | | | | | |
| 36.35 | 36.37 | 36.3 | 21.2 | .. | 24.9 |
| 36.39 | | | | | |
| 32.80 | 32.80 | 32.7 | 2.6 | .. | 49.9 |
| 32.80 | | | | | |

[a] Eastman Company sample numbers were from 102,429 to 102,434 in that order.

## Results and Discussion

The determination in the form described above has been carried out more than 60 times with various cellulose esters. Very few of the duplicate estimations differed from the average acyl content by more than ±0.15 per cent; the great majority agreed to within 0.1 per cent and a considerable number to within 0.05 per cent. The dominant cause of deviation between duplicates was probably connected with the final titration, in which a buret reading to 0.1 cc. was used.

Pure ethyl acetate and pure, recrystallized sorbitol hexa-acetate gave data within 0.1 per cent of the calculated acetyl values, but doubt about the purity of cellulose ester samples made it more difficult to assess the accuracy of the method in these cases. The article by Gardner and Purves (*3*), however, contains examples in which the acetyl substitutions of a series of esters containing increasing amounts of the *p*-toluenesulfonyl radical were calculated from simultaneous equations involving both the acetyl and sulfur analyses. The acetyl substitutions found did not differ from their average value of 2.44 by more than ±0.03 in 21 out of 22 cases, and this error included those inherent in both the sulfur and the acetyl estimations.

Table I summarizes the results obtained with a series of cellulose acetate-propionates and acetate-butyrates kindly supplied, together with their analyses, by C. R. Fordyce, of the Eastman Kodak Company. Only three of the twelve independent estimations of "apparent acetyl" differed from the Eastman figures by more than 0.2 per cent and this divergence corresponded to a titration difference of about ±0.1 cc. in the experiments. The average values were about 0.1 per cent higher than the Eastman data, for reasons that are still obscure. These observations suggest that the present estimation is at least as accurate as the Ost technique, for which a precision of ±0.2 per cent is quoted (*4*), and may be applicable to cellulose butyrates, with which the Ost method often gives low results (*4*). The latter method also requires at least 7 hours of intermittent attention instead of the 5 or 6 hours demanded by the present one.

Although the procedure analyzed sorbitol hexa-acetate perfectly, it was not applicable to the acetates of reducing sugars because pure β-glucose penta-acetate analyzed 56.2, 59.4, 58.6, and 58.5 instead of the proper value of 55.2 per cent acetyl. The 6-chloro- or 6-iodo-6-desoxy-cellulose acetates also gave high acetyl data because traces of halogen-containing substances passed into the standard caustic soda solution. Corrections for this effect by estimating halogen in the titrated solution were not very successful, although proper allowance could be made in this way for samples containing 5 per cent of chlorine added as sodium chloride. A few analyses of cellulose acetate-nitrates also gave high results.

When both the "apparent acetyl" and the actual percentage of acyl groups in a mixed ester are known, an easy simultaneous equation makes it possible to calculate the individual amounts of the acyl substituents, provided only two are present. All the mixed esters in Table I except the first were accordingly deacylated by a modification of Zemplén's procedure (*9*) and the loss in weight of each sample was taken as equivalent to its original acyl content. Although the final data for acetyl, propionyl, and butyryl were always within 1 per cent of those in Table I, the calculation ignored the fact that the percentage of acyl found in this way differs from other acyl values in being based upon the equivalent weight of the radical *minus unity*. The agreement therefore owed much to the cancellation of errors and the method in its present state, although fairly rapid, is probably inferior in accuracy to those described by Malm and his collaborators (*5*, *6*).

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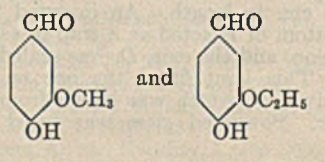
Contribution from the Research Laboratory of Organic Chemistry, Massachusetts Institute of Technology, No. 280.

Correction. In the article entitled "Detecting Adulteration of Ethylvanillin with Vanillin" [Ind. Eng. Chem., Anal. Ed., 15, 268 (1943)] the structures of vanillin and ethylvanillin were incorrectly given. They should have been printed as:




Howard Nechamkin

# Volumetric Determination of Bromide in Brines

HOBART H. WILLARD AND ARNO H. A. HEYN, University of Michigan, Ann Arbor, Mich.

CERTAIN difficulties are encountered in the determination of bromide in salt brines containing much calcium and magnesium by the method of van der Meulen (*3*).

This involves the oxidation of bromide to bromate by means of hypochlorite at 80° to 90° C. in a solution buffered with boric acid and borate. The excess of hypochlorite is destroyed by adding an excess of hydrogen peroxide and the hydrogen peroxide is removed by boiling in the presence of osmium tetroxide as catalyst. The bromate is then determined iodometrically. The use of formate for the removal of the excess hypochlorite is also suggested (*4*).

D'Ans and Höfer (*1*) pointed out that the reaction is quantitative only within a definite pH range, but gave no numerical values. They suggested the use of sodium dihydrogen phosphate as a buffer, but this precipitates calcium and therefore cannot be used.

Kolthoff and Yutzy (*2*) used essentially the same procedure, but unfortunately gave no data on the limits for the pH. They used an ammonium molybdate solution as catalyst to hasten the reaction between potassium iodide and bromate, a suggestion already made by van der Meulen (*4*).

The difficulty with all the methods lies in the uncertainty in maintaining the solution at the correct pH when the bromide is oxidized to bromate. Since a solution containing no precipitate is preferable, phosphate buffers were not used in this work. The solutions were adjusted to the correct pH, using acetic acid-sodium acetate buffers. Experiments showed that the presence of acetate does not interfere. To find the limits of pH for the quantitative oxidation of bromide to bromate, samples containing a known amount of bromide and 5 grams of reagent sodium chloride were subjected to the procedure of Kolthoff and Yutzy except that an acetic acid–sodium acetate buffer was used. The results are shown in Table I. The pH was measured with a Beckman pH meter.

It is evident that the oxidation of bromide to bromate with hypochlorite is quantitative between pH 5.5 and 7.0.

The procedure was tested by analyzing synthetic brines, which were prepared as follows: 2.5 grams of anhydrous calcium chloride, purified by recrystallization from methanol and subsequent drying at 130° C., were dissolved in 25 ml. of water and 5 grams of reagent grade sodium chloride were added, followed by a known amount of potassium bromide solution. After 10 ml. of the hypochlorite solution were added, the pH was adjusted to between 6.0 and 6.5. Since the presence of hypochlorite interferes with the use of common acidimetric indicators, a small amount of zinc acetate was added to the solution. After some zinc hydroxide was precipitated glacial acetic acid was added dropwise until all zinc hydroxide was just dissolved. At this point the pH was between 6.3 and 6.8.

The solution was acidified with hydrochloric instead of sulfuric acid because of the presence of calcium. The results obtained are shown in Table II.

TABLE I. INFLUENCE OF pH ON OXIDATION OF BROMIDE TO BROMATE WITH HYPOCHLORITE

(Each sample contained 2.911 mg. of bromine as potassium bromide and 5 grams of reagent sodium chloride.)

| pH | Bromide Found | Error | Average Error |
|---|---|---|---|
| | *Mg.* | *Mg.* | *Mg.* |
| 5.0 | 2.84 | −0.07 | 0.06 |
| | 2.88 | −0.03 | |
| | 2.83 | −0.08 | |
| 5.5 | 2.902 | −0.009 | 0.012 |
| | 2.902 | −0.009 | |
| | 2.928 | +0.017 | |
| 6.0 | 2.911 | 0.000 | 0.002 |
| | 2.911 | 0.000 | |
| | 2.904 | −0.007 | |
| 6.5 | 2.898 | −0.013 | 0.008 |
| | 2.907 | −0.004 | |
| | 2.904 | −0.007 | |
| 7.0 | 2.902 | −0.009 | 0.009 |
| | 2.906 | −0.005 | |
| | 2.924 | +0.013 | |
| 7.5 | 2.884 | −0.027 | 0.028 |
| | 2.883 | −0.028 | |
| | 2.882 | −0.029 | |
| 8.0 | 2.68 | −0.23 | 0.158 |
| | 2.82 | −0.09 | |
| | 2.76 | −0.15 | |
| 8.5 | 2.72 | −0.19 | 0.179 |
| | 2.72 | −0.19 | |
| | 2.76 | −0.15 | |
| 9.0 | Excess hypochlorite did not react with formate. | | |

TABLE II. DETERMINATION OF BROMIDE IN SYNTHETIC AND NATURAL BRINES

(Artificial brine contained 2.5 grams of anhydrous calcium chloride, 5 grams of sodium chloride, and 2.911 mg. of bromine as potassium bromide)

| | Bromide Found | | Error |
|---|---|---|---|
| | *Mg.* | *Mg./l.* | *Mg.* |
| Artificial brine | | | |
| pH 6.25, glass electrode used | 2.906 | ... | −0.005 |
| | 2.907 | ... | −0.004 |
| pH 6.3 to 6.8, zinc acetate used | 2.891 | ... | −0.020 |
| | 2.914 | ... | +0.003 |
| | 2.914 | ... | +0.003 |
| Natural Michigan salt brines containing 175,000 p. p. m. of chloride, 10.00-ml. samples | | | |
| Brine A, pH 6.25 | 6.080 | 608.0 | ... |
| | 6.081 | 608.1 | ... |
| Brine B, pH 6.25 | 7.486 | 748.6 | ... |
| | 7.475 | 747.5 | ... |

## Special Solutions

Sodium Hypochlorite Solution. Dissolve 7.0 grams of reagent sodium hydroxide in 150 ml. of water, cool to room temperature, saturate with chlorine gas, and after the solution is distinctly yellowish pass air through for 10 minutes. Add 1.8 grams of reagent sodium hydroxide and dilute to 200 ml.

This solution contains enough free sodium hydroxide to be stable for a few days if kept cold. Sodium hypochlorite solutions with a pH below 12 are too unstable.

Sodium Formate Solution. 25 grams of C. P. sodium formate dissolved and diluted to 50 ml.

## Procedure

Dilute or concentrate a sample containing no more than 13 mg. of bromide to 25 to 50 ml. in a 400-ml. beaker. If the solution is distinctly acid or alkaline, neutralize with acetic acid or sodium hydroxide solution. If there is not an excess of chloride in the sample, add 5 grams of reagent grade sodium chloride. After all solids have dissolved add 5 to 10 ml. of the sodium hypochlorite solution, depending on the amount of bromide present. Adjust the pH to between 6.0 and 6.5, using a glass electrode, by adding glacial acetic acid and, if necessary, sodium hydroxide solution. As alternative add a solution of 0.1 gram of zinc acetate in 5 ml. of water containing a few drops of acetic acid, and after obtaining a precipitate of zinc hydroxide add just enough glacial acetic acid dropwise to dissolve all zinc hydroxide. On swirling no zinc hydroxide should remain on the sides of the beaker.

Rinse down the sides of the beaker with water, heat over a small flame, and bring the solution to boiling in 5 to 10 minutes, then add 5 ml. of the sodium formate solution or 2.5 grams of solid C. P. sodium formate. This should cause an effervescence of carbon dioxide, indicating that a sufficient excess of the sodium hypochlorite was used. Rinse down the sides of the beaker and boil for a few seconds. Cool to room temperature, dilute to 150 to 200 ml., and add 5 ml. of a 20 per cent potassium iodide solution or 1 gram of solid potassium iodide. If free iodine is liberated at this point the sample must be discarded. Add 8 ml. of concentrated hydrochloric acid diluted to 30 ml. and 1 or 2 drops of 0.25 *M* ammonium molybdate solution. Titrate imme-

diately with a 0.01 *N* sodium thiosulfate solution, adding a little starch solution just before the end point, until the disappearance of the blue color.

Run a blank in the same manner, using the same amounts of reagents. The blank usually requires between 0.2 and 0.8 ml. of 0.01 *N* sodium thiosulfate solution.

One milliliter of 0.01 *N* thiosulfate solution corresponds to 0.00013319 gram of bromide.

The thiosulfate is conveniently standardized against analytical reagent potassium iodate.

## Summary

An improved method of determining bromide in brines is described. The bromide is oxidized to bromate with hypochlorite at a pH of 5.5 to 7.0 which is adjusted by using zinc hydroxide as indicator or by using a glass electrode. After the removal of excess hypochlorite by formate the bromate is determined iodometrically. This method is rapid and is suitable for amounts of bromide up to about 13 mg. with an accuracy of about 2.5 parts in 1000.

Iodide and all oxidizing reagents that will liberate iodine from iodide in an aqueous solution interfere.

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# Determination of Water in Aniline by a Cloud Point Method

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A METHOD has been reported for determining water in aniline by means of the cloud point of a mixture of the aniline with rapeseed oil (*2*, *3*). Because rapeseed oil is imported and its sale is subject to governmental priorities, it seemed desirable to attempt to substitute an oil more easily available to American chemists, so that the use of the method (which is a good one) in the wartime production of aniline would not be hindered. It was found that a mixture of cottonseed oil and heavy mineral oil could be used satisfactorily instead of rapeseed oil, with some points of advantage for the mixed oil over the rapeseed oil. The method using mixed oil has a standard deviation from the true value for a single determination of ±0.006 per cent of water up to about 1.5 per cent of water, and ±0.009 per cent from 1.5 to 4.2 per cent of water. The standard deviation using rapeseed oil was found to be ±0.015 per cent of water up to a water content of 2 per cent.

## Reagents

COTTONSEED OIL. The commercial "cooking oil" first used had a specific gravity (25° C./25° C.) of 0.9172 and a refractive index ($n_D^{20}$ ° C.) of 1.47324, and the insoluble fatty acids obtained upon saponification had a freezing point of 31.9° C. The oil gave a positive response to the Halphen test for cottonseed oil (red coloration upon heating in carbon disulfide with amyl alcohol and sulfur). Wesson oil, a brand of cottonseed oil, which was also used, had a specific gravity (25° C./25° C.) of 0.9171.

MINERAL OIL. The mineral oil first used was a heavy white oil that had a specific gravity (25° C./25° C.) of 0.8756 and a refractive index ($n_D^{20}$ ° C.) of 1.47916. Nujol, a brand of medicinal mineral oil, which was also used, had a specific gravity (25° C./25° C.) of 0.8757.

MIXED OIL. Five parts by volume of cottonseed oil and one part of mineral oil were used with the slight adjustment described below.

## Apparatus and Procedure

All apparatus must be dried by heating and cooling in a desiccator, by a stream of dried air, or, for the aniline pipet, by washing several times with the aniline sample to be analyzed.

With a 5-ml. Mohr pipet, transfer 3.5 ml. of the mixed oil to a test tube (2.5 × 15 cm., 1 by 6 inches) and then add (25-ml. Mohr pipet) 20 ml. of the aniline. Insert into the test tube a two-hole cork stopper containing in one hole a thermometer and in the other a stirrer with a loop to encircle the thermometer. (Any efficient stirrer can be used.) The thermometer may be of any type, but it should be calibrated in tenths of a degree centigrade and the temperature should be capable of being estimated to at least 0.05° C. There should be about 5-mm. clearance between the bottom of the test tube and the bottom of the thermometer bulb. When 20° range thermometers are used, two are necessary, one covering the range 30° to 50° C. for water up to about 2.5 per cent, and the other 40° to 60° C. for water from 2.5 to about 4.5 per cent.

With constant stirring of its contents, the tube is dipped into a warm water bath until the opaque emulsion just changes to a slightly turbid liquid. It is immediately removed from the bath in order to avoid too high a rise in temperature, and its contents, which are now clear, are stirred with long even strokes (about 50 to 80 per minute) until the cloud point is reached. This is recorded to the nearest 0.05° C. About 0.3° to 0.4° C. above the cloud point, a slight turbidity develops, which increases gradually upon cooling, giving an indication of the approach of the cloud point. Then suddenly, within a temperature drop of less than 0.05° C., the mixture becomes milky and practically opaque. This constitutes the cloud point.

The percentage of water may be calculated from the linear equation:

$$\text{Per cent water by weight} = \frac{\text{cloud point found (° C. corr.)} - \text{cloud point of dry aniline (° C. corr.)}}{\text{mean variation of cloud point for 1 per cent variation in water content}}$$

Substituting the proper constants, for cloud point values up to about 42.60° C. (1.15 per cent water) the formula becomes:

$$\text{Per cent water by weight} = \frac{\text{cloud point found (° C. corr.)} - 36.80}{5.043}$$

For values of the cloud point from 42.60° to 58.75° C. (4.2 per cent water) the value of the denominator changes and the equation becomes:

$$\text{Per cent water by weight} = \frac{42.60 - 36.80}{5.043} + \frac{\text{cloud point found (° C. corr.)} - 42.60}{5.263} =$$

$$1.15 + \frac{\text{cloud point found (° C. corr.)} - 42.60}{5.263}$$

## Duplication of Mixed Oils

When a fresh batch of mixed oil is made up, it can be adjusted by adding cottonseed or mineral oil, so that dry aniline will have a cloud point of 36.80° C. within the normal variation. The formulas could then be used without change, but it is advisable to check them with a few samples of aniline of known water content. About 7 or 8 ml. of mineral oil added to 1 liter of 5 to 1 mixed oil raise the cloud point by about 1°, whereas about 40 ml. of cottonseed oil lower the point by the same amount. It is thus easier to adjust finally by adding cottonseed oil rather than mineral oil.

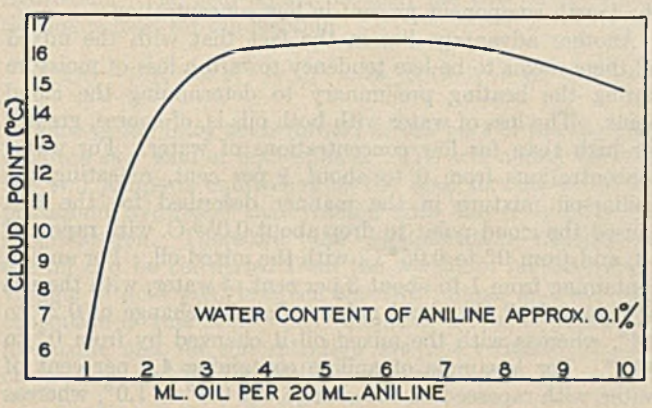


FIGURE 1. VARIATION OF CLOUD POINT WITH CHANGING RATIO OF COTTONSEED OIL TO ANILINE

In this way it is possible to adjust the proportions of cottonseed and mineral oils of different origin so that the given formulas may be used, but this adjustment must be made by trial and error. Merely to mix cottonseed and mineral oils in the same proportions will not necessarily give an oil which will fit the formulas. For instance, before any adjustment, two samples of mixed oil (made from cottonseed and mineral oils from different sources, but mixed in the same proportions as exactly as possible) gave, with the same sample of aniline, cloud points of 39.70° and 36.90° C., respectively.

Fairly dry oils should be used, but small variations in their water content are of slight influence. If water is present, it will effect the initial cloud point of the mixed oil, but since the latter is finally adjusted to a definite value, the adjustment automatically corrects for the water present. This would obviously not be possible with a single oil such as rapeseed oil.

A mixed oil was prepared from Wesson oil and Nujol, in order to determine whether adjustments of a fresh batch of oil, made with different samples of cottonseed oil and mineral oil, to a 36.80° C. cloud point for dry aniline would maintain the correctness of the formulas. Ten known aniline solutions containing from 0.00 to 1.18 per cent by weight of water were examined. The values found by using the formulas agreed with the known values as closely as the known accuracy of the method would lead one to expect.

## Precision and Accuracy

The formulas given above are based upon a plot of a total of 51 values for cloud points obtained with aniline samples of known water content.

The precision of the values obtained is less for samples with more than 1.15 per cent of water than for those with less water. Using solutions of water in aniline ranging from 0.000 to 1.199 per cent of water, the cloud points deviated from those calculated from the formula by from +0.07° to −0.05° C. for 34 values. The standard deviation of a single value from the arithmetic mean was ±0.03° C. (equivalent to ±0.006 per cent of water). (The standard deviation is the square root of the arithmetic mean of the squares of the deviations of the individual values from their arithmetic mean. It represents the range above and below the arithmetic mean within which about 68 per cent of the determined single values will lie. By determining more than one value, the standard deviation of the mean will be that of a single value divided by the square root of the number of values used in obtaining the mean value.)

In the range 1.514 to 4.230 per cent of water, the cloud points deviated from those calculated from the formula by from +0.08° to −0.07° C. for 17 values. The standard deviation was ±0.046° C. (equivalent to ±0.009 per cent of water). It is obvious that the precision of the values should decrease with an increase in the difference between the cloud point and room temperature, as occurs with the higher values, because of more rapid cooling.

If the aniline used for determining the constants of the equations can be shown to be free of water in so far as this can be proved, considering the precision of the method, the precision will then be synonymous with the accuracy. Mere distillation in a well-dried system and collection of a middle cut of constant boiling point was unsatisfactory, yielding aniline which had 0.02 per cent of water. The aniline which was finally used was obtained by distilling 2.5 liters of aniline, collecting 21 cuts, and determining cloud points on alternate cuts. It was assumed that constancy of the cloud point would indicate a dry aniline. This assumption was based on the high sensitivity of the method to the presence of small amounts of water. (It might, of course, be sufficient to dry the aniline merely by an efficient desiccant.) After the first cut of 350 ml. (cloud point 37.30° C.) the cloud points of all the other cuts (1965 ml.) varied between 36.77° and 36.87° C. for ten values, with no trend toward lower cloud points in the later cuts. The mean value was 36.80° C. with a standard deviation of a single value from the mean of ±0.048° C. Since 36.80° C. is a mean of ten values, its standard deviation would be $\frac{\pm 0.048°\text{ C.}}{\sqrt{10}}$, or ±0.015° C., which is less than the limit of reading the thermometer. Consequently, the value taken for dry aniline is of sufficient precision to indicate that the method is as accurate as it is precise.

## Search for Most Suitable Oil

Before deciding upon a mixed cottonseed-mineral oil to replace rapeseed oil, other possibilities were considered. Mineral oil alone was not satisfactory. When as little as 1 ml. was used with 25 ml. of stock aniline containing no added water, the mixture remained cloudy even in a boiling water bath. Castor oil alone was too viscous for satisfactory use; besides, mixtures with the stock aniline containing 1 ml. of the oil to 25 ml. of aniline or equal proportions of oil and aniline did not become turbid even in an ice bath. A mixture of 5 ml. of castor oil and 1 ml. of mineral oil with 25 ml. of aniline gave a fairly reproducible cloud point at

about 53.5° C., but the point was not so sharp as with rapeseed oil and, in addition, oily droplets persisted even above the temperature at which the main turbidity had vanished. Additions of 1 and 2 per cent of water caused increases of about 4.5° and 7.5°, respectively, in the cloud point, but the oily droplets still persisted.

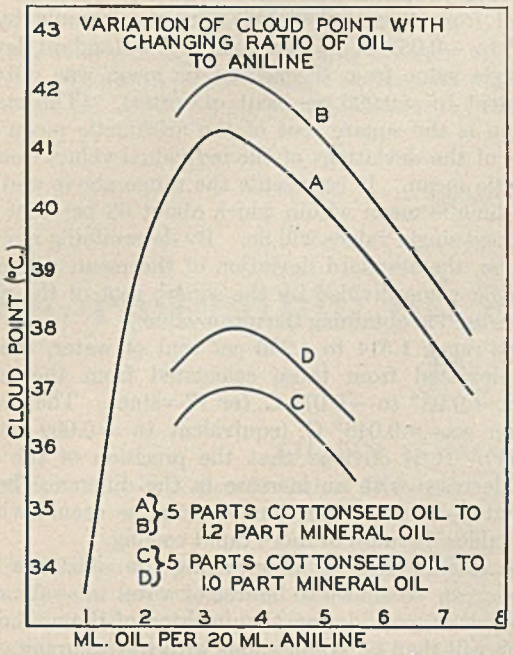


FIGURE 2. COTTONSEED-MINERAL OIL MIXTURES

Jansen and Schut (*3*) studied the cloud points of aniline containing water in solution, by means of rapeseed, soy, arachis, and maize oils added in the proportion of 1 part of oil to 4 parts of aniline (by weight). They reported cloud points for dry aniline with these oils as 37.6°, 8.7°, 26.2°, and 11° C., respectively.

Before fixing the proportions of mixed oil as 5 parts by volume of cottonseed oil to 1 part of mineral oil, the cloud points were determined with cottonseed oil alone added to aniline in various proportions. The oil was unsuitable in any proportion which was tried, because the cloud points obtained with it were not only too low for manipulative convenience, but could not be obtained with sufficient precision. Figure 1 illustrates the variations in cloud points of a stock sample of aniline with changes in the proportion of cottonseed oil used. Even the highest cloud point obtained is below the usual room temperature. The use of this oil would, therefore, necessitate a cooling bath that would have to be regulated to within a few degrees of the expected cloud point. Even then the precision was actually considerably poorer than with the mixed oil.

On the basis of the behavior of the aforementioned oils and with a consideration of their availability, mixtures of mineral oil and cottonseed oil were tried.

Figure 2 gives the variation in the cloud point with a changing ratio of mixed oil to aniline for a mixture of 5 parts (by volume) of cottonseed oil to 1.2 parts of mineral oil (curves A and B) and for another mixture of 5 parts of cottonseed oil to 1.0 part of mineral oil (curves C and D). A and C are for stock aniline, and B and D are for stock aniline with 0.2 per cent of added water. With both oil mixtures the cloud point reaches a maximum with 3.5 ml. of oil for 20 ml. of aniline, but with the 5 to 1.0 mixture the slope of the curve changes more slowly near the maximum than with the 5 to 1.2 mixtures. Because of this, the 5 to 1 mixture, when used in the ratio of 3.5 ml. of the mixture to 20 ml. of aniline, would suffer less change in cloud point because of small variations in the ratio of mixed oil to aniline, and so would be preferable to the 5 to 1.2 mixture.

## Comparison of Rapeseed Oil and Mixed Oil Procedures

The use of the mixed oil instead of rapeseed oil has several advantages.

With rapeseed oil the transition from a clear to a cloudy solution is abrupt, so that the analyst has no warning of the approach of the cloud point. With the mixed oil a region of about 0.3° to 0.5° C. occurs upon cooling, in which the mixture gradually assumes a turbid appearance, which is followed by the abrupt formation of a distinct opacity. The latter is taken as the cloud point and is reproducible. Dolique (*1*) has reported that a similar phenomenon takes place upon adding various substances to the phenol-water system when the added substances contain impurities. He considers this behavior to be characteristic of quaternary systems for low concentrations of the fourth component. In the analysis for water in aniline the water would serve as the fourth component present in low concentration.

Another advantage lies in the fact that with the mixed oil there seems to be less tendency toward a loss of moisture during the heating preliminary to determining the cloud point. The loss of water with both oils is, of course, greater for high than for low concentrations of water. For water concentrations from 0 to about 1 per cent, reheating the aniline-oil mixture in the manner described for the test caused the cloud point to drop about 0.05° C. with rapeseed oil, and from 0° to 0.05° C. with the mixed oil. For aniline containing from 1 to about 3 per cent of water, with the use of rapeseed oil the cloud point suffered a change of 0.2° to 0.4°, whereas with the mixed oil it changed by from 0° to 0.15°. For a sample of aniline containing 4.2 per cent of water, with rapeseed oil the change was 0.8° to 1.0°, whereas with the mixed oil the change was only 0.20° to 0.25°. This would indicate that the use of the mixed oil might offer less liability of error because of change in composition during the preparation of the mixture for the test.

Still another advantage of the mixed oil over the rapeseed oil is the possibility of adjusting any batch of oil to the value obtained with the previous batch, regardless of the differences in moisture content of the oil. With rapeseed oil, an entire new calibration might have to be made with every new batch of oil. For instance, the authors found when using a commercial rapeseed oil that the cloud points from 0 to 2 per cent of water were 35.90° to 47.75° C. corrected, respectively, about 1.4° C. lower than the corresponding values reported by Griswold (*2*). The slopes of the lines representing the two sets of data were, however, identical.

The mixed oil also appeared to have some advantage in precision. Nineteen cloud point values were obtained with rapeseed oil on solutions of water in aniline ranging from 0 to 2.2 per cent by weight. The values deviated from the best possible mean straight line by from +0.15° to −0.15° C. The standard deviation was ±0.087° C., equivalent to ±0.015 per cent of water. The standard deviation calculated from Griswold's data is ±0.027 per cent by weight of water for values up to about 2 per cent of water.

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# Determination of the Saponification Number of Fats and Oils

## Elimination of the Blank

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A "DOUBLE-indicator method" has been developed for determining the saponification number of fats and oils. For almost all oils this method gives excellent checks with the standard method and requires less time, reagents, and apparatus.

Figure 1 represents the potentiometric titration of the solution obtained by saponifying 5 grams of oil or fat with 50 ml. of alcoholic potassium hydroxide according to the standard procedure (*1*). The acid added up to the first point of inflection is used to neutralize the excess potassium hydroxide. The acid added between the two points of inflection is used for the reaction

$$RCOO^- + K^+ + H^+ + Cl^- \longrightarrow RCOOH + K^+ + Cl^-$$

Some of the fatty acids formed in this part of the titration separate as a solid or liquid phase. The acid added between the two jumps is equivalent to the soap formed or to the potassium hydroxide that reacted with the oil during the saponification. Therefore, the saponification number of the oil can be calculated from the weight of oil taken, the volume of acid used between the two jumps, and the concentration of this acid. The volume of alcoholic potassium hydroxide and its concentration are not required for the calculation, and the blank titration is eliminated.

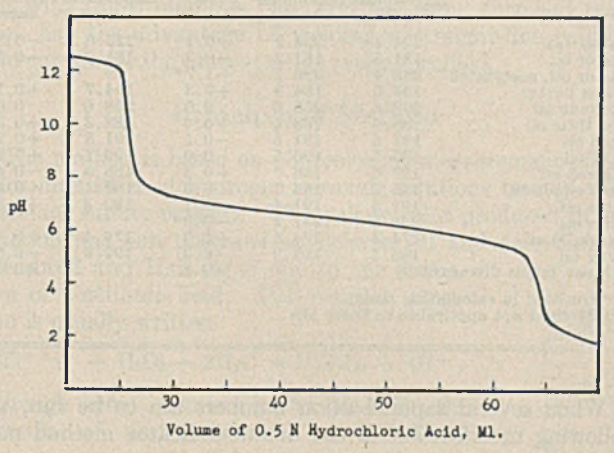


FIGURE 1. TITRATION GRAPH OF SAPONIFIED CORN OIL

However, the slope of the graph at the second equivalence point is ordinarily not great enough to permit an accurate determination of this point either with the potentiometer or with a suitable indicator. This slope is given by the equation (*2*, *3*)

$$\frac{d\text{pH}}{dv} \cong -0.22\,N\sqrt{\frac{1}{KLV}}$$

where $v$ is the buret reading, $N$ the normality of the hydrochloric acid, $K$ the ionization constant of the fatty acid, $V$ the total volume of the solution, and $L$ the number of millimoles of fatty acid in solution at the equivalence point. An accurate solution of this equation is not practical because the titration deals with a mixture of fatty acids rather than with a single acid and because the ionization constants of the acids in the mixture of alcohol and water are not known.

Nevertheless, the equation indicates that the slope can be increased (arithmetically) by decreasing the quantity of fatty acid dissolved in the alcohol-water mixture. This is readily accomplished by the addition of 10 ml. of benzene, which extracts most of the fatty acids from the aqueous layer. This removal of the fatty acid also raises the pH of the equivalence point. For example, in the titration of saponified corn oil without the addition of benzene, the second point of inflection occurred at a pH of 3.76 and had a slope of −2.1 pH units per ml.; in another titration performed by the same procedure except for the addition of 10 ml. of benzene, the pH at the second point of inflection was 4.31 and the slope was −6.9.

### Reagents

Alcoholic potassium hydroxide is prepared according to the standard procedure, the alcohol being previously distilled from a mixture of potassium hydroxide and aluminum (*1*). The solution is filtered to remove potassium carbonate. The storage bottle is fitted with a two-hole rubber stopper which carries an Ascarite tube and a siphon. Thus the solution is effectively protected against the absorption of carbon dioxide.

Bromophenol blue (0.010 *M*) is prepared by mixing 1.3 grams of the indicator with 2.0 millimoles of sodium hydroxide and diluting with water to 200 ml.

### Procedures

POTENTIOMETRIC METHOD. Five grams of oil are saponified according to the standard method (*1*), except that the volume of alcoholic potassium hydroxide is measured only roughly and a U-shaped Ascarite tube is attached to the upper end of the reflux condenser. This tube contains Ascarite in only one side, and the other side (inverted) is attached to the reflux condenser. Thus there is no possibility that any Ascarite will contaminate the solution.

After saponification, the solution is cooled to room temperature, or slightly above room temperature if necessary to prevent the crystallization of the soap. A cork stopper fitted with glass and calomel electrodes is placed in the neck of the flask. The solution is titrated with standard 0.5 *N* hydrochloric acid, the pH readings being taken on a Beckman pH meter, laboratory model. In the vicinity of the first equivalence point, the acid is added in increments of 0.10 ml. After the first equivalence point, 10 ml. of benzene are added, and the titration is continued. Near the second equivalence point, increments of 0.10 ml. are again added, and the flask is swirled vigorously after each addition to hasten the extraction of the liberated fatty acids by the benzene.

The inflection points are located by calculating the second differentials of the pH with respect to the buret reading, $\Delta^2\text{pH}/\Delta v^2$, and interpolating to find the buret reading at which this differential is zero. The slopes are considered to be ten times the maximum increment in pH.

DOUBLE-INDICATOR METHOD. The oil is saponified as in the potentiometric method. After cooling, 17 drops (0.3 ml.) of 1 per cent alcoholic phenolphthalein are added. Then 0.5 *N* hydrochloric acid is added until the color of the indicator dis-

appears. The volume of acid used to reach this end point need not be measured. Then 3 drops (0.2 ml.) of 0.010 *M* bromophenol blue and 10 ml. of benzene are added, the buret is refilled to the zero mark with standard 0.5 *N* hydrochloric acid, and the solution is titrated to a green color.

This end point is sharp. The solution is distinctly blue a drop or two before the end point, and distinctly yellow a drop or two beyond. Nevertheless a few cautions may be helpful. The solution ordinarily turns yellow several drops before the end point is reached. Then on agitation, the color changes to blue again as the fatty acids are extracted by the benzene. The end point is a green color that is not changed to blue by further swirling of the flask. The benzene also extracts the coloring matter (usually yellow) of the oils. Upon agitation of the flask before the end point is reached, the emulsion of the yellow benzene in the blue aqueous phase sometimes gives the appearance of the green end point. Fortunately, the two phases separate quickly when the agitation is stopped, and the color of the aqueous phase can be observed.

Table I. Summary of Potentiometric Titrations

| Oil | First Jump pH | First Jump Slope | Second Jump pH | Second Jump Slope |
|---|---|---|---|---|
| Butter fat | 10.70 | 9.6 | 3.89 | 2.9 |
| Castor oil | 10.03 | 9.6 | 4.49 | 7.1 |
| Castor oil, acetylated | 11.14 | 9.7 | 3.27 | 0.8 |
| Cocoa butter | 10.66 | 15.1 | 4.28 | 6.1 |
| Coconut oil | 10.62 | 9.4 | 4.03 | 3.9 |
| Cod liver oil | 10.50 | 9.7 | 4.45 | 6.8 |
| Corn oil | 10.13 | 8.5 | 4.31 | 6.9 |
| Lard oil | 10.49 | 8.7 | 4.46 | 6.0 |
| Linseed oil | 9.85 | 8.1 | 4.38 | 6.8 |
| Neat's-foot oil | 9.86 | 8.8 | 4.41 | 4.4 |
| Olive oil | 10.35 | 8.3 | 4.55 | 5.5 |
| Palm oil[a] | 10.00 | 5.5 | 3.33 | 0.8 |
| Rapeseed oil | 10.15 | 7.4 | 4.61 | 4.8 |
| Tung oil | 10.28 | 6.2 | 4.42 | 4.6 |

[a] See discussion.

## Results

The pH values and the slopes at the two equivalence points for the titrations of various oils are recorded in Table I. Ten milliliters of benzene were added after the first equivalence point in all these titrations.

A comparison of the results obtained by the recommended methods with those of the standard method (*1*) is given in Table II.

## Discussion

It is well known that the glass electrode fails to check the hydrogen electrode in solutions of high pH and also in solutions containing a large concentration of alcohol. Therefore the values read on the scale of the Beckman pH meter in this work should not be interpreted as exponents of hydrogen-ion activity or concentration. The uncertainty of the liquid-junction potential between the aqueous solution in the calomel electrode and the mixed solvent adds further doubt to the meaning of the pH values. Nevertheless, these values were perfectly reproducible and changed on addition of acid or base in the expected direction. They may be used to compare the acidity of two solutions that have approximately the same ratios of alcohol to water.

Varying quantities of phenolphthalein were added to some of the solutions that were titrated potentiometrically. It was found that the disappearance of the indicator color coincided best with the inflection point when 17 drops of 1 per cent phenolphthalein were added. Therefore this quantity was used in the double-indicator method.

Table I reveals that for all the oils studied, except acetylated castor oil and palm oil, the second inflection point occurs near a pH of 4.3. Bromophenol blue was chosen as the indicator for this end point because it shows a sharp color change at a pH of 4.3 in the mixed solvent.

Table II reveals that the recommended methods give excellent checks with the standard method for all the oils studied except acetylated castor oil and palm oil. The failure of the recommended methods with acetylated oils is to be expected because the benzene fails to extract acetic acid from the aqueous phase. This results in a low pH at the second equivalence point, a small slope of the graph at this point, and hence an unsatisfactory end point with the indicator.

The behavior of the sample of palm oil is more puzzling. The high saponification number obtained by the standard method indicates that the oil was not pure and that palm nut oil was a likely adulterant. The low pH and small slope at the second equivalence point indicate the presence of fatty acids of very low molecular weight.

The potentiometric method is time-consuming and is recommended only for highly colored oils. The double-indicator method, however, gives accurate results and requires less time, reagents, and apparatus than the standard method.

The double-indicator should be more easily adaptable to microprocedures than the standard method. In any micromodification of the standard procedure, it would be necessary either to measure accurately a very small volume of alcoholic potassium hydroxide or to use a very dilute solution of this reagent. In the first alternative, difficulty would be encountered because of the volatility and large expansion coefficient of the alcohol. In the second alternative, the time of saponification would be prolonged. In the case of the double-indicator method, a small volume of fairly concentrated alcoholic potassium hydroxide could be used without the necessity for accurate measurement or control of this volume.

Table II. Comparison of Results by Various Methods

| Oil | Standard Method Saponification No. | Potentiometric Method Saponification no. | Potentiometric Method Difference | Double-Indicator Method Saponification no. | Double-Indicator Method Difference |
|---|---|---|---|---|---|
| Butter fat | 224.1 | 224.2 | +0.1 | 223.8 | −0.3 |
| Castor oil | 181.5 | 181.2 | −0.3 | 181.0 | −0.5 |
| Castor oil, acetylated | 388.4 | 390.1 | +1.7[a] | [b] | ... |
| Cocoa butter | 194.6 | 194.9 | +0.3 | 194.7 | +0.1 |
| Coconut oil | 258.6 | 258.6 | 0.0 | 258.6 | 0.0 |
| Cod liver oil | 188.0 | 188.4 | +0.4 | 188.3 | +0.3 |
| Corn oil | 191.6 | 191.5 | −0.1 | 191.8 | +0.2 |
| Lard oil | 195.5 | 195.5 | 0.0 | 195.7 | +0.2 |
| Linseed oil | 187.0 | 186.7 | −0.3 | 186.6 | −0.4 |
| Neat's-foot oil | 186.7 | 186.7 | 0.0 | 187.1 | +0.4 |
| Olive oil | 191.5 | 191.4 | −0.1 | 192.3 | +0.8 |
| Palm oil | 238.0 | 237.1 | −0.9[a] | [b] | ... |
| Rapeseed oil | 174.3 | 174.5 | +0.2 | 174.4 | +0.1 |
| Tung oil | 193.5 | 193.2 | −0.3 | 192.9 | −0.6 |
| Mean (signs disregarded) | | | ±0.2 | | ±0.3 |

[a] Omitted in calculating mean.
[b] Method not applicable to these oils.

When several saponification numbers are to be run, the following modification of the double-indicator method may be advantageous:

Pipet 50.00 ml. of alcoholic potassium hydroxide into each of the flasks containing the weighed samples of oils. After saponification, titrate two or three samples by the double-indicator method, but measure the volume of standard hydrochloric acid used to reach both the first and the second end points. The total volume of acid is the same as the blank titration of the standard method. Then the remaining samples need to be titrated only to the phenolphthalein end point.

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# Colorimetric Determination of Hydrogen Peroxide

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DURING a study of the effect of humidity on the production of ozone from air by the action of the silent electric discharge, qualitative tests revealed the formation of hydrogen peroxide. Attempts at quantitative estimation by means of the commonly employed volumetric methods and by those few colorimetric procedures (*3*, *4*) reported in the literature were not entirely satisfactory. It was therefore necessary to develop a method which would be sensitive to very small amounts of peroxide. This procedure, in comparison with other methods based on the same chemical reaction, has the advantage of yielding a straight-line calibration curve over the concentration range specified.

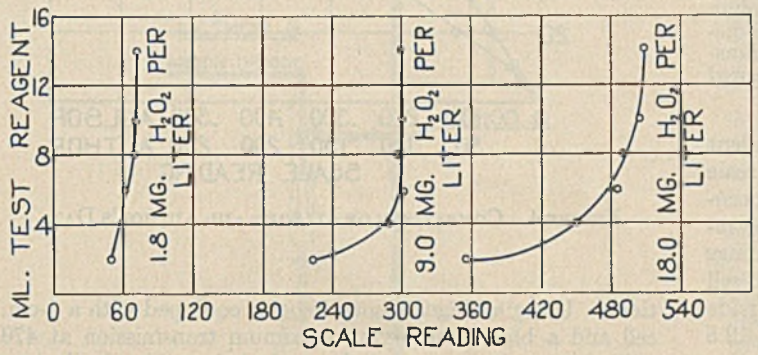


FIGURE 1. EFFECT OF VARYING QUANTITIES OF TEST REAGENT ON COLOR INTENSITY
K. & S. colorimeter, filter 40, cell depth 40 mm.

## Principle of Method

The method is based on photoelectric measurement of the color intensities of hydrogen peroxide solutions treated with titanium sulfate reagent. The yellow color produced in the reaction was first observed by Schonn (*5*) and according to Treadwell and Hall (*6*) is due to the formation of pertitanic acid. The reaction equation is usually written

$$Ti^{++++} + H_2O_2 + 2H_2O = H_2TiO_4 + 4H^+$$

although some chemists prefer to write the formula of pertitanic acid as $TiO_2.H_2O_2$, showing a true peroxide structure. It was Jackson (*2*) who pointed out the quantitative possibilities. Richardson (*4*) and Knecht (*3*) developed methods in which colors were compared visually. More recently Allsop (*1*) reported on a photoelectric method which is discussed in greater detail below.

## Reagents and Solutions

All reagents were of the purest grade obtainable.

The titanium sulfate test reagent was prepared by digesting 1 gram of anhydrous titanium dioxide (Schering-Kahlbaum) with 100 ml. of sulfuric acid (specific gravity 1.84) for 15 to 16 hours on a sand bath at a temperature of 150° C. The solution was cooled, diluted with 4 parts (by volume) of distilled water, and filtered through an asbestos mat prior to use. Preliminary experiments in which varying quantities of reagent, ranging from 2 to 50 ml., were diluted to 100 ml. with distilled water indicated no color or turbidity to be present.

A standard hydrogen peroxide solution was made by diluting 20 ml. of 30 per cent hydrogen peroxide solution to 1 liter with distilled water and standardized by titration with permanganate.

## Apparatus

The photoelectric colorimeter was a Klett-Summerson Model 900-3 in which was used a KS-40 filter (transmission limits 380 to 430 millimicrons). An unusual feature of this instrument is the scale on which readings are made. This scale is logarithmic and has been designed so that under certain specified conditions the scale readings are a measure of and are proportional to the optical density of the colored solution as determined by the photoelectric cell. Since the optical density is theoretically proportional to the concentration of the colored substance, the scale readings are likewise proportional to the concentration under the specified conditions.

## Optimum Quantity of Test Reagent for Maximum Color Development

To ascertain whether the intensity of color is a function of the quantity of titanium sulfate used, varying quantities of the test reagent were added to known quantities of hydrogen peroxide solution contained in a series of 100-ml. Nessler tubes. All solutions were then diluted to 100-ml. volume with distilled water and the intensity of color was measured by transferring to the solution cell of the colorimeter. In all, three such experiments were made in which concentrations of 18, 9, and 1.8 mg. of hydrogen peroxide per liter were used. Cell depth was 40 mm. Results are shown graphically in Figure 1.

It is evident that 1 volume of titanium sulfate reagent per 10 volumes of peroxide solution is required for maximum color development. As far as could be ascertained, the color is developed instantly and remains stable for at least 6 hours. In view of this finding, 10 ml. of reagent were used in all subsequent experiments.

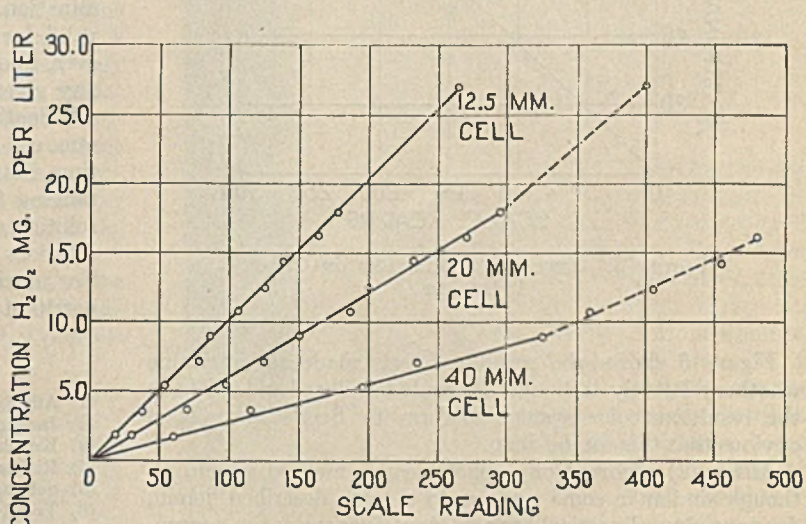


FIGURE 2. CONFORMITY TO BEER'S LAW

TABLE I. ACCURACY OF METHOD

(12.5-mm. cell)

| Scale Reading *Divisions* | Concentration of $H_2O_2$ *Mg./100 ml.* | | Deviation *Mg./100 ml.* |
|---|---|---|---|
| | Actual | Calculated | |
| 18 | 0.18 | 0.18 | 0.00 |
| 38 | 0.36 | 0.39 | +0.03 |
| 54 | 0.54 | 0.55 | +0.01 |
| 78 | 0.72 | 0.80 | +0.08 |
| 86 | 0.90 | 0.88 | −0.02 |
| 106 | 1.08 | 1.08 | 0.00 |
| 125 | 1.26 | 1.27 | +0.01 |
| 139 | 1.44 | 1.42 | −0.02 |
| 164 | 1.62 | 1.67 | +0.05 |
| 177 | 1.80 | 1.80 | 0.00 |
| 263 | 2.70 | 2.68 | −0.02 |
| | | | Av. ±0.03 |

## Conformity to Beer's Law

A series of solutions, containing from 1.8 to 27 mg. of hydrogen peroxide per liter, was prepared by placing the appropriate quantity of peroxide in 100-ml. Nessler tubes, adding 10 ml. of titanium sulfate reagent to each tube, and diluting to the mark with distilled water. After thorough mixing the solutions were transferred to the colorimeter cell. Color intensity was measured using three different cell depths: 40, 20, and 12.5 mm.

That Beer's law is followed by the color system is evident from the fact that a straight line is obtained when the scale readings are plotted against the hydrogen peroxide concentration (Figure 2). The deviation from the straight-line relationship for the higher color intensities (scale readings above 300) observed in the cases of the 20- and 40-mm. cell depths is probably due to the fact that a relatively wide band-pass filter was used. The use of a smaller cell, 12.5 mm., eliminates this deviation and increases the effective range of the method. In view of this, in order to obtain conformity to Beer's law throughout any given range of concentrations, it is necessary that the cell depth be adjusted to the colorimetric system.

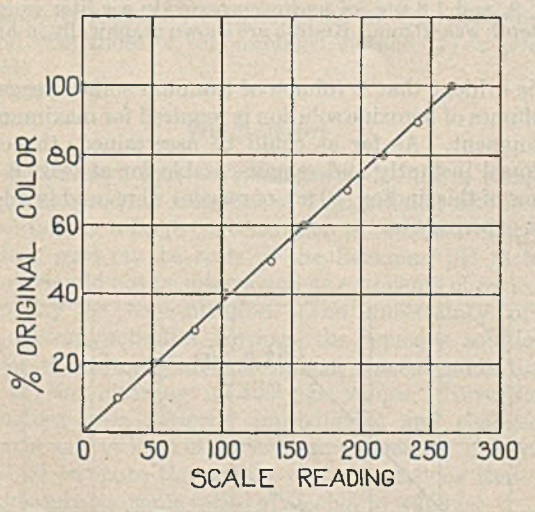


FIGURE 3. EFFECT OF DILUTION ON COLOR INTENSITY

Figure 3 shows the results of tests made to determine whether relatively dark solutions could be diluted and still have the resultant color system conform to Beer's law. It is obvious that this can be done.

Allsop (*1*) reported on a photoelectric method which, although similar in some respects to the one described herein, does not give a linear calibration curve over the entire concentration range nor does it cover the same range of concentrations. Using a Hilger absorptiometer equipped with a 4-cm. cell and a blue filter giving maximum transmission at 470 millimicrons, he measured the optical densities of solutions containing varying amounts of hydrogen peroxide treated with titanium sulfate. For purposes of comparison, his data are shown graphically in Figure 4, together with the results obtained by the author. The difference in the linear character of the two curves is obvious.

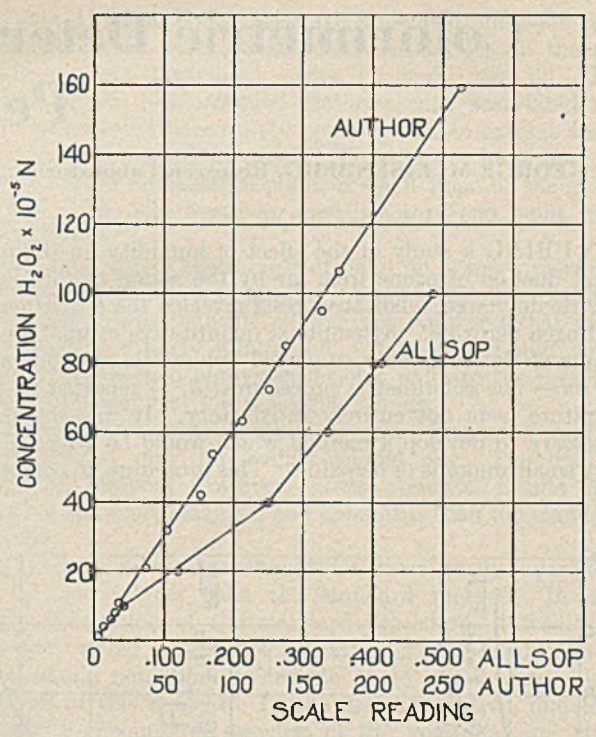


FIGURE 4. COMPARISON OF ALLSOP'S AND AUTHOR'S DATA

The accuracy with which hydrogen peroxide can be determined with this method is shown in Table I. With the 12.5-mm. cell it is possible to determine hydrogen peroxide in concentrations ranging from approximately 0.2 to 3.0 mg. per 100 ml. with an average deviation of ±0.03 mg. per 100 ml.

## Summary and Conclusions

A rapid and accurate colorimetric method for the determination of hydrogen peroxide is described. Beer's law is valid for measurements at 380 to 430 millimicrons. However, in order to obtain conformity to Beer's law throughout any given range of concentration, it is necessary that the cell depth be adjusted to the colorimetric system. A 12.5-mm. cell increases the effective range of the method, permitting determination of hydrogen peroxide concentrations ranging from approximately 0.2 to 3.0 mg. per 100 ml. of solution with an average deviation of 0.03. Relatively dark solutions can be diluted without invalidating Beer's law. For maximum color development 1 volume of test reagent per 10 of solution is required.

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# Current Methods of Measuring Foam

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WHEN methods of foam measurement were previously considered, an initial classification was based on the obvious differences between "static" and "dynamic" methods. Static methods are those in which measurements are made after the foam has been produced, with no reference to the manner of its production. In dynamic methods measurements are made while the foam is being formed, usually by a current of gas passing through the liquid.

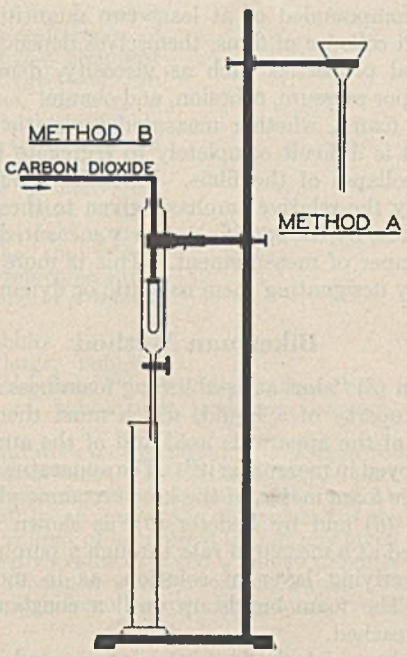


FIGURE 1. ROSS AND CLARK STATIC FOAM APPARATUS
Method A, applicable to carbonated liquids
Method B, applicable to uncarbonated liquids

It has already been shown that results obtained by the various methods do not necessarily agree (*5*). It is not generally possible, when dealing with a property of matter so complex as the foaminess of a liquid, to make classifications that can be universally applied. In the present case it is recognized that static and dynamic methods represent only an empirical distinction and that there exist more fundamental theoretical considerations upon which a final classification of methods must be based. Meanwhile the two terms are of value in providing a method of approach to the problem of foam measurement, especially with a view to unraveling the complex mass of results and conclusions contained in the literature on the subject.

It is proposed in the present paper to examine some of the recently published methods of both types, to obtain evidence of whatever correlations may exist, and to establish if possible a better understanding of the factors involved.

## Static Methods Involving Drainage

Static methods usually consist of forming a foam, in any one of a number of possible ways, and observing the rate at which liquid drains or collects from it, beginning the observations some time after cessation of foam formation. It has been discovered experimentally that for many foams the rate of drainage of liquid obeys the equation

$$V = V_0 e^{-kt}$$

or $$\ln V_0/V = kt$$

where $V$ = volume of liquid in the foam after time $t$, $V_0$ = volume of liquid in the foam when $t = 0$, $t$ = time, and $k$ is a constant characteristic of each liquid from which the foam is formed. This logarithmic equation has been found to hold for such diverse substances as beer (*4*), wine (*1*), saponin (*2*), Aerosol OT (*15*), and lauryl sulfonic acid (*15*). It would therefore appear to be a general law for the behavior of foams. Deviations are known to exist—e. g., foams of egg albumin (*5*)—but they can be explained on the basis of specific considerations for the case in point.

Using this mathematical relation, it was possible to establish a unit of foaminess for all static foams (*5*, *16*), which can be applied regardless of the way in which the foam is produced and independently of many empirical restrictions of former methods.

Figure 1 illustrates the Ross and Clark static method for both carbonated (method A) and uncarbonated liquids (method B). The foam is produced in the former case by pouring the liquid from a height and in the latter case by bubbling a gas through it. In both cases the same value for the foaminess was obtained, using the reciprocal of the constant of the exponential equation as a unit of foaminess. A modification of method B, capable of greater accuracy, is illustrated by Figure 2. This is the Stiepel foam meter and can be used to measure not only the rate of drainage of the liquid but also the volume of foam produced. In this way it can be applied as a dynamic method (with certain limitations because of the narrowness of the tube) as well as a static method.

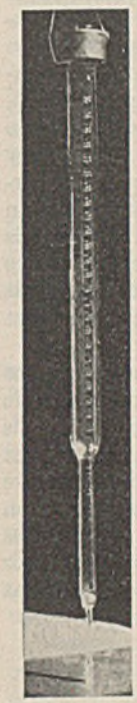

FIGURE 2. STIEPEL FOAM METER

Figure 3 represents the apparatus used by Arbuzov and Grebenshchikov (*2*) to measure the foam stability of saponin solutions. Ten cubic centimeters of the liquid are put in the buret, $D$, and go out at an even rate, one drop at a time, onto the porous filter. The container, $A$, is connected to a water pump, a mercury manometer, and an outlet valve, $E$. When the solution passes through the filtering medium it takes up air from the pores and a foam is produced in the cylinder. As soon as all the solution has thus been converted into foam, valve $E$ is opened and the drainage of the foam is allowed to proceed under atmospheric pressure. The rate at which the liquid drains from the foam is measured, using the narrow graduated tube at the bottom of the cylinder. The apparatus is kept in a thermostat during the whole process.

It is considered undesirable by these authors to observe the breakdown of a foam while the foam remains under the influence of the conditions which caused its formation. This is really an expression of preference for a static rather than for a dynamic method. More significant is the attention that they draw to the fact that, by their method, the surface-active substance is equally distributed through the whole mass of foam, because liquid is turned into foam one drop at a time until no more liquid remains. In other methods, where the entire remaining body of the liquid is present while

the foam is being formed, the liquid is continually depleted of surface-active agent, so that succeeding portions of the foam are progressively different, yielding a nonuniform foam. Hence, the method of foam formation may influence the experimental value of foam stability. However, it would seem that this is important chiefly in very dilute solutions of surface-active agents.

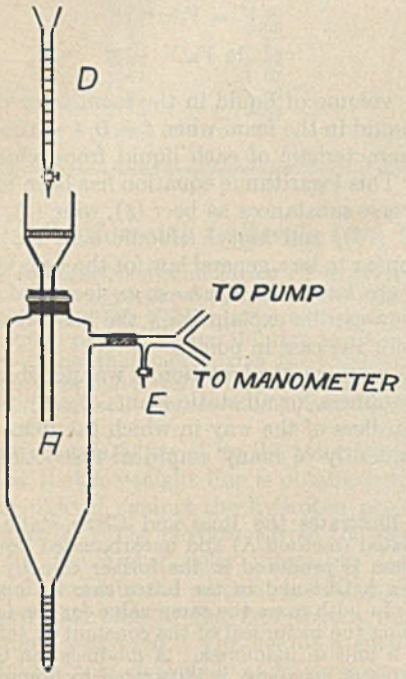


FIGURE 3. STATIC FOAM METER USED BY ARBUZOV AND GREBENSHCHIKOV (*2*, PAGE 33)

Arbuzov and Grebenshchikov (*2*) found that the exponential relation holds for the later half of the drainage of the foam, and they considered the reciprocal of the exponential constant to be a satisfactory unit of foam stability.

It would therefore appear that, with or without the conditions mentioned by Arbuzov and Grebenshchikov (*2*), the exponential relation is commonly obtained for drainage of a foam; moreover, results are reproducible within very wide experimental limits. There is little difficulty in correlating the results because of the same underlying relation for the drainage. In each case the total liquid collected is actually measured, and although collapse of the films takes place simultaneously, it is not measured as such but finds its place in the effect that a smaller volume of foam must exercise on the rate of drainage.

It is immediately recognized that where the foam possesses absolute stability, drainage of liquid is a purely hydrodynamic matter and has no relation at all to the foam stability. This has been pointed out by Ross and Miles (*14*), who suggest measuring the effect of an arbitrary standardized destructive mechanism acting upon a volume of foam during production under standard conditions and protected from adventitious destructive forces. This procedure is necessary only for foams of such stability that the films will drain without simultaneous or immediately subsequent collapse.

## Dynamic Foam Methods

The lack of mathematical relations to express the opposing rates of growth and decay of a dynamic foam makes the task of correlating these methods considerably more complex than for static foam methods. In dynamic methods the emphasis is taken off the question of drainage from the foam and focused only upon the position of the topmost films. The effects that are actually measured are either the rate of rise of the foam in the measuring cylinder or the maximum constant volume of foam obtained, in both cases when a gas is passed continuously through a foam-producing liquid. It is, of course, impossible to prevent draining of the liquid from the foam and the consequent thinning and ultimate collapse of the liquid films, but only the rate of collapse is actually measured in dynamic methods. The rate of drainage, although it is not measured directly, nevertheless operates in the effect that a more rapid rate of drainage has in altering the rate of collapse of films.

This is in direct contrast to the static methods and the two together lead to the conclusion that the foaminess of a liquid, as it is usually measured, is a derived or subsidiary property, compounded of at least two quantities, drainage of films and collapse of films, themselves dependent on more fundamental properties such as viscosity, density, surface tension, vapor pressure, cohesion, and charge.

In most foams, whether measured by static or dynamic methods, it is difficult completely to segregate the drainage from the collapse of the films. Methods should be characterized by the relative emphasis given to these two quantities—that is, by the specific property measured rather than by the manner of measurement. This is more satisfactory than merely designating them as static or dynamic.

## Bikerman Method

Bikerman (*3*) "aims at establishing foaminess as a definite physical property of a liquid, which must therefore be independent of the apparatus used and of the amount of material employed in measuring it". The apparatus he employed is a dynamic foam meter, of the kind recommended by Foulk and Miller (*6*) and by Lederer (*9*), as shown in Figure 4. Gas is forced at a measured rate through a porous membrane and an overlying layer of solution, as in most dynamic methods. The foam builds up until a constant maximum volume is reached.

The unit by which the foaminess is measured is designated as $\Sigma$ and described as "the average lifetime of a bubble in the foam". The derivation of $\Sigma$ throws some light on its character.

When dynamic equilibrium has been reached, the amount of foam maintains its constant maximum volume. At any time during this equilibrium the mass rate at which gas is being passed into the liquid is equal to the mass rate at which it is rising in the foam, and also to the mass rate at which it is escaping therefrom. In a tube of constant cross section linear rates can be used instead of mass rates. If the linear velocity of the gas in the foam is $u$ when a volume of gas, $V$, is forced through the membrane in $t$ seconds, then

$$u = V/tQ$$

where $Q$ is the cross section of the measuring tube. If now, at equilibrium, the average maximum height of the foam layer is $h$, the expression $h/u$ represents the time during which any arbitrary volume of gas is present in the foam. (The average maximum foam height is defined as the difference between the average maximum height attained by the liquid plus foam and the height of the liquid at rest.) It is this period of time that is $\Sigma$ and the equation $\Sigma = h/u$ gives a precise definition—namely, the average lifetime of the gas in the foam. This might better be designated as $L_g$, because it is only in the rare case where there is no coalescence of bubbles within the foam that $L_g$ becomes equal to the average lifetime of a bubble in the foam. It involves assumption and interpretation to take the next step, which is to call $L_g$ the average lifetime of a bubble in the foam.

$L_g$ is easily found experimentally. The quantities measured are the average maximum foam volume, $v$, and the volume of gas, $V$, passed through the membrane in time $t$. Then $L_g = h/u$, where $h = v/Q$ and $u = V/tQ$. Substitution leads to the expression $L_g = vt/V$.

The attainment of a constant volume of foam indicates equilibrium between building up of the foam by the in-going gas and the destruction of the top of the foam by the collapse of the topmost film or films. This is partly or often largely a matter of evaporation or contamination. With liquids of great foam-forming capacity the former process so far outweighs the latter that equilibrium, denoted by a constant maximum foam volume, is not practicable.

It can readily be seen that the actual average life of a bubble even in the foam cannot be defined by $L_\theta$ unless there is no coalescence of bubbles before they disappear at the top of the foam. In actual practice, however, in many cases a single bubble present at the top of the foam may have started out as a large number of individual bubbles that have united somewhere on their upward journey to the top. Here $L_\theta$ would not represent a true average lifetime of a bubble in the foam.

Smaller bubbles are more stable than larger bubbles. Hence $\Sigma$ has a definite value for a given liquid only for a definite average size of bubble and a definite average amount of coalescence.

Another limitation on the meaning of $\Sigma$ is introduced by its method of measurement. If, for illustration, a pure nonfoaming liquid is considered and air is passed through at a measured rate, there will be an increase in the volume of the sample due to the added volume of air present in it at any given time. This leads to a positive value of $h$ and $u$ and so provides a numerical value for the foaminess of a nonfoaming liquid. The error thus introduced is significant for poorly foaming or viscous liquids.

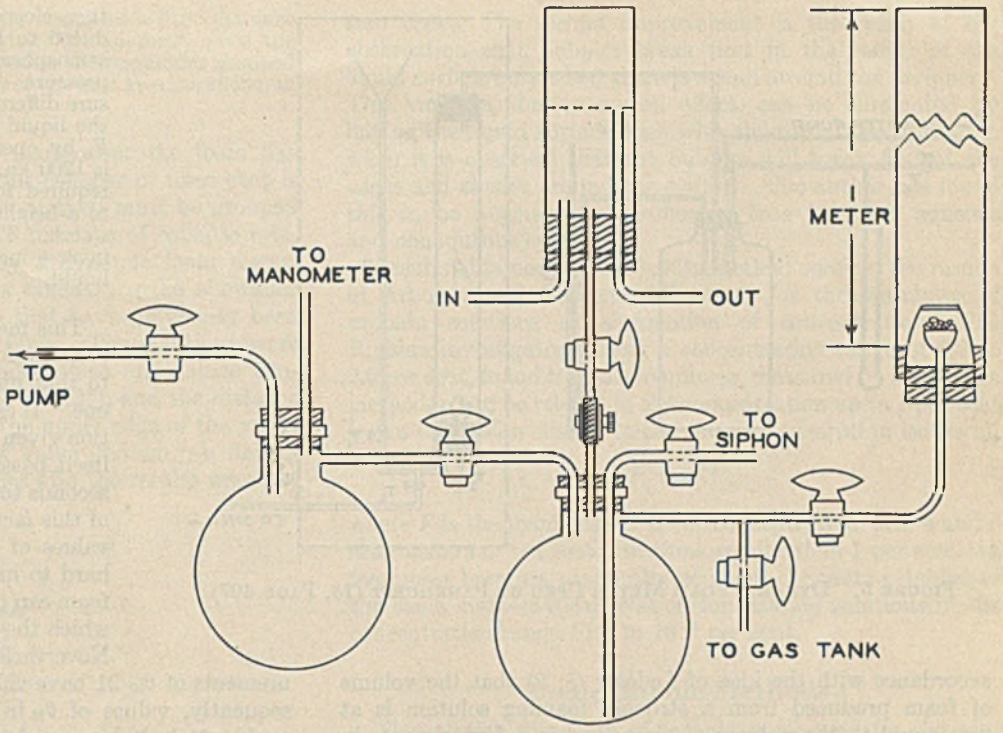


FIGURE 4. DYNAMIC FOAM METER

A paper by Hoffmann and Peter (*7A*) was not available until recently. Hoffmann and Peter used exactly the same unit as that originally proposed by Bikerman (*3*), though without any reference to his previous work. They used a capillary to produce a homogeneous foam of known bubble size. With hydrocarbon oils they observed only slight coalescence of bubbles in the body of the foam. For such foams it is permissible to interpret $L_\theta$ as the average lifetime of a bubble, although the same reservations must be placed on that interpretation in the general case as have already been mentioned for the Bikerman unit. The linear rate of increase of the foam volume observed at the beginning of the foam production, although used by Mischke (*10*) and by Ostwald and Mischke (*11*) as a measurement of foaminess, is here more properly as a measurement of the rate of air input.

## Effect of Size of Bubble

For a uniform film the chance of breaking is proportional to the exposed area, since breakage at one point causes collapse of the whole film; hence, the breakage of a bubble would depend upon the area exposed to the atmosphere or to other bubbles. Likewise, small bubbles are partly surrounded and protected by the mass of liquid in the Gibbs angle between them.

In general, therefore, the smaller a bubble the longer its life, which may be expected to be at least in inverse proportion to the square of the diameter of the bubble.

## Conditions for Defining Rate of Collapse of Films

If the rate of collapse of film, or the foam stability, for one and the same liquid is to have a definite and significant numerical value, the following factors must be taken into account:

1. The size of the bubble, which should be stated.
2. The age of the solution.
3. The rate at which bubbles are formed.
4. The time taken for the determination, because if the time is protracted the rate of film collapse becomes abnormally slowed on account of the accumulation of stabilizing material on the remaining films.
5. In some cases carbon dioxide or reactive gases should be excluded from the atmosphere which is in contact with the foam. Carbon dioxide has a pronounced effect on stability of foam prepared from soap solutions and this perhaps may be true of some other surface-active agents.

It has been previously shown (*17*) that in addition to Nos. 2 and 3 there are two further factors which in practice can be important in affecting the stability of exposed film in an insufficiently enclosed apparatus. These are:

6. Temperature of the gas above the foam.
7. Partial vapor pressure above the foam for appreciably volatile solvents or solutes, often determined by the distance from the top of the foam to the top of the containing vessel.

## Ostwald and Mischke Method

Ostwald, Siehr, and Mischke (*10–12*) have developed a dynamic foam meter in which gas bubbles of known size are passed into the liquid. For a constant flow of gas, the initial rate of rise of the foam in the measuring cylinder is taken as a measure of the foaming capacity of the liquid. In experiments with gelatin solutions between 10 and 0.1 per cent concentration Mischke found that the initial rate of rise is the same for all the samples and is constant for the first 3 minutes of foaming. This strongly suggests the possibility that it was characteristic of nothing more than the rate at which air was being passed into the liquid, in

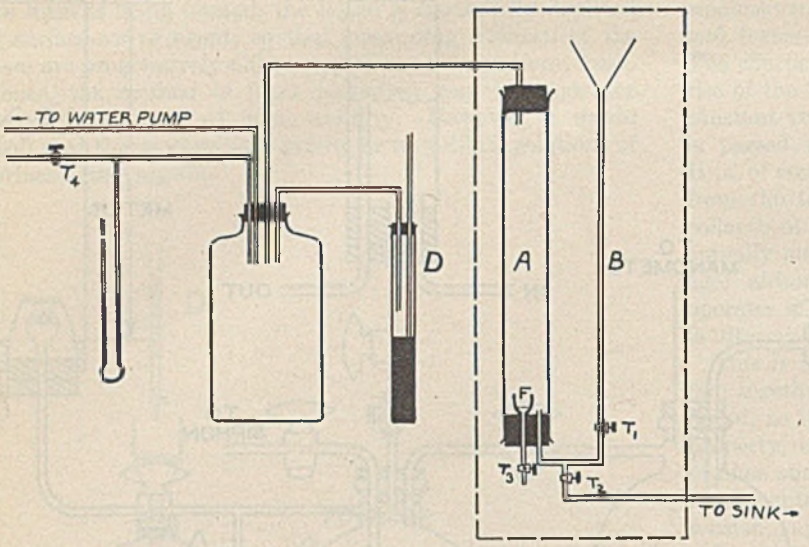


FIGURE 5. DYNAMIC FOAM METER USED BY PANKHURST (*13*, PAGE 497)

accordance with the idea of Lederer (*8*, *9*) that the volume of foam produced from a strongly foaming solution is at first equal to the volume of air passed in. Experiments in this laboratory with dilute solutions of Aerosols have also proved the validity of this suggestion. It presents a formidable objection to their method, which is seen to be inapplicable to strongly foaming solutions.

The rate of formation of bubbles depends solely on the rate at which gas is bubbled through the liquid, itself determined by the difference in pressure, diameter of inlet tubes or pore diameters, viscosity of the gas and liquid, etc. Subtracted from this arbitrarily determined rate is the rate of collapse of films, the resulting rate being the experimentally determined quantity. The rate of film collapse cannot be a constant quantity, as not only must it depend on the amount of foam present, which is increasing steadily with the time, but also on propinquity (an isolated submerged bubble cannot break, even if infinitely unstable), and drainage, which is a nonlinear function of time (*15*). (Film collapse may indeed appear to be a linear function of time at the outset. Many complex functions can be approximated by a straight line when near the origin.) Consequently, if both kinetic factors are in simultaneous operation the experimentally determined rate of foam rise would not be constant, except possibly for a short time near the origin. It could be constant for a longer range only when film collapse is so delayed that it does not take place to any appreciable extent while the foam is being formed.

## Pankhurst Method

Pankhurst (*13*) adopted a dynamic foam meter, a modification of that made by Foulk and Miller (*6*). A diagrammatic scheme of the apparatus is given in Figure 5.

The liquid under test is admitted to the large Pyrex tube, *A*, through the side tube, *B*. Tap $T_1$ is then closed and the level of the liquid in *A* is adjusted to an arbitrarily chosen zero mark above the porous septum, *F*, by means of tap $T_2$. Taps $T_3$ and $T_4$ are then closed and the pressure inside *A* is reduced to 10 cm. of mercury below that of the atmosphere and maintained by a constant-pressure device, *D*. When a steady pressure difference is obtained, air is admitted to the liquid in *A* through the porous septum, *F*, by opening $T_3$. The rate of intake of air is 1200 cc. per minute (±15 cc.). The time required for the advancing foam front to rise to a height of 50 cm. is measured with a stop watch. This value, $\theta_{50}$ seconds, is taken as an inverse measure of the foaming power of the liquid.

This method is similar in principle to that of Ostwald and Mischke and the objections to that method are also applicable to this one. It can be calculated from the information given about the apparatus that the air itself passing into tube *A* would require 99 seconds to rise 50 cm. in the tube. (Because of this fact one would not expect to find any values of $\theta_{50}$ less than 99 seconds, as it is hard to image any mechanism whereby the foam can rise at a faster rate than the rate at which the air is being passed into the liquid. Nevertheless, out of the 37 reported measurements of $\theta_{50}$ 21 have values less than 99 seconds.) Consequently, values of $\theta_{50}$ in the neighborhood of 99 seconds could not be taken as characteristic of the foaminess of the liquid under test.

## Schütz Method

The gas is introduced by means of a single capillary, ending underneath the surface of the liquid (*17*). The amount of gas bubbled through the liquid is the same in every case, and corresponds to a reduction of pressure of the gas from 18.80 to 15.40 cm. of mercury. The apparatus is shown schematically in Figure 6.

*E* is a device for obtaining the same initial pressure of the gas and *J* is a sintered-glass filter to retain fine dust particles. The tube, *G*, has at its end a "thermometer capillary" ground to the shape shown in the inset of Figure 6. The period between the stoppage of the gas entry and the time when half the foam-covered surface of vessel *F* is free from bubbles, was called the "foam time", $T_f$, and used as a measure of foaminess. It is perhaps an unfortunate choice to use dissimilar quantities to define the extremities of the time period. To determine when exactly half the foam surface is gone, a wire circle of calculated

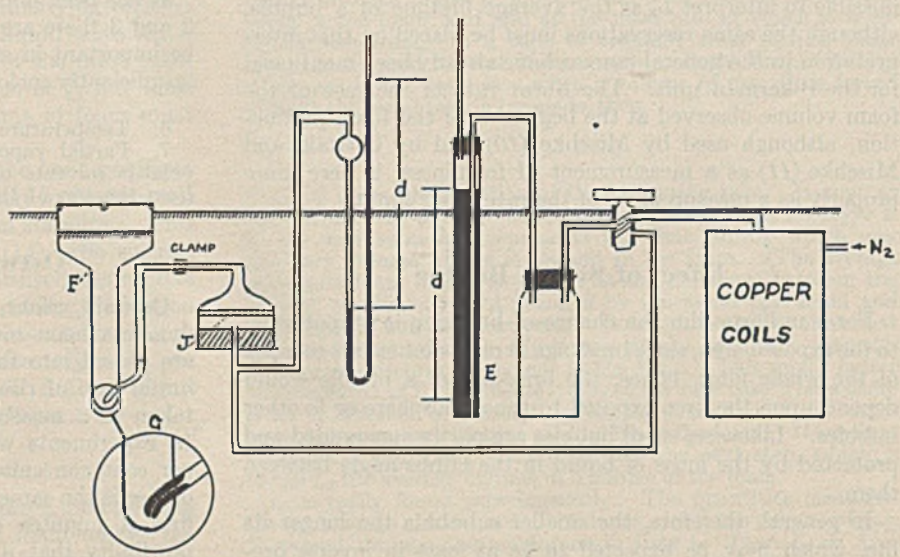


FIGURE 6. APPARATUS USED BY SCHÜTZ TO MEASURE FOAM TIME (*17*, PAGE 89)

diameter is placed on the edge of the vessel, at a fixed distance from the eye of the observer (Figure 7). In most cases the bubbles break first in the center, leaving the remainder grouped around the wall, so that observation of the half area, as shown in Figure 7, offers no difficulty.

Although the measurement is made after the foam has formed, yet it is the time taken for collapse of films that is actually measured, and hence this method must be grouped along with those that emphasize a function of collapse rate, as do most methods that employ a dynamic foam meter.

In this method unusual care is taken to make allowance for the influence of the variables that have previously been noted to affect the stability of films. The solutions were always of equal age, the temperature was maintained constant by a water bath at 21.4° C. (±0.2°), and the distance from the surface of the liquid to the upper edge of the vessel (*a* in Figure 7) was kept at a low value (2.5 cm.), it having been shown that with higher values of *a* the results were not reproducible.

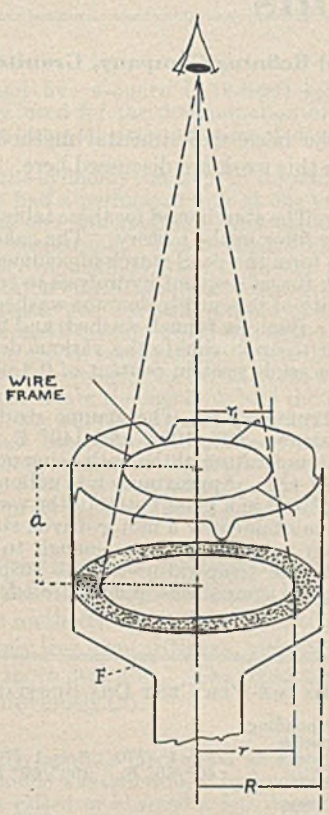


FIGURE 7. DEVICE USED BY SCHÜTZ TO OBSERVE WHEN HALF-SURFACE AREA IS CLEAR OF FOAM (*17*, PAGE 87)

As with many of the methods previously discussed, this one too was inapplicable to strongly foaming solutions. For solutions of saponin greater than 0.001 per cent the values of $T_f$ exceeded 50 minutes and, because of the difficulties of keeping conditions constant for so long a period of time, reproducible results could not be obtained. This particular method, however, would appear to be abnormal in this respect, as very much higher concentrations of saponin have previously been measured by other methods without undue difficulty (*2*, *13*).

Two improvements on the Schütz method are suggested. One of them is to restrict the amount of foam to a single ayer of bubbles on the surface, in order to dispose of the effect of drainage as much as possible; and to take this as zero time. The second improvement is the result of the observation that bubbles break first in the center of the liquid surface only when there is a wall around the periphery. This variable, due to a wall effect, can be eliminated by having the liquid surface flush with the top of the container, when it is observed that the bubbles will break first at the edges and cluster around the center. The author has found this to be a general phenomenon, true for both aqueous and nonaqueous systems.

Results obtained by the Schütz method confirm the results of Arbuzov and Grebenshchikov (*2*) for the foaminess of saponin solutions as a function of concentration. The Russian investigators, using a concentration range of 0.2 to 2.0 per cent, found that the foaminess, measured by a drainage method, could be related to the concentration up to 1 per cent by an expression similar to the classical adsorption isotherm,

$$F = aC^b$$

where $F$ is the foaminess, $C$ is the concentration, and $a$ and $b$ are constants. At concentrations greater than 1 per cent the foaminess becomes practically constant. Schütz established the same mathematical relation for saponin solutions in the concentration range $10^{-3}$ to $10^{-5}$ per cent.

## Single Bubble Methods

The relative simplicity of technique required for the production and observation of single isolated bubbles floating on a liquid surface, as well as the fact that a single bubble is more amenable to a theoretical mathematical treatment than is a bulk foam, makes it likely that single bubble methods are due to receive a greater prominence than heretofore. Such a method has already been put to good use, as, for instance, by Hardy (*7*) and by Talmud and Suchowolskaja (*18*), to gain information about the nature of interfaces. There is no doubt that reproducible results can be obtained, which have been of value in research.

It is known, and can readily be demonstrated, that several bubbles together are more stable than each separately, possibly on account of the entrained liquid in the rounded corners and edges between them, discussed by Gibbs. Nevertheless, experimental results obtained by C. G. Lindquist and the author in this laboratory indicate a correlation between the results of single bubble observations and some foam-measuring methods for certain nonaqueous systems. A difference in the order of magnitude of the numerical result is to be expected but the same sequence of stabilities for a series of different samples can be obtained. A more extensive report of those experiments cannot be given at the present time.

## Acknowledgment

Thanks are due to James W. McBain for many helpful discussions.

## Summary

1. The chief methods, static and dynamic, currently employed to measure foaminess of liquids have been examined to ascertain the factors involved, since each measures a somewhat different complex of properties, such as drainage, apart from mere collapse or coalescence of bubbles.

2. For a film of a given stability the average life of a bubble is greater the smaller the bubble, increasing at least in inverse proportion to the square of the diameter of the bubble.

3. Methods have been suggested for examining the stability of foams with a minimum of complications through other factors.

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# Baumé–Dry Substance Tables for Starch Suspensions

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**No tables for Baumé–dry substance starch have been published in this country, although it is a large producer of starch. Such tables are presented for cornstarch, together with a new method for moisture on starch suspensions. The experimental evidence indicates that such tables can be constructed mathematically if the absolute density of the specific starch is known.**

FOR many years the wet-milling industry in this country has employed the hydrometer for measuring the density of starch suspensions or slurries in their manufacturing operations. Various companies have adopted dry substance tables for these Baumé values, but no common table exists for use within or without the industry where starch is employed.

The use of a hydrometer for the measurement of solids in a suspension or slurry may seem strange to chemists who regard it solely from the standpoint of determination of density in true solutions. Its success in starch suspensions is based on the relatively slow settling of the starch and on the fact that the concentration of the starch suspensions at the hydrometer bulb is substantially constant. An analyst familiar with these properties acquires the technique readily and results on a common sample agree within 0.1 Bé. or 0.2 per cent dry substance.

Tables have been published for the Baumé–dry substance relationship for potato starch suspensions (*4*, *5*, *6*). Other papers have been published on the densities of the various starches (*3*).

The purpose of this paper is to present such tables for cornstarch, known in the trade as pearl starch, for the hydrometer in use in this country—145 Modulus, standardized at 60° F.

## Experimental

This work is based on methods previously published on the determination of the Baumé–dextrose equivalent–dry substance for corn sirup and corn sugar (*1*, *2*) which should be consulted for the basic experimental methods, as only the departures from this work are discussed here.

CORNSTARCH. The starch used for these tables was taken from the final washing filter in the factory. The cake from this filter is either dried to form the pearl starch of commerce or suspended in softened water for subsequent hydrolysis to corn sirup or corn sugar. A quantity of this suspension was washed by decantation or dewatered on a Büchner funnel, washed, and finally suspended in distilled water to approximate the various densities required. The starch had a crude protein content of 0.3 per cent on a dry basis.

BAUMÉ BY HYDROMETER. The Baumé readings were made at three temperatures—60°, 100°, and 140° F. (15.56°, 37.78°, and 60° C.), the temperature of the bath being maintained within ±0.02° F. (0.011° C.). Approximately a gallon (3.785 liters) of the sample was placed in a glass bottle in the water bath and the suspension was maintained by a motor-driven stirrer so adjusted that the agitation was not vigorous enough to cause air to be occluded. When the temperature of the suspension equalled that of the bath, the sample was quickly transferred to hydrom-

TABLE I. BAUMÉ–PER CENT DRY SUBSTANCE STARCH

| Baumé | Corresponding Specific Gravity (Air) | $F$ = 1.7770 60°/60° F. | $F$ = 1.7700 100°/60° F. | $F$ = 1.753 140°/60° F. |
|---|---|---|---|---|
| −2.01 | 0.9864 | ... | ... | 0.00 |
| −0.71 | 0.9951 | ... | 0.00 | 2.28 |
| 0.00 | 1.0000 | 0.00 | 1.26 | 3.52 |
| 1.00 | 1.0069 | 1.78 | 3.03 | 5.28 |
| 2.00 | 1.0104 | 3.55 | 4.80 | 7.02 |
| 3.00 | 1.0211 | 5.33 | 6.57 | 8.78 |
| 4.00 | 1.0285 | 7.11 | 8.34 | 10.54 |
| 5.00 | 1.0358 | 8.89 | 10.11 | 12.29 |
| 6.00 | 1.0433 | 10.66 | 11.87 | 14.04 |
| 7.00 | 1.0568 | 12.44 | 13.65 | 15.79 |
| 8.00 | 1.0585 | 14.22 | 15.42 | 17.55 |
| 9.00 | 1.0663 | 15.99 | 17.19 | 19.30 |
| 10.00 | 1.0742 | 17.77 | 18.96 | 21.05 |
| 11.00 | 1.0822 | 19.55 | 20.73 | 22.81 |
| 12.00 | 1.0903 | 21.32 | 22.50 | 24.56 |
| 13.00 | 1.0986 | 23.10 | 24.27 | 26.31 |
| 14.00 | 1.1071 | 24.88 | 26.04 | 28.08 |
| 15.00 | 1.1156 | 26.66 | 27.81 | 29.82 |
| 16.00 | 1.1242 | 28.43 | 29.58 | 31.57 |
| 17.00 | 1.1330 | 30.21 | 31.35 | 33.32 |
| 18.00 | 1.1419 | 31.99 | 33.12 | 35.08 |
| 19.00 | 1.1510 | 33.76 | 34.89 | 36.83 |
| 20.00 | 1.1602 | 35.54 | 36.66 | 38.58 |
| 21.00 | 1.1696 | 37.32 | 38.43 | 40.34 |
| 22.00 | 1.1791 | 39.09 | 40.20 | 42.09 |
| 23.00 | 1.1888 | 40.87 | 41.97 | 43.84 |
| 24.00 | 1.1986 | 42.65 | 43.74 | 45.60 |
| 25.00 | 1.2086 | 44.43 | 45.51 | 47.35 |

[1] Present address, Goodyear Tire & Rubber Co., Akron, Ohio.

Table II. Corrections to Be Subtracted from Assigned Specific Gravity to Give True Specific Gravity in Vacuum

| Baumé | Assigned Specific Gravity (Air) | Corrections to Be Subtracted 60°/60° F. | 100°/60° F. | 140°/60° F. |
|---|---|---|---|---|
| −2.01 | 0.9864 | ... | ... | 0.00206 |
| −0.71 | 0.9951 | ... | 0.00105 | 0.00213 |
| 0.00 | 1.0000 | 0.0000 | 0.00111 | 0.00216 |
| 5.00 | 1.0358 | 0.00005 | 0.00120 | 0.00233 |
| 10.00 | 1.0742 | 0.00012 | 0.00130 | 0.00233 |
| 15.00 | 1.1156 | 0.00019 | 0.00143 | 0.00265 |
| 20.00 | 1.1602 | 0.00025 | 0.00154 | 0.00281 |
| 25.00 | 1.2086 | 0.00031 | 0.00165 | 0.00298 |

Table III. Temperature Corrections to Be Added

| Baumé | 100° to 60° F. | 140° to 60° F. | 120° to 60° F. | 120° to 100° F. |
|---|---|---|---|---|
| 0.00 | 0.71 | 1.98 | 1.24 | 0.52 |
| 5.00 | 0.69 | 1.92 | 1.20 | 0.51 |
| 10.00 | 0.67 | 1.85 | 1.17 | 0.49 |
| 15.00 | 0.65 | 1.78 | 1.13 | 0.48 |
| 20.00 | 0.63 | 1.72 | 1.09 | 0.46 |
| 25.00 | 0.61 | 1.65 | 1.05 | 0.44 |

eter cylinders and five 4-ounce (0.12-liter) screw-top bottles. The former were used for the determination of Baumé by hydrometer and the latter for determination of density by pycnometer.

The starch in the cylinders was kept in suspension by means of a long rod which had a perforated disk at one end, and by carefully introducing and manipulating this agitator, no air was carried into the suspension. The hydrometer to be used was kept in a cylinder of distilled water adjacent to the cylinders containing the starch suspension, and when the temperature of the starch suspension was the same as that of the bath, the hydrometer was removed, quickly wiped free from the film of water, and placed in the starch suspension. When the spindle was at rest a small drop of methylene blue solution (in water) was added at the stem to accentuate the meniscus and the reading made at the upper edge of the blue line around the stem. Previous work had indicated that the differences between the reading of this point and the plane surface of the liquid (correct reading) was 0.08° Bé. Thus 0.08° Bé. was added to the observed reading and this value appears in all tabulated data for Baumé.

Density by Pycnometer. Settling difficulties presented a problem in the determinations of density by pycnometer and as a result three methods were used to overcome this trouble. None is completely free from criticism, although the latter two are regarded as more desirable. The pycnometers used have been described previously (*2*).

*a.* The 4-ounce samples mentioned above were kept in the bath while the Baumé was determined by hydrometer. Then the temperature was raised or lowered a few degrees, depending on relationship of bath to room temperature, to compensate for heat changes during the transfer of the sample to the pycnometer. The bottle was agitated every 5 minutes, then returned to the bath, reagitated, etc. After five such agitations, the sample was transferred rapidly to the pycnometer, stoppered, and placed in the bath which was now set at the desired temperature. The pycnometers were kept in the bath for 30 minutes, then removed and weighed.

Using this method, difficulty was encountered at the higher concentrations in that starch collected at the ground surfaces. The precision was about 0.05° Bé. and as a result this method was discarded for the heavier Baumés and method *b* used instead.

The moisture on the starch was determined on one of the remaining 4-ounce samples, as described below.

*b.* The pycnometers were filled about three-fourths full at room temperature, heated to about 130° F., and boiled very gently under high vacuum to remove occluded gas. The starch was then allowed to settle and the remaining space was filled with cool, recently boiled distilled water. The pycnometer was placed in the water bath and weighed at the end of an hour. From this weight it was possible to calculate the specific gravity on the basis of complete suspension.

This method eliminated the difficulty of the collection of starch between seals and also enabled any loss through the capillary of the stoppers to be taken as pure water—i. e., no solids. The moisture test was made on a separate bottle as before.

*c.* The pycnometer was used with an added feature consisting of a test tube with standard-taper joint as shown in Figure 1. The starch suspension was run into the pycnometer until it was nearly full and then allowed to settle. When the top part of the liquid was essentially clear, the stopper was inserted and the apparatus placed in the bath. After an hour the pycnometer was removed from the bath and weighed. Then the stopper and outer cap were removed and the test tube was applied. The suspension was shaken until uniform and a moisture determination made on this material.

This method eliminated the difficulties of starch at the seals; the liquid that was lost through the capillary was essentially pure water; and settling was not a dominant factor, since the moisture determination was made on the suspension actually present in the pycnometer. Tests were made to estimate the error introduced by loss of the film of water left on the stopper and cap which were removed when the test tube was attached for resuspending the starch. This was found to be 20 to 30 mg. on a 95- to 100-gram sample weight. This involves an error of 0.02 to 0.03 per cent and can be neglected for all ordinary purposes or be compensated for in work of highest precision.

Determination of Moisture. The method used is basically the same as that employed for the determination of moisture in corn sirup and corn sugar (*1*) which should be consulted for details.

*Diatomaceous Silica*, prepared Johns-Manville Hy-Flo (*1*, *2*).

*Apparatus.* Wide-mouth Erlenmeyer flasks, 250-ml. capacity, with 40/12 standard taper, with stoppers. Pyrex test tubes 100 × 15 mm. (*1*).

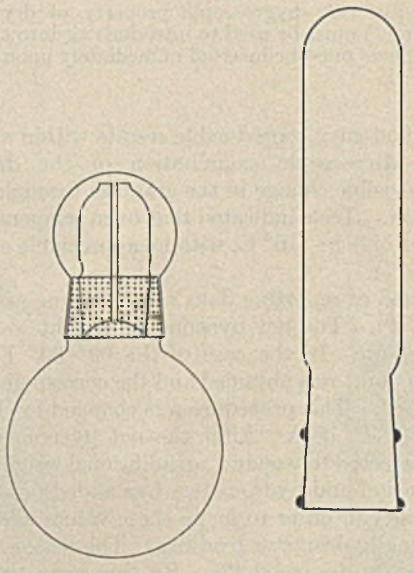

Figure 1

*Procedure.* Twenty-five to 35 grams of diatomaceous silica were run into duplicate flasks, the test tube was introduced, and weight constancy was obtained under oven conditions identical with those of the test. Weighings were made with an empty flask as a tare. The starch samples, either in the 4-ounce bottles or the pycnometer equipped with test tube, were shaken until homogeneous suspensions were obtained. Sample portions, sufficiently large to yield 5 to 8 grams of dry substance, were transferred quickly to the flasks by means of a pipet from which the tip had been cut. The sample was then worked into the diatomaceous silica by means of the test tube, which yielded a damp powdery mass.

The flasks were placed in a vacuum oven at 100° C., and the pressure was reduced first by an efficient water pump until most of the water was removed and then by means of a Megavac pump. After approximately 4 hours of drying the flasks were removed

TABLE IV. BAUMÉ–DRY SUBSTANCE STARCH

(100°/60° F., $F$ = 1.7700, 10° hydrometer)

| Baumé | Specific Gravity (Air) | Dry Substance | Pounds per Gallon | Pounds Dry Substance per Gallon |
|---|---|---|---|---|
| 0.00 | 1.0000 | 1.26 | 8.328 | 0.105 |
| 1.00 | 1.0069 | 3.03 | 8.386 | 0.254 |
| 2.00 | 1.0140 | 4.80 | 8.445 | 0.405 |
| 3.00 | 1.0211 | 6.57 | 8.504 | 0.559 |
| 4.00 | 1.0285 | 8.34 | 8.565 | 0.714 |
| 5.00 | 1.0358 | 10.11 | 8.626 | 0.872 |
| 6.00 | 1.0433 | 11.88 | 8.689 | 1.032 |
| 7.00 | 1.0508 | 13.65 | 8.751 | 1.195 |
| 8.00 | 1.0585 | 15.42 | 8.815 | 1.359 |
| 9.00 | 1.0663 | 17.19 | 8.880 | 1.526 |
| 10.00 | 1.0742 | 18.96 | 8.946 | 1.696 |
| 11.00 | 1.0822 | 20.72 | 9.013 | 1.867 |
| 12.00 | 1.0903 | 22.50 | 9.081 | 2.043 |
| 13.00 | 1.0986 | 24.27 | 9.150 | 2.221 |
| 14.00 | 1.1071 | 26.04 | 9.220 | 2.401 |
| 15.00 | 1.1156 | 27.81 | 9.291 | 2.584 |
| 16.00 | 1.1242 | 29.58 | 9.362 | 2.769 |
| 17.00 | 1.1330 | 31.34 | 9.436 | 2.957 |
| 18.00 | 1.1419 | 33.11 | 9.510 | 3.148 |
| 19.00 | 1.1510 | 34.88 | 9.586 | 3.344 |
| 20.00 | 1.1602 | 36.65 | 9.662 | 3.541 |
| 21.00 | 1.1696 | 38.42 | 9.741 | 3.742 |
| 22.00 | 1.1791 | 40.19 | 9.820 | 3.947 |
| 23.00 | 1.1888 | 41.96 | 9.900 | 4.154 |
| 24.00 | 1.1986 | 43.73 | 9.982 | 4.365 |
| 25.00 | 1.2086 | 45.50 | 10.065 | 4.580 |

from the oven and cooled and the mass in the flasks was reworked into a fine powder. The flasks were returned to the oven, and a pressure of less than 1 mm. maintained until weight constancy was obtained. An overnight period has been found adequate. Because of the very hygroscopic property of dry starch, an efficient train (*1*) must be used to introduce air into the oven and the flask stoppers must be inserted immediately upon opening the oven.

This method gave reproducible results within very narrow tolerance. Microscopic examination of the dried starch revealed no visible change in the granules through rupture or gelatinization. Tests indicated that oven temperatures could be carried as high as 110° C. with no appreciable effect on the results.

The method of reporting data was the same as that previously used (*2*). The net pycnometer weight was corrected first to vacuum. In the case of the 60°/60° F. data, the density (vacuum) was obtained and the corresponding Baumé value assigned. This procedure was changed for 140°/60° F. and 100°/60° F. data. After the net pycnometer weights had been corrected to vacuum, an additional weight correction (glass expansion and hydrometer) was added before calculating the density in order to make these values agree with the corresponding hydrometer readings. The reason for this has been previously discussed (*2*). By this correction observed Baumé readings and Baumé values obtained by pycnometer always agreed within 0.05° Bé. and with an average deviation of 0.03° Bé.

The Baumé–dry substance data were plotted in two ways: Baumé *vs.* dry substance and Baumé *vs.* factors. In both cases the best curve was a straight line, as it should be if the slurry were a true suspension. The previously used method of factors was employed for purposes of tabulation.

The factors were:

$$\text{Factor for } 60°/60° \text{ F.} (F60°/60° \text{ F.}) = \frac{D.S.}{\text{Bé.}} = 1.7770$$

$$\text{Factor for } 100°/60° \text{ F.} (F100°/60° \text{ F.}) = \frac{D.S.}{\text{Bé.} + 0.71°} = 1.7700$$

$$\text{Factor for } 140°/60° \text{ F.} (F140°/60° \text{ F.}) = \frac{D.S.}{\text{Bé.} + 2.01°} = 1.753$$

In Table I, the Baumés in all cases are the observed values and the specific gravities (air) are those corresponding to the Baumés.

In the above data the specific gravities at 60°/60° F. in air can be converted to vacuum by the usual calculations. The 100°/60° F. and the 140°/60° F. data require not only the calculation for air to vacuum but also those for glass expansion and for hydrometer. This has been discussed previously (*2*) and an abridged table (Table II) is given here for those who may desire to express the data on some other basis.

Since most Baumé tests in factory processes are not made at a fixed temperature, a table of temperature corrections is necessary (Table III).

The determination of each point in the table furnished enough data for calculation of the density of the actual starch solids. Hence a series of independent starch density values were obtained over the range of suspension gravities. These were observed to check very closely in the 60°/60° F. series and to average 1.636. Employing this value for the Baumé–dry substance relationship, a calculated table was found to check the experimental table within the limits of the experimental methods used. It would appear, therefore, that tables for water suspensions of the whole gravity range could be prepared for any starch from an accurate density value of starch solids determined at a single point. Each temperature, of course, would require a separate determination.

A table has been prepared for general factory use based on 100° F (Table IV). In this table the Baumés are the observed readings, the specific gravities are those in air assigned to the Baumé values and the weights per gallon are based on these specific gravities.

Briefly, the corrections (Table V) mean that when the 100°/60° F. table is used: for each 5° F. below 100° F., subtract 0.1° Bé. from the reading; for each 4° F. above 100° F., add 0.1° Bé. to the reading.

TABLE V. TEMPERATURE CORRECTIONS IN BAUMÉ

(To be added to or subtracted from observed Baumé to adjust to 100° F.)

| Temperature, ° F. | Observed Baumé (10° Hydrometer) 0 | 5 | 10 | 15 | 20 | 25 |
|---|---|---|---|---|---|---|
| | Subtracted from Observed Baumé | | | | | |
| 80 | 0.40 | 0.39 | 0.38 | 0.37 | 0.36 | 0.35 |
| 85 | 0.31 | 0.31 | 0.29 | 0.29 | 0.28 | 0.27 |
| 90 | 0.21 | 0.21 | 0.20 | 0.20 | 0.19 | 0.19 |
| 95 | 0.11 | 0.11 | 0.11 | 0.10 | 0.10 | 0.10 |
| | Added to Observed Baumé | | | | | |
| 104 | 0.09 | 0.09 | 0.09 | 0.08 | 0.08 | 0.08 |
| 108 | 0.19 | 0.19 | 0.18 | 0.17 | 0.17 | 0.16 |
| 112 | 0.30 | 0.28 | 0.28 | 0.27 | 0.26 | 0.25 |
| 116 | 0.41 | 0.39 | 0.38 | 0.37 | 0.36 | 0.34 |
| 120 | 0.52 | 0.51 | 0.49 | 0.48 | 0.46 | 0.44 |

## Conclusions

Experimental results indicate that tables for Baumé–dry substance starch can be made mathematically, provided the density of any starch is known.

A procedure for drying starch slurries or suspensions is presented.

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# Dual Alternating Current Titrometer

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**An instrument is described which comprises two complete titration stands and an alternating current–operated electrometer which allows the use of any electrode system in aqueous or nonaqueous solutions. Meter scales and potentiometer circuits are provided which give the instrument a range of −1.65 to +1.65 volts, readable to 0.5 millivolt, and with a calomel–glass electrode system, a full pH range readable to 0.02 pH unit. The stability is excellent and the grid current of the order of $10^{-12}$ ampere.**

THE increased use of sturdy glass electrodes and nonaqueous, high-resistance titration media in potentiometric titrations has emphasized the need for a versatile potentiometer, or millivoltmeter (*5*), that will satisfactorily measure the potential between electrode terminals of a cell having a resistance up to 5000 megohms. The final step in the development of an ideal laboratory titrometer—namely, the elimination of batteries with their attendant replacement problem and constant drift—is the subject of the present paper.

The dual alternating current titrometer, like the battery-operated instrument previously described, is continuously indicating and employs a step potentiometer in combination with the vacuum tube electrometer. The use of a continuous indicating meter not only requires less manipulation by the operator, but also enables him easily and definitely to ascertain when the potential reaches equilibrium. The step potentiometer makes it possible to meet the requirement of wide range along with high sensitivity in a single indicating meter.

Figure 1 is a photograph of the instrument showing the housing which contains the electrometer circuit and supports the titration stands.

## Accessory Equipment

The convenient titration stand previously described (*3*) has been incorporated to provide a complete titration unit which can be used for routine as well as special determinations.

## Electrical Circuit

The electrometer circuit, shown in Figure 2, is of the single-stage, balanced-input type (*2*, *7*). Careful circuit balance and large values of negative feedback have resulted in a highly stable circuit which has negligible zero-drift when operated under moderate conditions of line voltage variation.

The input grid current is of the order of $10^{-12}$ ampere. A minimum value of grid current is obtained by varying the plate voltage of the electrometer tubes so that the grid voltage–grid current curve is shifted along the voltage axis until zero grid current is obtained for an input voltage of plus 100 to 150 millivolts. When this condition is fulfilled the maximum grid current for both positive and negative input voltages is approximately equal and occurs at full-scale deflection. A typical $E_g$–$I_g$ curve for an average tube is shown in Figure 3; the grid current does not exceed $3.7 \times 10^{-12}$ ampere in any part of the range from −250 to +250 millivolts.

The calibration of the linear scale is within the 2 per cent error of the meter used. Individual calibration of the scale would materially increase the accuracy of the instrument.

Two Type 38 tubes are used as the electrometer tubes. The rectifier is a type 6ZY5G, and a VR105 serves as a voltage regulator. Total power consumption is approximately 10 watts. The vacuum tubes may be expected to have long life with this instrument because they are all operated well below their normal ratings. The replacement of the rectifier tube (6ZY5G) should require no circuit adjustments. Replacement of the voltage regulator (VR105) may require an adjustment of the potentiometer circuit.

## Controls

There are six panel controls: three switches and three variable resistors. The switches, requiring more frequent manipulation than the resistors, are placed at the bottom of the sloping front panel where they are convenient to reach. The 12-point switch,

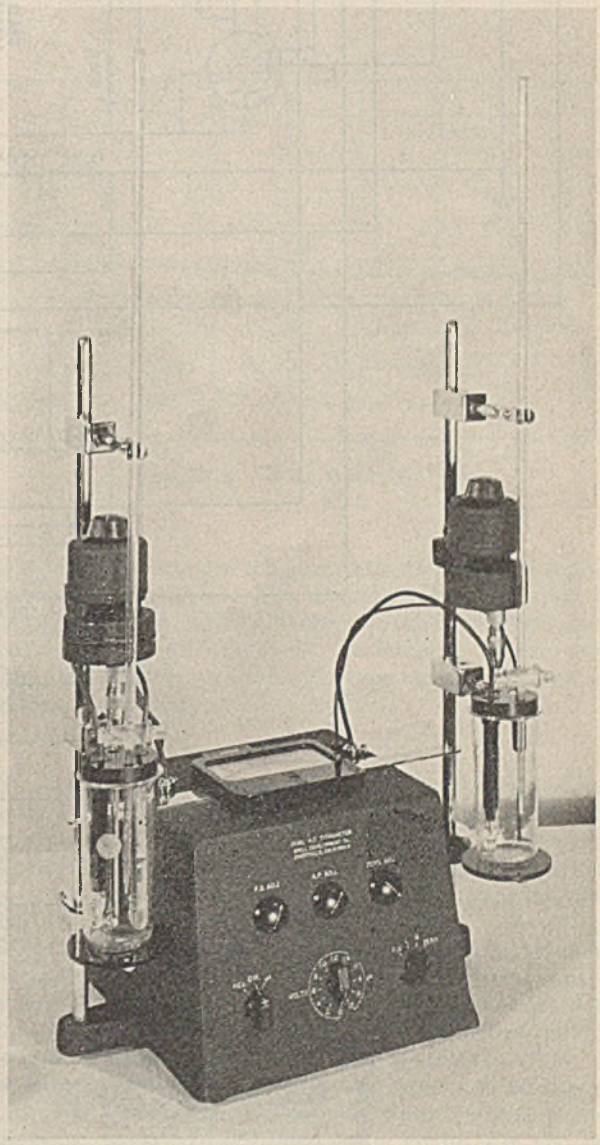

FIGURE 1. DUAL ALTERNATING CURRENT TITROMETER

$S_3$, controls the potentiometer circuits for both the millivolt and pH ranges. Switch $S_2$ reverses the meter and also sets up the circuit for the pH calibration. Switch $S_1$ shorts the electrode leads for zero adjustment, and connects them to a source of 250 millivolts for full-scale adjustment, and to either the right- or left-side electrodes for potential or pH measurements. The variable resistor controls are for zero and full-scale adjustment and for compensation of variations in asymmetry potential of individual glass electrodes.

In addition to the three panel controls, there are three controls mounted on the chassis inside the cabinet. One of these is a filament series resistor, *FIL.*, for tube balance, another aids in obtaining minimum grid current by adjusting the plate voltage, *P.V.*, and a third, *STD*, adjusts the main voltage divider to standard voltage.

## Power Supply

All the direct current voltages are obtained from a voltage divider which is maintained at constant potential by the regulator tube. Plus or minus 10 volts' change in line voltage (115 volts) results in a change in direct current voltage of ±0.1 per cent.

No power switches are provided; the rheostats on the motors have "off" positions against the counterclockwise stop, and it is intended that the electrometer circuit be operating continuously where it is in steady or intermittent use. The permanent tem-

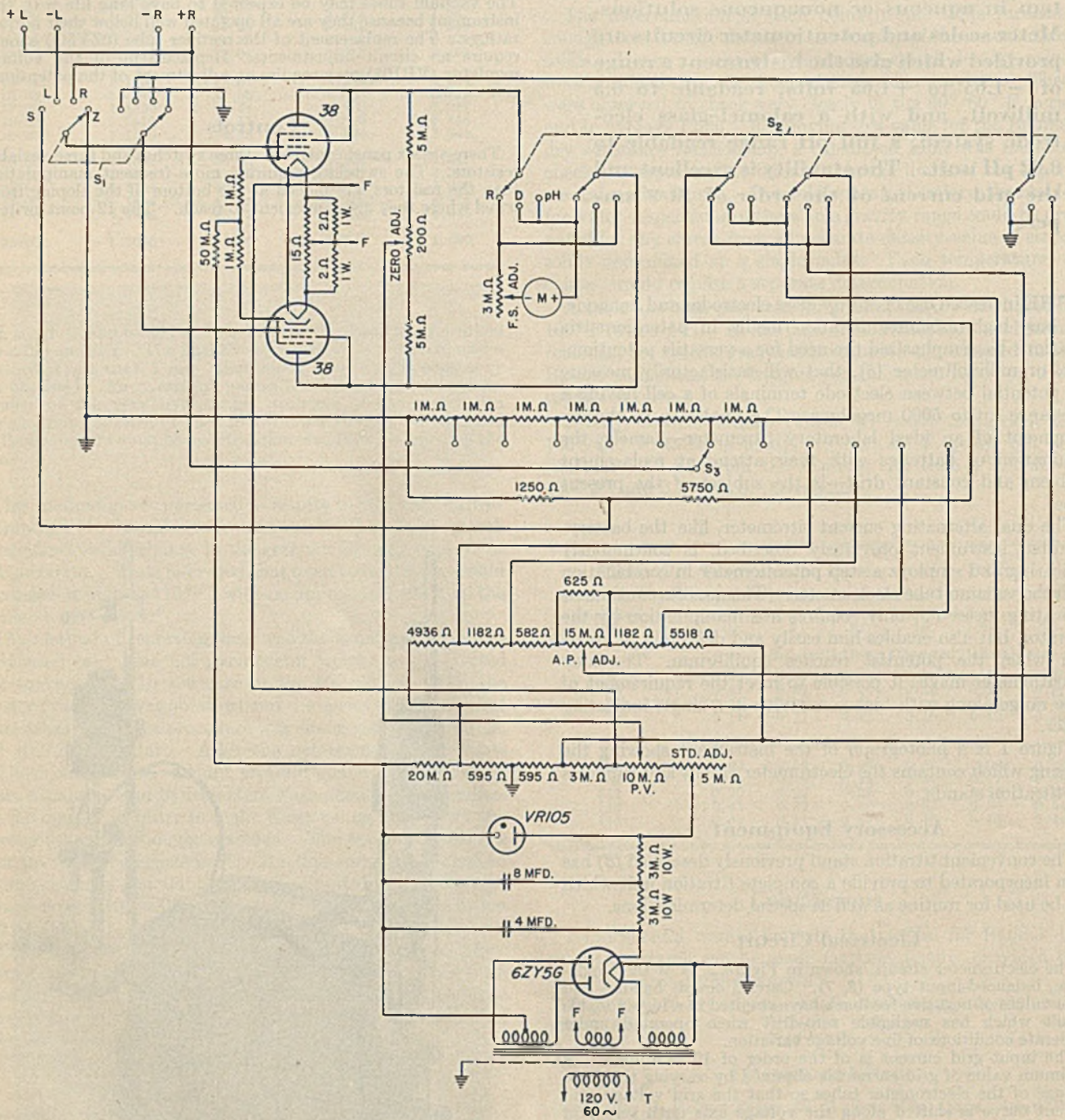


FIGURE 2. WIRING DIAGRAM OF TITROMETER

*M.* Simpson Model 29, 0- to 25-milliampere direct current meter
*S1.* Centralab Isolantite switch 2505
*S2.* Centralab Bakelite Switch 1417
*S3.* Yaxley switch 32112J
*T.* Specialty Division, Gardner Electrical Mfg. Co., Emeryville, Calif., transformer S 3690
Variable resistors, Yaxley Type 4 MP
Fixed resistors, Type R, Precision Resistor Co. W. W. ±1 per cent unless otherwise noted

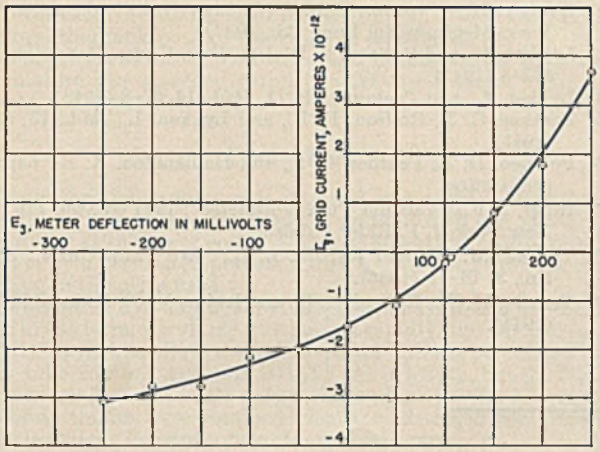


FIGURE 3. GRID CURRENT TYPE 38 TUBES

perature equilibrium thus established results in more stable operation, and the very low power consumption makes continuous operation entirely practical.

## Constructional Details

In order that the instrument may function on a high input resistance, all connections to the "high" tube must be exceedingly well insulated. Switch $S_1$ should be Isolantite-insulated, the spring contact for the glass electrode connection should be supported on Isolantite or polystyrene, and the interconnecting leads should be of rigid bus-bar suspended in air between end terminals.

The envelope of the 38 electrometer tube is coated with ceresin wax to prevent surface leakage. The envelopes are first carefully cleaned and then immersed in the molten wax (approximately 140° C.) until all bubbling stops; the grid cap must be freed of wax before the tube is placed in service.

The instrument must be completely enclosed in a metal box, with the high input terminal located well within the box. A shielded-lead type of glass electrode is necessary (*1*). Sheet iron can be used for all the box except the meter panel, which must be of nonmagnetic material because of the shunting effect of steel on the meter.

Lubricating the wires and sliding contacts of all the variable controls with a good grease is necessary to prevent fluctuations caused by poor contact.

## Circuit Adjustments

After the tubes are installed the instrument is plugged into the power outlet and allowed to reach temperature equilibrium. As the tubes warm up, the meter needle will swing across the dial once or twice and then approach a zero reading.

ZERO ADJUSTMENT. Switch $S_1$ is placed on *ZERO*, the panel knob, *ZERO ADJ.*, is set in the center of its travel, and the chassis-mounted filament rheostat, *FIL.*, is adjusted until the meter reads zero deflection. It may be necessary to interchange the tubes (Type 38) to complete this adjustment. The needle may be adjusted to an exact zero reading by means of the *ZERO ADJ.* knob. Further zero adjustments will be required at longer intervals until the instrument has reached a temperature equilibrium.

STANDARD ADJUSTMENT. One thousand millivolts from an accurate external potentiometer are applied to the input leads and switch $S_2$ is set at 1.0 volt. The meter should indicate zero deflection and may be adjusted to that value by means of the chassis-mounted control, *STD.*

FULL-SCALE ADJUSTMENT. Switch $S_1$ is placed on *F.S.* and the *F.S. ADJ.* rheostat varied until the meter indicates full scale.

MINIMUM GRID CURRENT ADJUSTMENT. (1) The glass electrode terminals are removed from their sockets.

2. Switch $S_1$ is placed on *R* or *L*, and switch $S_2$ on *REV.*, and the electrometer circuit is then completely shielded.

3. Potentiometer *P.V.* is adjusted so that the meter needle floats near mid-scale (100 to 150 mv.). This is done by means of a screw driver inserted through holes provided in the bottom plate of the cabinet. Since there is a slight interlocking of controls, it is advisable to check the full-scale adjustment. *F.S.*, after setting *P.V.*

If e. m. f. is to be measured, no other adjustments are required, but if a glass electrode system is to be used for pH readings, it will be necessary to make an adjustment for the asymmetry potential of the particular glass electrode used. To make this adjustment, switch $S_2$ is placed in the pH position, and if the usual pH 7 buffer is used, switch $S_3$ in placed on 4 pH. Switch $S_1$ is next placed on *L* if the left-side electrodes are used, or on *R* if the right-side electrodes are used, and the *A.P. ADJ.* knob is varied until the meter indicates 3 pH (a total reading of 7 pH—i. e., 4 pH on the potentiometer switch and 3 pH on the meter). Only one asymmetry potential adjustment is provided, since potential equilibrium is ordinarily reached so rapidly in aqueous solutions that there is no advantage in making pH determination alternately in two separate systems.

When potential measurements are made, the potentiometer switch is advanced from the zero position until the meter needle deflects on scale. The measured potential is then the sum of the potentiometer setting plus the meter reading.

If the meter indication is below zero when the potentiometer is on zero, the reversing switch, $S_2$, must be placed in its alternative position. When this switch is in the *D* (direct) position, the glass electrode is negative—that is, negative in the usual sense as applied to batteries and indicating electrical meters.

## Operating Characteristics

Because of the electrical characteristics of the circuit, the meter may safely be used to measure any potential without previous knowledge of the magnitude or sign of the potential being measured, and without danger of polarization of the electrode system.

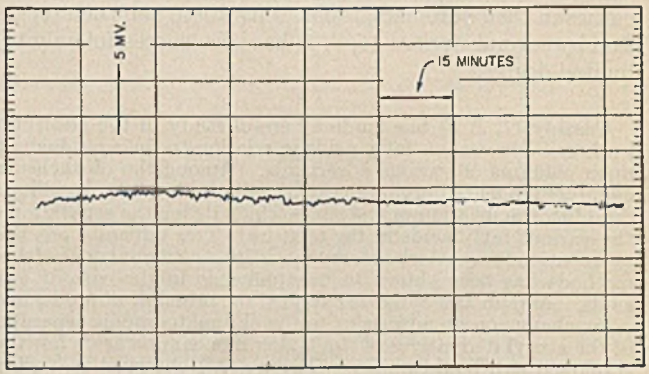


FIGURE 4. ZERO STABILITY RUN

Figure 4 is a record of a 2-hour stability run made without line voltage regulation. The maximum deviation over this period is less than ±1.5 millivolts and the momentary deviations are about ±0.5 millivolt. In locations having severe voltage fluctuations a transformer-type regulator will provide a perfectly stable zero.

## Uses

In addition to all the usual applications involving electrometric titrations using high-resistance glass, silver, platinum, antimony, and tungsten electrodes with their corresponding reference electrodes, and for spot determinations of pH, the instrument has been particularly useful in mercaptan determinations (*4, 8, 9*).

The dual feature permits two titrations to progress simultaneously, which approximately doubles the work capacity for lengthy titrations in nonaqueous media, such as determination of neutralization number of lubricating oils (*1*). Alternatively two diverse electrode systems may be left permanently set up, so that, for example, both acidity and sulfur titrations may be made without delay on the single unit.

SPECIAL APPLICATIONS. The entire circuit has also been built into the case of a standard Model R, Leeds & Northrup,

recorder-controller. It has a range of 8 pH units, adjustable within the range 0 to 14 pH.

The circuit has also been successfully used for some time in a semiautomatic titrating device (*6*).

### Acknowledgment

The authors wish to express their appreciation of the encouragement of L. Lykken and D. J. Pompeo.

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# Colored Chromatograms with Higher Fatty Acids

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THE higher fatty acids, being colorless compounds of similar chemical and physical properties, present difficulties in their chromatographic separation, and such separation by several workers (*5*, *6*, *7*) has been successful only to a limited degree.

Cassidy (*1*, *2*, *3*) has made a careful study of the adsorption isotherms for various fatty acids in relation to their resolvability upon columns of various adsorbents. Among the obstacles encountered in using chromatographic columns has been inability to establish the location of the zones which define the separation of the various fatty acids in the mixture. This difficulty has been overcome in some colorless compounds (*10*, *11*, *13*), but none of these methods has been shown to be applicable in the case of fatty acids. Martin and Synge (*8*) applied the principle of producing a color change on an adsorbent to the chromatographic separation of the acetyl derivatives of the higher monoamino acids but they employed two liquid phases. Trappe (*12*) observed that silica-gel columns became transparent or translucent when wetted by certain solvents, and under these conditions it was possible to observe zones formed in the study of lipid mixtures.

This paper describes a method for separating mixtures of certain higher fatty acids into their components with the aid of adsorption columns, using an adsorbent impregnated with a dye to serve as an indicator. To be suitable for following the adsorption of fatty acids on an adsorbent, an indicator must change color on the column when in contact with the fatty acid, it must be insoluble in both the solvent for the fatty acid and the eluting agent and, in addition, it must revert to its original color after elution of the fatty acid. Preliminary experiments showed that phenolsulfonphthalein (phenol red) as manufactured by the Eastman Kodak Company satisfies the above conditions. Columns prepared with heavy magnesium oxide impregnated with this indicator proved satisfactory in bringing about and observing a separation. (Good results were obtained only when the heavy magnesium oxide manufactured by the J. T. Baker Chemical Company and the phenol red manufactured by the Eastman Kodak Company were used.)

The fatty acids were recovered from the adsorbent by dissolving the magnesium oxide in particular sections of the column (*4*) in concentrated hydrochloric acid and water, and subsequently extracting the fatty acid with diethyl ether. Magnesium oxide was found to be particularly adaptable to this procedure, in that it is readily soluble in the acidified water.

### Reagents

Magnesium oxide, U. S. P. Baker's heavy powder; phenolsulfonphthalein (phenol red), Eastman Kodak Company; petroleum ether, Skellysolve F having a boiling range of about 30° to 60° C. The petroleum ether was distilled before use.

### Fatty Acid Samples

Stearic acid, Eastman Kodak Company, m. p. 69.8° C.; myristic acid, Eastman Kodak Company, m. p. 54.6° C.; oleic acid, Baker's U. S. P.

### Preparation of Impregnated Magnesium Oxide

About 0.5 gram of phenol red was dissolved in 10 to 15 ml. of 95 per cent alcohol. A few grams of magnesium oxide were added and stirred into a paste, which was mixed thoroughly

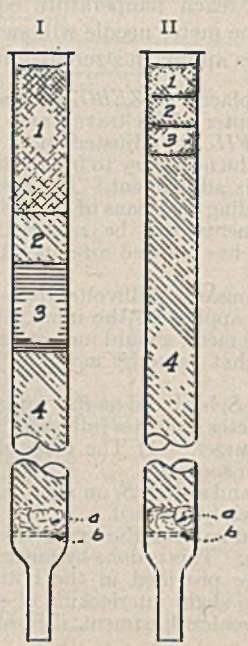


FIGURE 1. ABSORPTION COLUMNS
I, Oleic and stearic acids. II. Myristic and stearic acids.
*a*. Cotton plug. *b*. Perforated porcelain disk.

| Zone | Length of Zone I | Length of Zone II |
|---|---|---|
| | *Mm.* | *Mm.* |
| 1 | 35 | 8 |
| 2 | 12 | 7 |
| 3 | 21 | 7 |
| 4 | 125 | 175 |

| | Color I | Color II |
|---|---|---|
| 1 | Yellow pink | Yellow pink |
| 2 | Original pink | Original pink |
| 3 | Light pink | Yellow pink |
| 4 | Original pink | Original pink |

while the alcohol was being evaporated on an electric hot plate. The resulting pink powder was dried in an oven at 100° C. The dried powder was mixed with 100 grams of magnesium oxide and the mixture was screened through a 200-mesh sieve. The adsorbent was then ready for use.

## Procedure

The adsorption column was retained in a vertical cylindrical glass tube 11 to 12 mm. in diameter and 60 to 70 cm. in length, constricted at the lower end. A plug of absorbent cotton held in position by a perforated porcelain disk was tamped over the constriction in the glass tube.

A mixture of dye-impregnated magnesium oxide and petroleum ether in the form of a slurry was poured rapidly into the tube and the adsorbent was allowed to settle as the solvent percolated out of the tube, forming a column 20 to 22 cm. in length, but the liquid was never allowed to fall below the surface of the adsorbent. The best columns were obtained when the adsorbent was allowed to settle under a layer of solvent for several hours.

The fatty-acid mixtures were dissolved in about 50 ml. of petroleum ether and added to the column. The column was then cautiously filled with petroleum ether and connected by means of a siphon to a reservoir-flask of petroleum ether at a higher level. When the fatty-acid mixture came in contact with the adsorbent, a bright yellow band was formed which separated into zones upon development. The chromatogram was developed by allowing petroleum ether to percolate through the adsorbent continuously until clearly defined zones were obtained. The volume of petroleum ether used and the rate of flow varied, depending on the packing of the column.

After formation of clearly defined zones, the column was permitted to run dry, forced from the tube, and then cut into sections as defined by the change in the color of the adsorbent. The cuts were made in order best to demonstrate the separation of the constituents of the mixture. The fatty acids were recovered from the adsorbent by dissolving the latter in hydrochloric acid and then extracting with diethyl ether. The ethereal extracts were washed free of indicator and acid by water in a separatory funnel and subsequently dried over anhydrous sodium sulfate and evaporated to dryness under a stream of nitrogen. The residues were dried in a vacuum over phosphorus pentoxide to constant weight. Samples were removed for iodine number (*9*) and melting point determinations.

Table I. Adsorptive Separation of a Mixture of Oleic and Stearic Acids (Commercial Grades)

| Section | Weight of Fraction *Mg.* | Iodine No. | Melting Point ° *C.* |
|---|---|---|---|
| 1 | 110.2 | 1.6 | 68–69 |
| 2 | 21.6 | 72.8 | ... |
| 3 | 71.3 | 91.6 | ... |
| 4 | 1.4 | ... | ... |

## Experimental

By means of the above procedure it was possible to demonstrate a separation of (I) an unsaturated fatty acid from a saturated fatty acid of the same number of carbon atoms and (II) two saturated fatty acids differing in length of chain by 4 carbon atoms.

In (I) a mixture of 100 mg. of stearic acid ($C_{18}H_{36}O_2$), melting point 69.8° C., and 100 mg. of oleic acid ($C_{18}H_{34}O_2$), iodine number 86.4, was used. The development of the chromatogram required 17 days. The appearance of the column and the definition of the zones are sketched in Figure 1. The analytical data shown in Table I indicate an almost complete separation of the fatty acids in the mixture. Practically all of the stearic acid was retained in section 1 and the oleic acid had moved down the column.

The fractions recovered from sections 2 and 3 were pale yellow oils.

In (II) a mixture of 50 mg. of stearic acid ($C_{18}H_{36}O_2$), m. p. 69.8° C., and 50 mg. of myristic acid ($C_{14}H_{28}O_2$), m. p. 54.6° C., was used. The development of the chromatogram required 14 days. The appearance of the column and the definition of the zones are sketched in Figure 1 and the analytical data are tabulated in Table II.

The fractions recovered on evaporation of the ether solutions from the different sections to dryness, without recrystallization, gave successively lower melting points from the top to the bottom of the column, indicating that a separation had been accomplished. These fractions probably contained traces of solvent or other accumulated impurities. By recrystallization from acetone it was possible to show that the acids recovered from section 1 and section 2 consisted mainly of stearic acid and myristic acid, respectively.

The generality of the application of this method to chromatographic separation of a mixture of fatty acids is indicated by the fact that definite zones have been obtained with other mixtures of fatty acids. In the case of palmitic and stearic acids, where the length of chain differs by only two carbon atoms, zones were developed, but it was difficult to establish definite proof of a separation using melting points as criteria because of the proximity of their melting points.

Table II. Adsorptive Separation of a Mixture of Myristic and Stearic Acids (Commercial Grades)

| Sections | Weight of Fraction *Mg.* | Melting Point ° *C.* | Melting Point after Recrystallizing 1st time ° *C.* | Melting Point after Recrystallizing 2nd time ° *C.* |
|---|---|---|---|---|
| 1 | 44.7 | 56–57 | 63 | 68 |
| 2 | 36.5 | 51–52 | 54 | 54 |
| 3 | 31.5 | 44–46 | .. | .. |
| 4 | 0.8 | ... | .. | .. |

## Summary

A method has been described for separating mixtures of higher fatty acids by Tswett adsorption analysis, whereby separation into zones was observed on a column of heavy magnesium oxide impregnated with a suitable indicator. The fatty acids were recovered by dissolving the magnesium oxide in particular sections of the column in acid and extracting with ether. By this procedure it was possible to demonstrate a separation of an unsaturated fatty acid from a saturated fatty acid of the same number of carbon atoms and of two saturated fatty acids differing in chain length by four carbon atoms.

## Acknowledgment

The authors wish to thank H. R. R. Wakeham for his interest and cooperation in the course of this work.

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# Determination of Fluorine in Organic Compounds with Cerous Nitrate

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THE increasing importance of organic fluorine compounds makes a simple, reliable method for the determination of fluorine in such compounds extremely desirable. Reliable methods for the determination of chlorine, bromine, and iodine in organic compounds have been available for many years. However, fluorine differs considerably in its chemical behavior and the usual halogen methods cannot be applied for its determination.

Halogens are generally determined in organic compounds by decomposition of the compound and then determination of the halide ion. The fluorine-to-carbon bond is much more stable than the other halogen-to-carbon bonds and this makes the problem of decomposing organic fluorine compounds, to transform the fluorine to the fluoride ion, somewhat more difficult than in the case of the other halogen compounds.

In the case of compounds of the less stable type, where the fluorine is on a side chain or in certain aliphatic compounds, the decomposition may be accomplished by refluxing with metallic sodium or potassium in absolute alcohol or any other suitable inert solvent. Drogin and Rosanoff (*15*) applied this method for chlorine, bromine, and iodine, but Whearty (*59*) was not very successful in applying it to the analysis of several fluorine compounds. Vaughn and Nieuwland (*57*) used sodium in liquid ammonia or if the compound was insoluble in liquid ammonia, added ether. In a few cases where the carbon bonded to the fluorine is also bonded to oxygen, as with carbonyl fluorides, fluorinated alcohols, or sulfonyl fluorides, decomposition may be accomplished by refluxing with aqueous or alcoholic potassium hydroxide. This was used by Morgan and Tunstall (*42*) with boron beta-diketone fluorides.

Piccard and Buffat (*46*) heated fluorobenzene in a glass tube with potassium at 400° C. and obtained a rapid removal of the fluorine. Similarly Simons and Block (*53*) and Elving and Ligett (*17*) decomposed fluorocarbons and other fluorine compounds by heating with sodium or potassium in an evacuated tube.

Kimball and Tufts (*32*) recommended thermal decomposition. Although chlorine, bromine, and iodine can be transformed to free ions by this method at moderately high temperatures, Meyer and Hub (*40*) found that fluorine bonded to aromatic rings required 2 hours' heating at 1000° C. in a nickel or platinum bomb tube for complete transformation into the fluoride ion.

Bockemüller (*9*) considered the general methods of decomposition and used a combustion method. The fluorine compound was burned in an atmosphere of oxygen at 900° C. in a platinum tube with the exit end of the tube packed with granular calcium oxide. Hubbard and Henne (*30*) used a similar method in a silica tube packed with granular quartz. Bigelow (*8*) modified the method slightly. The method appears to be very useful for gaseous compounds and very volatile liquids, but has not been used for stable solid substances.

Another method which appears to have general application is sodium peroxide fusion using a Parr bomb (*36*, *45*). Hahn and Reid (*24*), Bigelow (*8*), and Locke, Brode, and Henne (*37*) have reported analyses of fluorine by this method.

After the fluorine is in the ionic state, it may be determined either gravimetrically or volumetrically. The gravimetric methods most generally used are those of Berzelius (*7*, *9*), Starck (*55*) and Hawley (*25*), Pisani (*47*), and Allen and Furman (*2*). Adolph (*1*) obtained reliable results with both the calcium fluoride method and lead chlorofluoride method, which was also used by Elving and Ligett (*17*), but the thorium fluoride method of Pisani gave low results. The triphenyl tin fluoride method of Allen and Furman is somewhat inconvenient, since the precipitation is made in boiling 70 to 80 per cent alcohol.

The most important volumetric methods are those of Willard and Winter (*60*), Kurtenacker and Jurenka (*34*), Batchelder and Meloche (*5*), Gyot (*23*), Greef (*22*), Offerman (*44*), Hempel and Scheffler (*26*), Wagner and Ross (*58*), and Hoffman and Lundell (*28*).

Willard and Winter titrated with thorium nitrate using a zirconium-alizarin mixture as indicator, and Langer (*35*) made this titration polarographically. Gyot and Greef titrated the fluorine with ferric chloride, using thiocyanate as indicator, and Treadwell and Köhl (*56*) determined this equivalence point potentiometrically. Hoffman and Lundell precipitated the fluorine as lead chlorofluoride, filtered, dissolved in nitric acid, and determined the chloride by the Volhard method.

Kurtenacker and Jurenka and Batchelder and Meloche proposed the titration of the fluoride with cerous nitrate. The titration must be started with the solution about neutral and at the equivalence point there is a rapid change in the pH which can be determined by using methyl red as indicator or potentiometrically (*3*). The titration has also been made with yttrium nitrate (*20*).

The remaining methods depend upon the evolution of fluorine as silicon tetrafluoride, absorbing it in water or alkali, and titrating. Comprehensive surveys of the methods of determining fluorine are given by Meyer (*39*) and Hernler and Pfeningberger (*27*).

## Experimental

The titration of fluorine with cerous nitrate after the decomposition of the organic compound with sodium peroxide has the possibility of an accurate, rapid, and convenient method for the determination of fluorine but has been found to give erratic results. Therefore, this work was undertaken to make a systematic study of the effect of the initial pH, initial volume, concentration of fluorine, temperature, presence of neutral salts, and use of alcohol as solvent to determine the optimum conditions and limitations of this method.

Pure sodium fluoride was prepared by evaporating reagent hydrofluoric acid in a platinum dish to about three-fourths its volume, neutralizing with sodium carbonate solution, making just acid with hydrofluoric acid, and cooling. The sodium fluoride was filtered off on a sintered-glass funnel, washed with 50 per cent alcohol, dried at 110° C., and ignited to a dull red. This pure sodium fluoride was analyzed for fluorine by the lead chlorofluoride method (*25*) and three analyses gave results of 45.26 ± 0.24 per cent fluorine. A spectroscopic examination showed only a trace of silicon and less than 0.01 per cent of potassium.

Pure cerous nitrate was prepared by the method of Smith, Sullivan, and Frank (*54*). One thousand grams of 40 per cent crude ceric oxide were added slowly to 2 liters of hot concentrated nitric acid in a large evaporating dish on a steam bath. After all the oxide was added the gelatinous residue was filtered off, 400 grams of ammonium nitrate were added to the clear, deep red filtrate, and the solution was evaporated slowly. When the supernatant liquid became light yellow, the crystals of ceric ammonium nitrate were filtered off on a sintered-glass funnel. This salt was about 95 per cent pure and when ignited to the oxide was dark brown, which is characteristic of ceric oxide contaminated with other rare earth oxides (*18*). Five recrystallizations from hot concentrated nitric acid gave pure ceric ammonium nitrate. The absorption spectrum of a 0.3 *M* solution of this salt with a glass-prism Hilger spectrograph showed that praseodymium and neodymium were present in less than 2 parts per thousand (*4*, *10*, *49*, *50*).

The purified ceric ammonium nitrate was dissolved in water, reduced with perhydrol, and precipitated with oxalic acid. The cerous oxalate was washed several times by decantation with a hot, saturated ammonium oxalate solution, filtered, washed free from ammonium oxalate, and dissolved in nitric acid. It was necessary to digest the solution on a steam bath for some time to destroy the oxalate completely. The cerium was again precipitated with sodium carbonate and the cerous carbonate filtered and washed thoroughly with hot water. The cerous carbonate was added to enough water to form a slurry and nitric acid added in an amount not quite sufficient to dissolve

it. This is essential, as it is impossible to remove the nitric acid if an excess is added. The excess cerous carbonate was filtered off and the solution evaporated until it became sirupy. After crystallization, the cerous nitrate was removed by heating the dish for a few minutes, pulverized, and stored in glass-stoppered bottles.

The potassium dichromate was prepared according to Kolthoff and Sandell (*33*) and all other reagents were of reagent grade and used without further purification.

Approximately 0.01 *N* solutions of sodium fluoride, cerous nitrate, ferrous ammonium sulfate, and potassium dichromate were prepared. The sodium fluoride and potassium dichromate solutions were standardized by direct weighing, the ferrous ammonium sulfate by titration against dichromate using diphenylamine sulfonic acid, and the cerous nitrate by titration (*61*) against the ferrous ammonium sulfate, using *o*-phenanthrolineferrous ion.

FIGURE 1

Batchelder and Meloche (*5*) tried to carry out the titration of sodium fluoride with cerous nitrate potentiometrically but obtained unsatisfactory results due to adsorption by the hydrous cerous fluoride. However, a few preliminary experiments with the glass electrode showed that it gave reproducible results, and that a definite change in pH occurred at the equivalence point.

The glass electrode was of the bulb type, constructed from Corning 015 glass tubing, containing an internal silver–silver chloride electrode (*11*, *13*, *38*) immersed in 0.1 *N* hydrochloric acid. The calomel cell (*11*) was made from 12-mm. Pyrex tubing with a fine Pyrex sintered-glass plate sealed into the lower end. Over this was placed a 4-cm. layer of agar to reduce diffusion. The cell proper was made of 5-mm. soft-glass tubing containing a small hole for contact with the outer potassium chloride solution. The assembled electrode is shown in Figure 1. The electrometer used was a Leeds & Northrup, Model 7660, single vacuum tube instrument.

Since the glass electrode is subject to sodium-ion errors, some means of estimating or compensating for them was necessary. The original data of Dole (*11*, *12*) and Jordan (*31*) on these errors were examined and by changing the constants in Jordan's equation to fit the data of Dole and Weiner (*14*) an empirical equation was found from which these errors could be calculated at sodium-ion concentrations of 0.1, 1.0, and 3.5 *M*. Using this equation, $\log \Delta E = 0.54\ pH' + 0.60\ (\log \text{concentration } Na^+) - 4.35$, the agreement between calculated and experimental data was good and showed that this error did not exceed 3 millivolts up to a pH of 9.0 and a sodium-ion concentration of 1.0 *M* at 25° C. The sodium-ion errors are greatly affected by change in temperature (*21*, *48*). The glass electrode was also calibrated and checked daily against buffer solutions at 25° C. to correct for asymmetry potential.

A study of the effect of the initial pH upon this titration showed that consistent results were obtained only when the initial pH was between 7.0 and 9.0. Under these conditions the calculated and theoretical volumes of cerous nitrate agreed within 4 parts per thousand.

A determination of the effect of the initial volume and the amounts of fluorine that can be determined was made by titrating solutions containing 1, 2, 4, 8, 16, and 30 mg. of fluorine with 0.01 *M* cerous nitrate at various dilutions. The solutions were always adjusted to a pH of 8 and dilutions giving a change of less than 7 millivolts at the equivalence point were not considered. The results for the titration of 2, 4, and 8 mg. of fluorine in 10 ml. are given in Table I and Figure 2. These data are the average of three titrations.

The results indicated that the amount of fluorine is best kept between 1 and 10 mg. and that the initial volume should be 10 to 20 ml. The theoretical volumes are based on independent standardization and the assumption that $CeF_3$ is formed.

Since the organic compounds are to be decomposed with sodium peroxide and then neutralized with nitric acid, the most important salt to be considered for the effect of neutral salts is sodium nitrate. This was determined by titrating 2 mg. of fluorine in 10 ml. of water at 25° C. in the presence of varying amounts of sodium nitrate. The sodium nitrate effectively reduced the magnitude of the change in potential at the equivalence point. A satisfactory change in potential at the equivalence point and stoichiometric results were obtained up to a concentration of 1.0 *M* in sodium nitrate, but at higher concentrations the volume of cerous nitrate used was about 1 per cent too small.

TABLE I. DETERMINATION OF FLUORINE

| Cerous Nitrate Added *Ml.* | E. m. f. | Cerous Nitrate Added *Ml.* | E. m. f. | Cerous Nitrate Added *Ml.* | E. m. f. |
|---|---|---|---|---|---|
| 0.00 | 0.3620 | 0.00 | 0.3600 | 0.00 | 0.3550 |
| 1.00 | 0.3000 | 3.00 | 0.2980 | 4.00 | 0.2960 |
| 2.00 | 0.2740 | 6.00 | 0.2360 | 8.00 | 0.2760 |
| 3.00 | 0.2250 | 6.20 | 0.2190 | 12.00 | 0.2250 |
| 3.10 | 0.2120 | 6.30 | 0.2060 | 12.50 | 0.2040 |
| 3.15 | 0.2040 | 6.35 | 0.2000 | 12.60 | 0.1960 |
| 3.19 | 0.1910 | 6.40 | 0.1880 | 12.70 | 0.1860 |
| 3.23 | 0.1780 | 6.44 | 0.1800 | 12.80 | 0.1730 |
| 3.28 | 0.1720 | ... | .... | 12.90 | 0.1660 |
| Fluorine present, mg. | 2.0 | | 4.00 | | 8.00 |
| Cerous nitrate used, ml. | 3.18 ± 0.02 | | 6.37 ± 0.03 | | 12.75 ± 0.05 |
| Cerous nitrate, theoretical, ml. | 3.17 | | 6.35 | | 12.70 |
| pH at equivalence point | 5.10 | | 5.19 | | 5.02 |

Sodium chloride, bromide, and iodide showed the same effect but, in general, the amount of these salts introduced from the organic compounds will be small and their contribution to the total salt concentration can be neglected. Sulfates interfered badly and must be absent. The presence of 2 mg. of sodium sulfate in 10 ml. made it impossible to detect the equivalence point. Perchlorates interfered seriously when there was more than 0.04 gram of sodium perchlorate in 10 ml. The presence of perchlorates did not elimi-

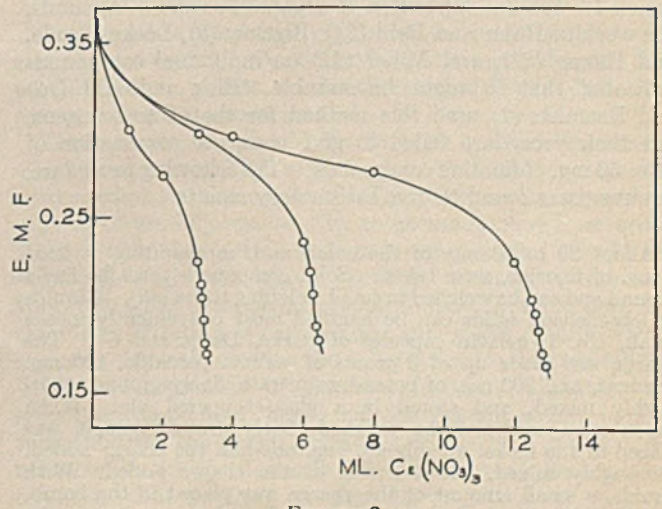


FIGURE 2

nate the potential change at the equivalence point but displaced it so as to give high results. Carbonates interfered, giving high results due to the precipitation of the cerous ion, but can be removed by careful neutralization and boiling. Any other ion which acts as a buffer or precipitates either fluoride or cerous ion will also cause trouble, but the above ions are the only ones which will ordinarily be encountered.

Kurtenacker and Jurenka (*34*) recommended that the titration be made at 80° C. The effect of temperature was examined by comparing the titration of like amounts of fluorine at 25° and 90° C. The change in e. m. f. at the equivalence point at the higher temperature was about twice that at 25° C. and the equilibrium was established much more rapidly, but the volume of cerous nitrate used was about 1 per cent too large. While the titration gave good results with pure solutions, the presence of neutral salts presented a serious interference, especially with visual titrations. However, this effect was lessened by titration in hot solution.

If 50 per cent ethyl alcohol was used as the solvent and the titration made at 90° C. the change in pH at the equivalence point was increased again to a surprising degree and equilibrium was quickly established. This was shown by two titrations of 1.5 mg. of fluorine in the presence of 1.7 grams of sodium nitrate at 90° C. in 20 ml. of water and in 20 ml. of 50 per cent alcohol. Both solutions were adjusted initially to a faint pink color with phenolphthalein. The change in e. m. f. at the equivalence point with alcohol was twice that with water, but the volume of cerous nitrate used was 5 per cent higher than the theoretical volume with water. No satisfactory means exists of correlating the e. m. f. of the glass electrode with the absolute pH in nonaqueous solvents but it can be used to follow changes in pH, as has been shown by Schicktanz and Etienne (*51*) and Evans and Davenport (*19*). These titrations indicated that, if a compensation is made for the nontheoretical relationship by standardizing the cerous nitrate solution under the same conditions, the addition of ethyl alcohol is desirable whenever appreciable amounts of sodium nitrate are present.

## Decomposition of Compound

An attempt to devise a general method for the decomposition of stable, solid, organic fluorine compounds on the combustion method of Hubbard and Henne (*30*) was unsuccessful. The results obtained were invariably low.

Although no single comprehensive investigation has been made to test the general applicability of the Parr bomb fusion for the decomposition of organic fluorine compounds, the work of Hahn and Reid (*24*), Bigelow (*8*), Locke, Brode, and Henne (*37*), and Miller (*41*) on individual compounds indicated that it might be suitable. Elek and Hill (*16*) and Beamish (*6*) used this method for the other halogens, but their procedure failed to give complete combustion of 25 to 50 mg. of fluorine compounds. The following procedure, however, was found to give satisfactory results.

About 20 to 50 mg. of the compound, representing at least 5 mg. of fluorine, were taken. Solid compounds must be finely ground and can be weighed in small weighing tubes (*43*). Liquids or low-melting solids can be handled most conveniently in the small, No. 1, gelatin capsules of Parke, Davis and Co. The charge was made up of 3 grams of sodium peroxide, 100 mg. of sugar, and 300 mg. of potassium nitrate, finely ground, thoroughly mixed, and stored in a glass-stoppered vial. When solids were analyzed the weighed, finely ground sample was placed in the nickel microbomb cup, one-half the charge added, thoroughly mixed, and the rest of the charge added. With liquids, a small amount of the charge was placed in the bomb, and the closed capsule containing the liquid was seated in the center of the charge, pressed to the bottom of the cup, and then covered completely with the peroxide charge. Normally the complete charge was not used, but the cup should be filled to within about 0.3 cm. (0.125 inch) of the top. The lid was clamped firmly in position, the bottom of the bomb was held in a strong Bunsen flame, and after a disturbance was felt the bomb was cooled with tap water. If no disturbance was felt the bomb was heated about 20 to 30 seconds before cooling. With the semimicrobomb a sample of 100 to 150 mg. was used with a charge of 8 to 9 grams of sodium peroxide and 300 mg. of sugar. The bomb was heated over a strong Bunsen flame or Fisher burner until the water in the cooling ring evaporated.

After the bomb was cooled and rinsed, the contents were dissolved in a small amount of water in a 125-ml. Erlenmeyer flask and the solution was boiled to destroy the peroxide. A slight residue of carbon apparently does not affect the accuracy of the determination. The solution was adjusted to the pale pink color of cresol red with strong nitric acid and sodium hydroxide and heated, until the color no longer changed on boiling, to remove the carbon dioxide.

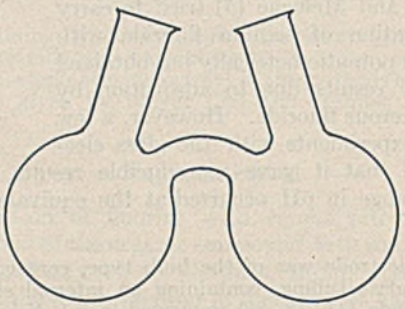

FIGURE 3

Practically all the nickel precipitated during this process. The nickel precipitate was filtered off and washed thoroughly, the filtrate was adjusted to a volume of 20 to 30 ml., and the neutralization was completed with 0.1 *N* nitric acid. During this neutralization the solution should be alternately adjusted to a faint pink color and boiled until 3 to 4 drops of the nitric acid change the color to a clear yellow and the color remains unchanged after 2 minutes' gentle boiling. The solution was cooled and transferred directly to a 50-ml. measuring flask, if no more nickel had precipitated; otherwise it was filtered again, as the titration fails in the presence of any nickel precipitate. The solution was diluted to 50-ml. and 10-ml. aliquot portions were taken for the titration.

Attempts to carry out the decomposition by the above procedure in a steel semimicrobomb were unsuccessful, as the iron was not removed completely. Some further modification, which will completely remove the iron, is necessary before this procedure can be used with a steel bomb.

The fluoride ion may now be titrated either electrometrically or visually.

## Electrometric Titration

The 10-ml. portion of the neutralized solution of fluoride, containing between 1 and 6 mg. of fluorine so as to be able to titrate with a 10-ml. buret, was placed in a 50-ml. beaker. The concentration of sodium nitrate should not exceed 2 *M*. Ten milliliters of alcohol were added and the beaker was placed on a small electric hot plate clamped to a ring stand. A small electric glass stirrer and the electrodes were introduced into the solution. While the solution was heating, one drop of phenolphthalein was added and the solution was adjusted with 0.001 *N* sodium hydroxide and nitric acid to a faint pink color. After the solution began to boil, the hot plate was adjusted to low heat and the pH of the solution readjusted if necessary. When the initial e. m. f. had become steady the fluorine was titrated with 0.01 *N* cerous nitrate until the maximum change in e. m. f. per unit volume of cerous nitrate was obtained.

Table II. Analysis of Organic Fluorine Compounds

| Compound | Fluorine Theoretical | Visual Titration No. of detns. | Visual Titration Fluorine found | Electrometric Titration No. of detns. | Electrometric Titration Fluorine found |
|---|---|---|---|---|---|
| | % | | % | | % |
| 4,4-Difluorodiphenyl | 20.00 | 4 | 19.9 ± 0.2 | 1 | 19.7 |
| Fluorobenzoic acid | 13.57 | 2 | 13.75 ± 0.05 | 1 | 13.6 |
| Fluorobenzene | 19.80 | 2 | 19.6 ± 0.1 | 1 | 19.6 |
| 2,5-Dichloro-1-fluorobenzene | 10.62 | 2 | 10.85 ± 0.05 | 2 | 10.7 ± 0.1 |
| Octachlorodifluorobutane | 10.27 | 1 | 10.3 | 2 | 10.1 ± 0.0 |
| Tetrachlorodifluoroethane | 18.62 | 2 | 18.65 ± 0.05 | 2 | 18.7 ± 0.1 |
| Trichlorotrifluoroethane (Freon) | 30.30 | 2 | 30.0 ± 0.0 | 2 | 30.2 ± 0.0 |
| Benzotrifluoride | 39.03 | 1 | 39.1 | 2 | 39.1 ± 0.1 |
| Nitrobenzotrifluoride | 29.80 | 2 | 29.9 ± 0.1 | 3 | 29.6 ± 0.1 |

## Visual Titration

The titration of pure sodium fluoride with cerous nitrate using methyl red gives little trouble when the solution is kept near the boiling point. However, the presence of neutral salts such as sodium nitrate in 1 molar concentration in aqueous solution makes the equivalence point very difficult to detect and renders the titration practically useless. Under the same conditions as used for the electrometric titration the visual titration with methyl red gave satisfactory results.

For this titration, 0.1 *N* and 0.001 *N* solutions of both nitric acid and carbonate-free sodium hydroxide, a special titration flask, a flashed opal screen with a daylight light bulb, a saturated solution of methyl red in ethyl alcohol, a 0.02 per cent solution of cresol red, and a 0.04 per cent solution of bromocresol green are required.

The titration flask (Figure 3), suggested and designed by W. T. Miller, is made of two 200-ml. round-bottomed Pyrex flasks, connected with a short curved tube which allows the solution to be poured back and forth between the two flasks. The complete titration must be carried out in front of the flashed opal screen, as the color changes of the indicators are too delicate to be observed clearly in ordinary light.

A 10-ml. aliquot portion of the neutral fluoride solution was placed in one side of the double flask and 10 ml. of 95 per cent alcohol were added. Three drops of cresol red were added, and the solution was adjusted to a definite pink color, and then brought to a gentle boil. The solution was then made acid with 1 or 2 drops of 0.1 *N* nitric acid in excess and the solution was boiled gently for 2 minutes, to remove all carbon dioxide. If the solution remains clear yellow during this period, the carbonate has been removed. The solution was then adjusted to a very faint pink color with the 0.001 *N* solutions of acid and base. The solution should be poured freely from one side of the double flask to the other, so that it remains constant in composition.

Two drops of the saturated methyl red and one drop of bromocresol green were added. This mixed indicator was recommended by Hubbard and Henne (*30*). The initial color of the solution should be a clear green. The solution was now titrated drop by drop, being boiled and agitated at frequent intervals. When the color became a light orange, the solution was divided evenly between the two flasks and one more drop of cerous nitrate added to one portion of the solution. The color difference between the two halves was noted. The two portions were reunited, poured back and forth several times, brought to a boil, and divided equally again. Another drop of cerous nitrate was added and the difference in color again noted. This procedure was repeated until a maximum color difference was observed.

The results in the previous sections confirm those of Kurtenacker and Jurenka that when fluoride, in hot 50 per cent alcohol and in the presence of sodium nitrate, is titrated with cerous nitrate either with a glass electrode or with an indicator, the result is not the stoichiometric relationship found in pure aqueous solution. Therefore, the cerous nitrate solution must be standardized against a known amount of sodium fluoride under the same conditions of concentration of sodium nitrate, temperature, and solvent. The potassium nitrate in the fusion mixture and any halogen contained in the organic compound may be neglected with regard to their contribution to the total salt concentration.

Using the above outlined procedure nine organic fluorine compounds were analyzed. The results obtained are given in Table II and in many cases are the average of several analyses on varying weights of samples.

Since the fluoride ion cannot be titrated with cerous nitrate in the presence of the sulfate ion, attempts were made to modify the proposed procedure to secure a separation. The distillation from perchloric acid, as advocated by Willard and Winter (*60*), was tried with *p*-methylbenzenesulfonylfluoride after decomposition in a microbomb, but low results were always obtained. Evidently the presence of a large amount of sodium perchlorate makes the quantitative recovery of fluorine impossible. Others have reported this same difficulty with natural waters when the salt concentration was appreciable. However, Scott and Henne (*52*) claimed a successful distillation from biological material ashed with calcium oxide.

Attempts to separate the sulfate ion by precipitation as barium sulfate always gave low results, probably because of the precipitation of barium fluoride in the 50 per cent alcohol solutions. Hoskins and Ferris (*29*) reported the same difficulty in the titration of fluorine with thorium nitrate in the presence of the barium ion.

The best method, at present, for determining fluorine in the presence of sulfate appears to be that of Starck (*55*) and Hawley (*25*) with the titration by the Volhard method (*28*). An analysis of *p*-methylbenzenesulfonylfluoride by this method gave 11.2 ± 0.1 per cent fluorine.

Two different explanations have been offered for the observed color change of methyl red in this titration. Kurtenacker and Jurenka (*34*) stated that this is due to a change in pH at the equivalence point caused by the hydrolysis of the excess cerous nitrate. Batchelder and Meloche (*5*) think that the methyl red behaves as an adsorption indicator. During the titration there is a definite flocculation of the gelatinous cerous fluoride precipitate and these experiments have shown that a clearly defined change in pH occurs at the equivalence point. However, the pH change obtained is somewhat greater than can be accounted for on the basis of hydrolysis, even assuming that the cerous ion undoubtedly exists as a coordination complex. Moreover, considering the effects of alcohol and temperature, it is evident that a rather complex condition exists and neither of the above explanations is entirely adequate.

## Summary

The effects of the initial pH, initial volume, temperature, and the presence of alcohol and several neutral salts, especially sodium nitrate, on the titration of fluoride ion with cerous nitrate have been studied.

The equivalence point of the titration can be determined either electrometrically with a glass electrode or visually with methyl red as indicator. This titration in general is capable of yielding results with an accuracy of ±1 per cent. The electrometric determination is superior and should be employed where the highest possible accuracy is desired. However, when little or no neutral sodium salts are present, the visual titration is satisfactory and yields results which compare favorably with the electrometric determination.

The determination of fluorine, in both aliphatic and aromatic compounds, based on the decomposition of the compound by the sodium peroxide fusion in a nickel Parr microbomb and titration with 0.01 *N* cerous nitrate has been outlined. The accuracy of the determination is ±1 per cent.

Sulfate and perchlorate ions interfere. The titration of fluorine with cerous nitrate cannot be carried out in the presence of the sulfate ion. An alternate method for this type of compound has been suggested. The presence of perchlorate is neither desirable nor necessary.

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# Extraction and Colorimetric Estimation of Certain Metals as Derivatives of 8-Hydroxyquinoline

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ALTHOUGH methods involving the use of 8-hydroxyquinoline as a quantitative precipitant have been extensively investigated and widely used (*5*), but little attention has been devoted to colorimetric estimations based upon this reagent. The lack of general applicability of colorimetric procedures is probably traceable to the complexity or indirectness of those methods which have been suggested.

Among the colorimetric methods proposed are the following:

Aluminum and magnesium through coupling of the 8-hydroxyquinoline derivatives with sulfanilic acid, followed by colorimetric estimations in terms of the dyes so produced (*2, 3*); bismuth through extraction of the 8-hydroxyquinoline complex with a 3 to 1 acetone–amyl acetate mixture and color comparison of the extract (*19*); iron through solution of the precipitated ferric complex in ethanol followed by colorimetric observation of the resulting solution (*15*); magnesium (*6, 22*), calcium (*6, 22*), and bismuth, aluminum, and zinc (*21*) through the colors produced by reactions of the 8-hydroxyquinoline derivatives with Folin's reagent (*10*); magnesium by addition of excess 8-hydroxyquinoline and colorimetric estimation of the excess after removal of the precipitated magnesium complex (*14*); magnesum by development of color through reaction of the precipitated derivative with ferric chloride in acetic acid (*12*); and iron (*1*), aluminum (*1*), and indium (*16*) through extraction of the colored complexes with chloroform and direct colorimetric observation of the diluted extracts.

For simplicity of operation, the last of these methods would appear particularly advantageous, consisting as it does in a mere extraction of an aqueous solution, adjusted to an appropriate pH value, with a solution of 8-hydroxyquinoline in chloroform, layer separation, and observation of the spectral transmittancy of the extract at a predetermined wave length. The sensitivity of the method for the accurate estimation of small quantities of iron, aluminum, and indium (*1, 16*) has been demonstrated.

It is the purpose of this paper to present data relative to the necessary conditions for the extraction of the complexes of cobalt, nickel, bismuth, and cupric copper, spectrophotometric data for solutions of these complexes in chloroform and data relative to the possible estimation of small amounts of these elements by this procedure. Inasmuch as a reinvestigation of the method as applied to iron and aluminum has yielded results significantly different from those reported

by Alexander (*1*), it has been deemed advisable to include these elements as well.

## Apparatus and Materials

All colorimetric measurements were made with the Cenco-Sheard spectrophotelometer (*20*) previously employed (*16*), using cells of 1-cm. (±0.5 per cent) thickness with a slit width of 5 mμ. Measurements of pH were made with a Beckman Laboratory Model G pH meter (*16*).

Chloroform and 8-hydroxyquinoline were of the same purities as previously reported (*16*), and chloroform was recovered by the same procedure (*16*), except that 12 *N* hydrochloric acid proved to be a better extracting agent. All other chemicals were of analytical reagent quality and were used without further purification.

## Spectrophotometric Examination of Chloroform Solutions of 8-Hydroxyquinoline Derivatives

Since in a preliminary investigation it was found that of the 8-hydroxyquinoline derivatives of aluminum, ferric iron, cupric copper, bismuth, cobalt, and nickel prepared and dried by the recommended methods (*5*) only the first three dissolved at all readily in chloroform, spectrophotometric studies were made upon solutions of these materials as extracted from aqueous salt solutions at appropriate pH values.

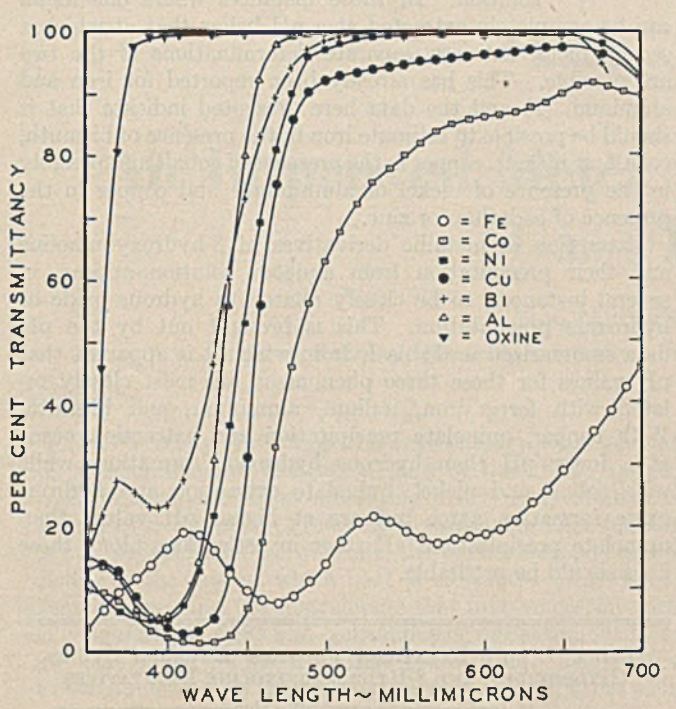


Figure 1. Transmittancy Curves for Chloroform Solutions of 8-Hydroxyquinoline and Certain Metallic Derivatives

In Figure 1 are given curves showing the spectral transmittancies of these complexes over the wave length range 350 to 700 mμ. In all cases except ferric iron, solutions contained the equivalents of 20 mg. of the metals per liter of chloroform; for ferric iron it was 10 mg. Included for purposes of comparison is a curve obtained for a solution prepared by dilution of 20 ml. of 0.01 *M* 8-hydroxyquinoline ("oxine") in chloroform to 50 ml.

Although the absorption bands for the metallic derivatives are all broad, maximum absorption is noted definitely at the following wave lengths: iron, 470 and 570 mμ; aluminum, bismuth, and nickel, 395 mμ; cobalt, 420 mμ; and copper, 410 mμ. Furthermore, in the region around 400 mμ, chloroform solutions of 8-hydroxyquinoline, although apparently

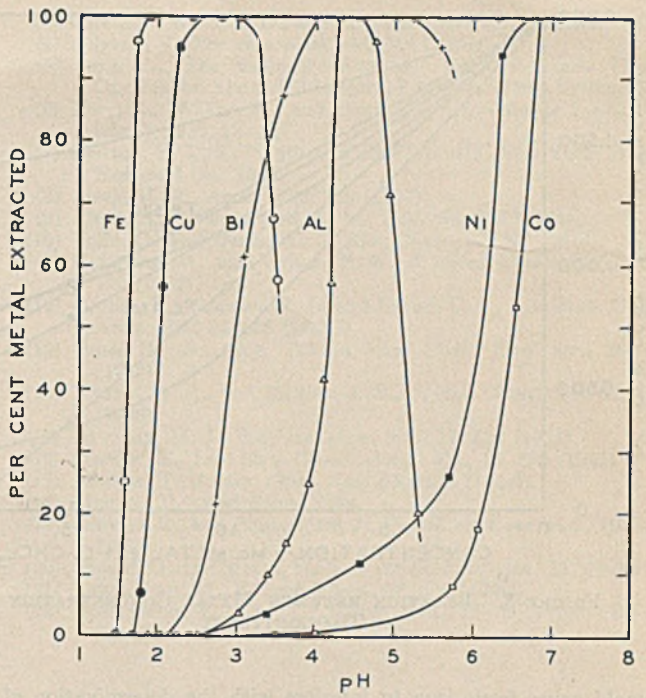


Figure 2. Effect of pH upon Metal Extraction

colorless, show measurable absorptions (*1*). However, as has been pointed out (*1*, *16*), the use of constant and controlled conditions of extraction obviates this difficulty.

## Extraction and Colorimetric Estimation

Extraction of metallic ions from aqueous solutions as derivatives of 8-hydroxyquinoline is as closely dependent upon pH (*1*, *16*) as is their precipitation by that reagent (*8*, *9*, *13*). Optimum pH ranges for extraction were determined as previously reported (*16*), using nitrate, sulfate, or chloride solutions containing in 25 ml. the equivalent of 20 mg. (10 mg. for iron) of the metal per liter of chloroform and adjusted to appropriate pH values with 0.1 *N* sulfuric acid or sodium acetate, extracting four times with 5-ml. portions of 0.01 *M* 8-hydroxyquinoline, and examining the extracts at appropriate wave lengths after dilution to 50 ml., the per cent extraction then resulting from application of calibration curves (Figure 3). Data so obtained are plotted in Figure 2.

Transmittancy data obtained at the wave lengths previously given for extracts made within the optimum pH ranges indicated in Figure 2 and containing the equivalent of 5, 10, 15, and 20 mg. of metal per liter of chloroform are plotted in Figure 3. Lack of intersection with the logarithm of 100 per cent transmittancy at zero concentration is due to the measurable transmittancy of the reagent in chloroform.

Iron. Data in Figure 2 confirm the observation of Alexander (*1*) that ferric iron is completely extracted at pH 2.0, although complete extraction was obtained here at pH 1.9. Inasmuch as completeness of extraction falls off above pH 3.0, owing to hydrous oxide formation (*16*), optimum conditions for the removal of iron are in the pH range 1.9 to 3.0.

Although Alexander (*1*) reported the colorimetric estimation of iron at 540 mμ, data in Figure 1 indicate the greater desirability of using the 470 and 570 mμ bands. At either of these wave lengths, the color system obeys Beer's law rigidly in a concentration range at least up to 20 mg. of the metal per liter of chloroform (or up to 1 mg. of iron in 25 ml. of aqueous solution), although absorption at the lower wave length is so strong as to make reading difficult at higher concentrations (Figure 3). The extensive absorption of these black solutions over the entire visible spectrum (Figure 1) coupled with the low extraction pH

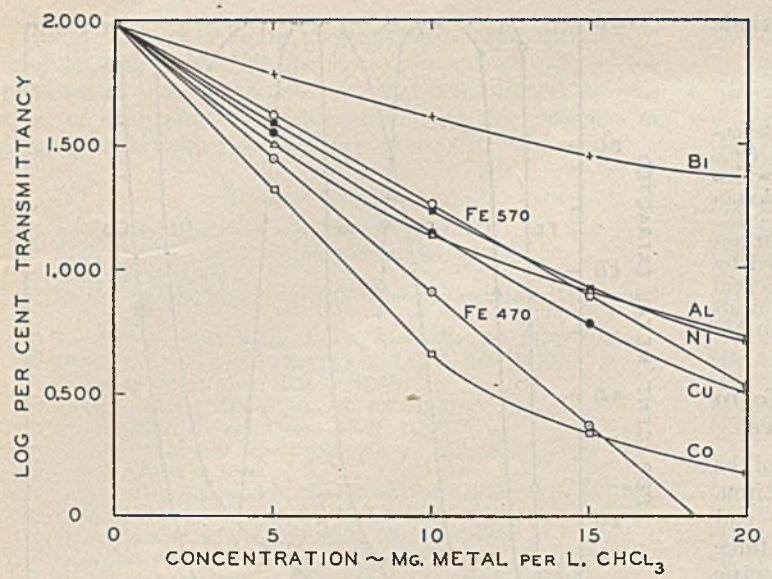


FIGURE 3. RELATION BETWEEN METAL CONCENTRATION AND TRANSMITTANCY

would cause ferric iron to interfere with the determination of certain other metals—e. g., copper, bismuth, and indium (*16*).

ALUMINUM. Although Alexander (*1*) reported complete extraction of aluminum at pH 3.5, data in Figure 2 indicate complete removal only in the pH interval 4.3 to 4.6, extraction again falling off at higher pH values. Partial extraction was noted in the pH interval 2.6 to 4.3, and within the optimum range complete extraction was attained only with difficulty. The use of higher reagent concentrations might improve the results.

Strict conformity to Beer's law at 395 mμ is not shown by chloroform solutions of the aluminum complex, although deviation in the range up to 20 mg. is insufficient to interfere with the method. Estimation of iron and aluminum in the same sample as reported by Alexander (*1*) appears to offer no difficulty, since iron extracts completely at pH values below those for aluminum extraction.

BISMUTH. Extraction of the yellow bismuth complex is complete in the pH range 4.0 to 5.2 and proceeds readily. At higher pH values, incomplete extraction is again noted. Reference to Figure 3 indicates but little deviation (at 395 mμ) from Beer's law up to 10 mg. of the metal per liter of chloroform, although differences become more marked at higher concentrations. Use of this method for the estimation of bismuth should present no difficulties.

COBALT. Although extraction of the cobalt complex begins around pH 3.5, complete removal occurs only at pH 6.8 or above. At appropriate pH values, extraction offers no difficulties, and complete removal of cobalt from the aqueous layer can usually be effected by two extractions. At 420 mμ, the slightly orangish chloroform solutions of the complex conform exactly to Beer's law up to 10 mg. of the metal per liter, but deviations at higher concentrations are marked.

NICKEL. Extraction of the nickel derivative begins just above pH 2.5 but is complete only at pH 6.7 or above. Deviations of the yellow chloroform solutions of the complex from Beer's law at 395 mμ are minor up to 10 mg. of the metal per liter but become more pronounced at higher concentrations. Quantitative estimation of nickel by this method should offer few difficulties.

COPPER. As is shown in Figure 2, extraction of the copper complex was complete at all investigated pH values in the range 2.7 to 7.0. Qualitative observations have indicated the complete removal of copper from ammoniacal solutions as well, an indication of the stability of the resulting compound.

At 410 mμ, the yellow chloroform solutions of the copper derivative approach Beer's law closely up to concentrations of 10 mg. of the metal per liter, with deviations becoming somewhat greater at higher concentrations.

Estimation of copper by this method is readily and accurately effected in the absence of other extractable cations, and the wide pH range of copper extraction permits a considerable choice of suitable pH values. Inasmuch as the estimation of copper in the presence of cadmium and zinc is often necessary and the cadmium and zinc derivatives of 8-hydroxyquinoline have been found not to extract at least below pH 5.5 (*18*), it was of interest to apply the above procedure to copper-cadmium and copper-zinc solutions. The accuracy and feasibility of the estimation of small amounts of copper in the presence of at least forty times as much of these elements are indicated by data obtained by the extraction of 25-ml. aqueous sulfate solutions adjusted to pH 4.0 and containing in all cases the equivalent of 10.0 mg. of copper per liter of chloroform plus a varying quantity of the other metal. The transmittancy of the solution containing copper (at 410 mμ) was unaffected by as much as 400 mg. of cadmium or zinc. It appears not unlikely that the method would be applicable in the presence of even larger amounts of cadmium or zinc.

Although all the elements except iron here investigated give 8-hydroxyquinoline derivatives the chloroform solutions of which show maximum absorption in the region around 400 mμ, thereby imparting to the method a lack of specificity, it is apparent that through proper pH control and in the absence of interfering cations a method is available for the accurate determination of iron, aluminum, bismuth, cobalt, nickel, and copper in amounts up to at least 1 mg. in 25 ml. of aqueous solution. In those instances where one metal can be completely extracted at a pH below that at which a second metal extracts, separate determinations of the two are possible. This has already been reported for iron and aluminum (*1*) and the data here presented indicate that it should be possible to estimate iron in the presence of bismuth, cobalt, or nickel; copper in the presence of cobalt and possibly in the presence of nickel or aluminum; and copper in the presence of cadmium or zinc.

Extraction of metallic derivatives of 8-hydroxyquinoline and their precipitation from aqueous solution appear, in several instances, to be closely related to hydrous oxide or hydroxide precipitation. This is brought out by the pH data summarized in Table I, from which it is apparent that pH values for these three phenomena are most closely related with ferric iron, indium, aluminum, and bismuth. With copper, quinolate precipitation and extraction occur at a lower pH than hydrous hydroxide formation, while with cobalt and nickel, quinolate extraction and hydrous oxide formation agree but are at higher pH values than quinolate precipitation. Further investigations along these lines should be profitable.

TABLE I. pH RELATIONS BETWEEN HYDROUS OXIDES (HYDROXIDES) AND 8-HYDROXYQUINOLINE DERIVATIVES

| | pH Value | | |
|---|---|---|---|
| Ion | Hydrous oxide (hydroxide) formation | Quinolate precipitation | Quinolate extraction |
| $Fe^{+++}$ | 2 (*7*) | 2.8 (*13*) | 1.9 |
| $Cu^{++}$ | 5.3 (*7*) | 2.7 (*13*) | 2.7 |
| $In^{+++}$ | 3.4 (*17*) | 3.0 (*11*) | 3.2 (*16*) |
| $Bi^{+++}$ | 4.2 [a] | 4.8 (*13*) | 4.0 |
| $Al^{+++}$ | 4.1 (*7*) | 4.2 (*13*) | 4.3 |
| $Ni^{++}$ | 6.7 (*7*) | 4.6 (*13*) | 6.7 |
| $Co^{++}$ | 6.8 (*7*) | 4.2 (*13*) | 6.8 |

[a] Calculated from solubility product data at 0.1 *M* (*4*).

## Summary

Spectrophotometric examinations of chloroform solutions of certain metal derivatives of 8-hydroxyquinoline indicate the following absorption maxima: ferric iron, 470 and 570 mμ; aluminum, bismuth, and nickel, 395 mμ; cobalt, 420 mμ; and copper, 410 mμ.

Agitation of aqueous solutions containing these metallic ions with a chloroform solution of 8-hydroxyquinoline leads

to complete extraction of the metallic complexes in the following optimum pH ranges: iron, 1.9 to 3.0; aluminum, 4.3 to 4.6; bismuth, 4.0 to 5.2; cobalt, 6.8 or above; nickel, 6.7 or above; and copper, 2.7 to at least 7.0. At pH values below the lower limits, partial extraction to no extraction will occur.

Chloroform solutions containing the equivalents of up to 20 mg. of these metals per liter (or 1 mg. of the metal in the aqueous solution extracted) show little or no deviation from Beer's law at the wave lengths given and thus lend themselves to use in accurate colorimetric determinations.

Copper can be determined in the presence of at least forty times as much cadmium or zinc by extraction at pH 4.0 and transmittancy measurement at 410 mμ.

An interesting correlation between pH values for hydrous oxide or hydroxide formation, quinolate precipitation, and quinolate extraction is given.

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# An Improved Meter for the Measurement of Gas Flow Rates


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IN THE study of reaction kinetics in flow systems, accurate measurement of the rate of flow of the intake and exit gases is essential. Flow-measuring instruments in common use are of two types: (A) meters that measure some function of the gas velocity—for example, the pressure drop across an orifice or the rate at which heat is conducted away from a heated wire—and (B) instruments that involve displacement of a liquid by the gas and continuous or discontinuous measurement of the volume displaced in unit time. The familiar wet-test meter is an example of this type. Both types of instruments, as commercially available, have disadvantages, particularly if flow rates of the order of 25 cc. per minute or smaller are to be measured. Type A requires a separate calibration curve for each kind of gas, hence is not suited for use with mixtures of unknown composition, such as the gaseous product of a thermal reaction. Type B requires estimation of and correction for the effect of the vapor pressure of the displaced liquid and for the solubility of some components of the mixture in the liquid. Meters of this type are therefore unsuitable for the measurement of intake rates.

The flowmeter shown diagrammatically in Figure 1 is an improved form of a meter described by Marsh (*1*) for measurement of very slow flow rates. The new type of meter is free from the disadvantages of the usual instruments in common use. The operation is clearly shown by the diagram.

Referring to the side view, gas enters at *A*, passes down the vertical tube, and forces a drop of mercury, *D*, to the right in the horizontal tube. Stopcocks $S_1$ and $S_2$ are open; $S_3$ is closed. When the drop reaches the end of the tube at *E*, the gas trapped behind the drop is discharged. However, as soon as the drop

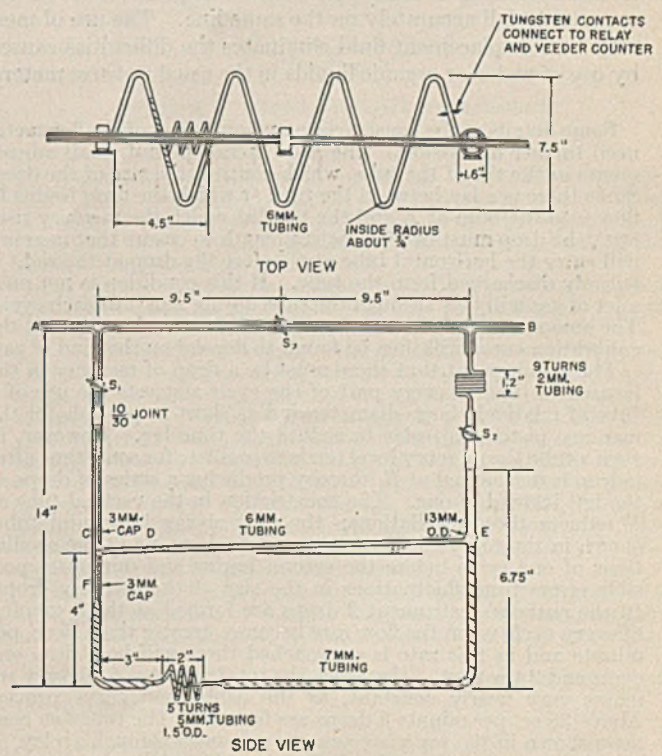


FIGURE 1. DIAGRAM OF FLOWMETER

begins to flow into the 13-mm. bulb at *E*, the mercury level is pushed up at *C*, so that a new drop is forced into the horizontal tube, trapping a new segment of the gas stream. This drop then moves to the right and the cycle is repeated.

Thus, each cycle represents the flow of a definite volume of gas and the time between cycles is a measure of the flow rate. For low flow rates the time required for movement of the drop between two fixed points may be a more convenient measure of the rate.

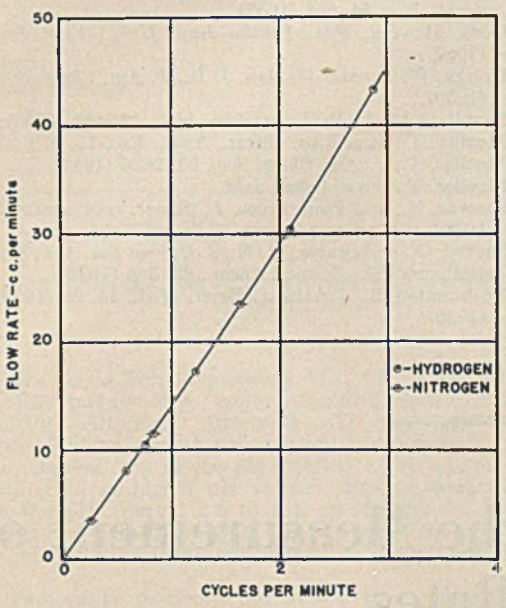


FIGURE 2

Figure 2 shows a calibration curve obtained with hydrogen and with nitrogen, when gas volumes were measured by collection over water in suitable burets. The points for the gases are seen to fall accurately on the same line. The use of mercury as a displacement fluid eliminates the difficulties caused by use of water or organic liquids in the usual wet-test meters.

Some details of the construction and operation of the flowmeter need further discussion. The glass spirals permit small adjustments in the tilt of the tube, which controls the size of the drop. Since there is a lag between the time at which the drop begins to flow into the bulb at *E* and the time at which the mercury rises at *C*, the drop must be of sufficient length to ensure that mercury will enter the horizontal tube at *C* before the drop at the right is entirely discharged from the tube. If this condition is not met, a jet of gas will flow through the tube during a part of each cycle. The amount of gas will be a function of the gas viscosity and the calibration curve will then be found to depend on the kind of gas.

The requirement that there must be a drop of mercury in the horizontal tube at every part of the cycle suggests the use of a tube of relatively large diameter and as short as possible for the mercury piston, in order to reduce the time lag. However, in such a tube the mercury level tends to oscillate for some time after a drop is discharged at *E*, thereby producing a series of drops at the left instead of one. The constriction in the vertical tube at *F* reduces these oscillations; the long zigzag horizontal tube, shown in the top view, ensures complete damping of the oscillations of one cycle before the second begins and minimizes possible errors from fluctuations in the size of the mercury drops. In the authors' instrument 2 drops are formed at the beginning of every cycle when the flow rate becomes greater than 15 cc. per minute and as this rate is approached there will be 1 drop one cycle and 2 the next. However, the total volume of mercury remains very nearly constant, as the calibration curve proves. Above 35 cc. per minute 3 drops are formed. The tungsten contacts shown in the top view actuate a counter through a relay, so that the instrument may be used to record total flow as well as to measure the rate.

The surface of the tubing through which the gas and mercury droplets flow should be free of any oil or water film or dust particles, and the mercury should be pure; otherwise there will be small fluctuations in the rate of gas flow which may give pressure variations as high as 1 or 2 mm. of mercury. This effect can be eliminated in any case, however, by having a surge chamber in the line following the meter.

Since the volume displacement per cycle does not depend on the pressure or temperature of the gas, the instrument may be used at subatmospheric pressures. During any particular run, however, the temperature and pressure should have constant values; otherwise it would be difficult to convert the gas volumes to standard conditions. The meter could also be adapted for use at superatmospheric pressures by using metal parts or suitable plastic tubing and connections.

### Acknowledgment

The authors are greatly indebted to Harold Gershinowitz for his interest and advice.

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## Forceps with Platinum-Covered Tips

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THE usual platinum-tipped forceps commercially available have each platinum tip fastened to nickel-plated steel by means of two rivets in a simple scarf joint. During the past five years, three or four of these expensive tools have failed here, always owing to corrosion or oxidation where the two metals join. Even moderate attack of the steel soon renders the forceps unfit for use because the platinum alone is too weak to withstand the flexing that occurs at the rivet holes.

Forceps that are stronger, cheaper, and more durable than those just described have been made in this laboratory by covering the tips of ordinary steel forceps with 5-mil platinum foil. The tips of the steel forceps were filed to the shape desired and a piece of foil (0.8 × 2.5 cm.) was fitted to each, so that the seam would fall outside. A close fit was obtained by slitting the lower edge of the foil so that it could be folded around the end of the tip; excess foil was then carefully trimmed away. Spot-welding to form a practically continuous seam completed the assembly. The welding operation is simple with the proper equipment. In this case, a low inertia bench welder with synchronous thyratron control was used under the following conditions: 350 amperes for 6 cycles, or 0.1 second; electrode pressure, 20 pounds; flat copper electrodes of diameters $^3/_{16}$ inch (lower) and $^3/_{32}$ inch (upper).

The forceps thus constructed have been used constantly during the last six months and have given satisfactory service without deteriorating noticeably. There is no reason why the tips of a wide variety of tongs and forceps could not be covered with platinum foil to great advantage. The implements to be covered should preferably be made of stainless alloys.

# A Large-Scale Laboratory Extractor of the Soxhlet Type

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IN CHEMICAL investigations of agricultural products it is frequently necessary to extract large quantities of material. This problem was encountered by the authors in an investigation on tobacco and its solution may be of use in other laboratories.

The extractor was designed and constructed from two alcohol drums and other materials obtained from a plumbing shop.

In Figure 1 the distilling pot, 1, is made from a 55-gallon drum. Heat is supplied by a steam coil entering at valve 2. The solvent vapor tube, 3, made of 2-inch iron pipe and fittings, is welded to the drums. A 30-gallon drum comprises the extraction chamber, 4, with 0.5-inch pipe and fittings for the siphon tube return, 5. Coupling joints are provided for both vapor tube and siphon tube for ease in handling. Condensation is maintained by a spiral cone condenser head, 6, with auxiliary straight tube condenser, 7, connected in series to the water line. A steam-radiator safety valve, 8, is attached to 7 when the air has been forced from the system. This prevents air from being drawn in by the partial vacuum created each time the extract siphons over, resulting in the loss of solvent.

A detailed diagram of the condenser-head assembly is shown in Figure 2. The drumheads are cut out and flanges welded to the sides. The extractor heads are bolted to these flanges, being sealed with braided graphite-impregnated packing. Thus they

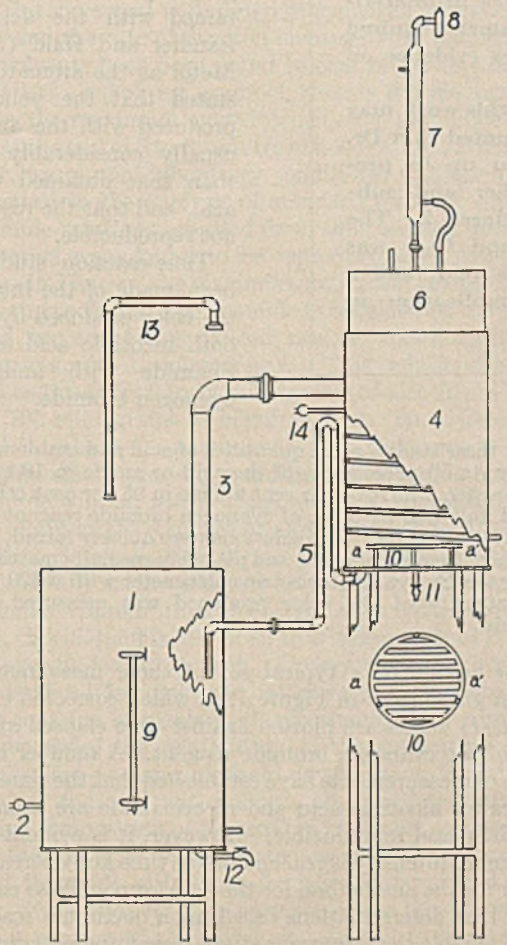



FIGURE 1. DIAGRAM OF APPARATUS

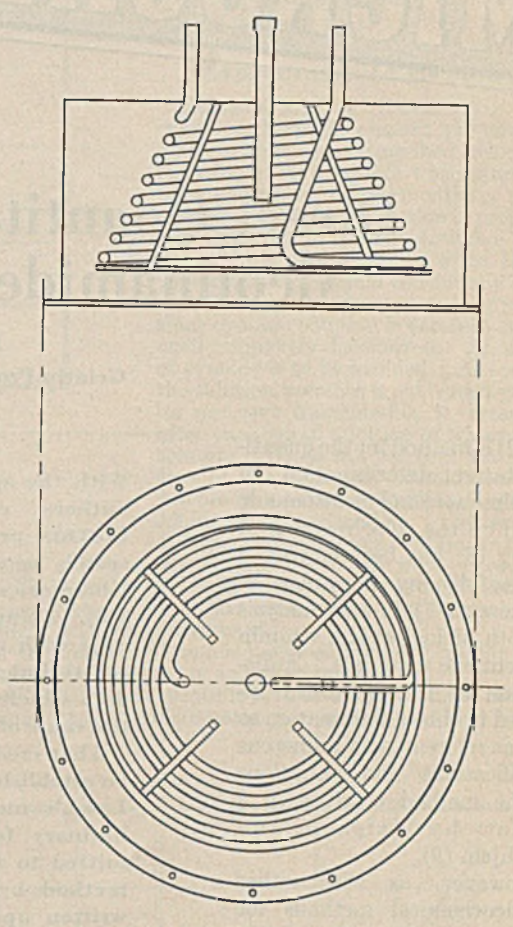


FIGURE 2. CONDENSER-HEAD ASSEMBLY

are removable for charging and cleaning. An ordinary 18-inch boiler gage, 9, provides a means for following the solvent level in the pot. The pot steam coil is so placed that the solvent level never falls below about 5 gallons, automatically preventing the extract from becoming overheated.

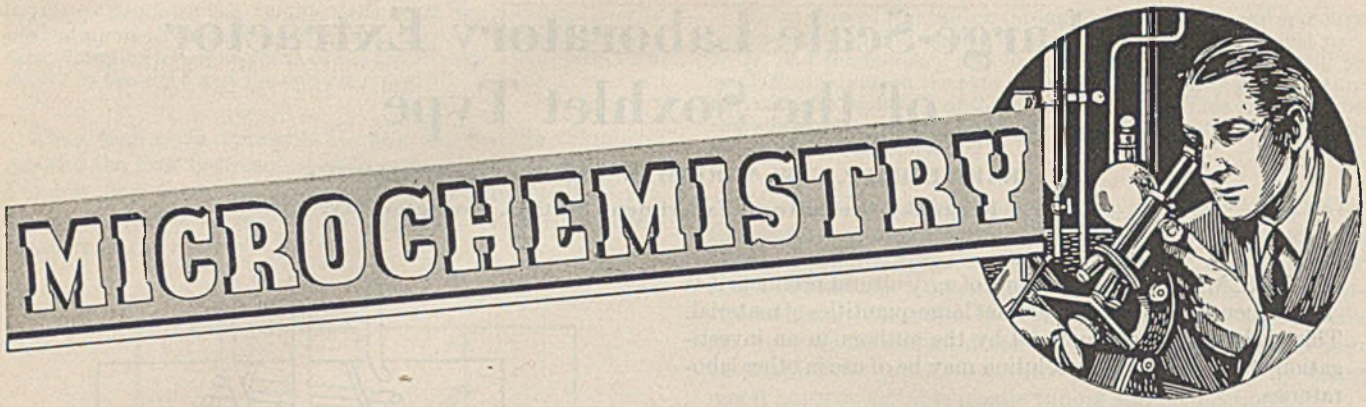
The ground material to be extracted is held in a heavy cotton bag double-sewn at the seams, which is supported on a small rack, 10, made of iron piping welded at the joints.

When extraction is complete the distilled solvent and concentrated extract solution are drawn off at taps 11 and 12, respectively. Following this operation the straight tube condenser, 7, is connected for distillation by means of adapter 13, the spiral cone condenser being disconnected from the water line. Steam through valve 14 is then used to distill the solvent from the extracted residue in the bag. Solvent vapor passes to the condenser and also in reverse direction through the vapor tube, 3, and is condensed. That returning to the distilling pot serves to rinse down the sides of the drum. It is drawn off and added to the concentrated extract for final solvent recovery.

The capacity of this apparatus is approximately 50 pounds of dried plant material. With this charge the solvent requirements are 25 to 35 gallons, most of which is recovered after extraction.

Experiments in the last year have proved the efficiency of the extractor.

THE investigation reported in this paper is in connection with a project of the Kentucky Agricultural Experiment Station and is published by permission of the Director




# Direct Quantitative Determination of Nicotinamide in Vitamin Mixtures

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**With the approval and cooperation of the authors concerned, the ANALYTICAL EDITION presents here the results of research conducted independently in two laboratories on the same problem (see page 355). Using the same fundamental procedure with minor modifications in analytical technique, the investigators acquired data which offer confirmatory evidence on the value of their method.**

**That credit for priority in this work may be established, it should be noted that Dr. Lamb's method was written up in preliminary form last November and submitted to this journal on March 2. The method by Drs. Melnick and Oser was written up in February this year, and a manuscript submitted for publication on March 11.**

THE method for the quantitative determination of nicotinic acid and nicotinamide based on the color reaction of König (*3*) has been employed successfully by a number of workers (*4–7*) in the analysis of both biological and vitamin concentrate materials. A discussion of the chemistry involved in this determination as well as references to numerous modifications and variations of the method is given in a review by Waisman and Elvehjem (*9*).

However, as with other physicochemical methods for the determination of nicotinic acid, the method is not specific, since other pyridine compounds also produce the same color upon treatment with cyanogen bromide and an aromatic amine. For this reason the usual practice is to hydrolyze the sample with either strong acid or alkali to convert other nicotinic acid derivatives to the free nicotinic acid. In this connection Melnick, Robinson, and Field (*6*) have reported that in the analysis of urine it is possible to obtain a semiquantitative analysis of the amounts of nicotinic acid, nicotinamide, nicotinuric acid, and trigonelline present by determining the apparent "nicotinic acid" values after various hydrolysis treatments.

Since today approximately 90 per cent of the compounded synthetic vitamin mixtures contain nicotinamide rather than nicotinic acid, it is highly desirable to have a direct quantitative method for the analysis of vitamin mixtures for nicotinamide. The microbiological method is not satisfactory for this purpose, since equimolecular weights of nicotinic acid, nicotinamide, and nicotinuric acid have equal activities for the organism used (*8*). In the case of the colorimetric method Melnick and Field (*5*) stated that the amide reacted with cyanogen bromide and aniline to give the same yellow color but the intensity of the color produced was about half that produced by an equal amount of nicotinic acid. Shaw and Macdonald (*7*) reported that when using nicotinamide the results were neither constant nor reproducible, and were generally lower than those obtained with the acid, while Bandier and Hald (*1*), using Metol as the aromatic amine, stated that the yellow color produced with the amide was usually considerably stronger than that obtained with the acid, and that the results were not reproducible.

Time-reaction studies have been made of the intensity of the color produced by treating both nicotinic acid and nicotinamide with aniline and cyanogen bromide.

For these studies equal quantities of acid and amide were used. Briefly, to 50 micrograms of the acid or amide in 12 ml. of distilled water, 1 ml. of 4 per cent aniline in 95 per cent ethanol was added, followed by 6 ml. of cyanogen bromide reagent (approximately 4 per cent). The solutions were quickly mixed, stirred to free them from air bubbles, and placed in specially matched tubes. Using the Evelyn photoelectric colorimeter with a 420 mμ filter, the intensity of the color produced was measured every 15 seconds.

The results of a typical set of these measurements are shown graphically in Figure 1, in which corrected extinction ($\log I_0/I$) values are plotted against time elapsed after addition of the cyanogen bromide reagent. A number of similar series of measurements have established that the time-reaction curves for nicotinic acid and nicotinamide are equally characteristic and reproducible. However, it is evident that the maximum intensity is reached in less time and decreases much faster for the amide than for the acid form. These curves also show that determinations based upon maximum readings are more reliable than determinations based upon readings taken at an arbitrary elapsed time of 5 minutes, since the readings

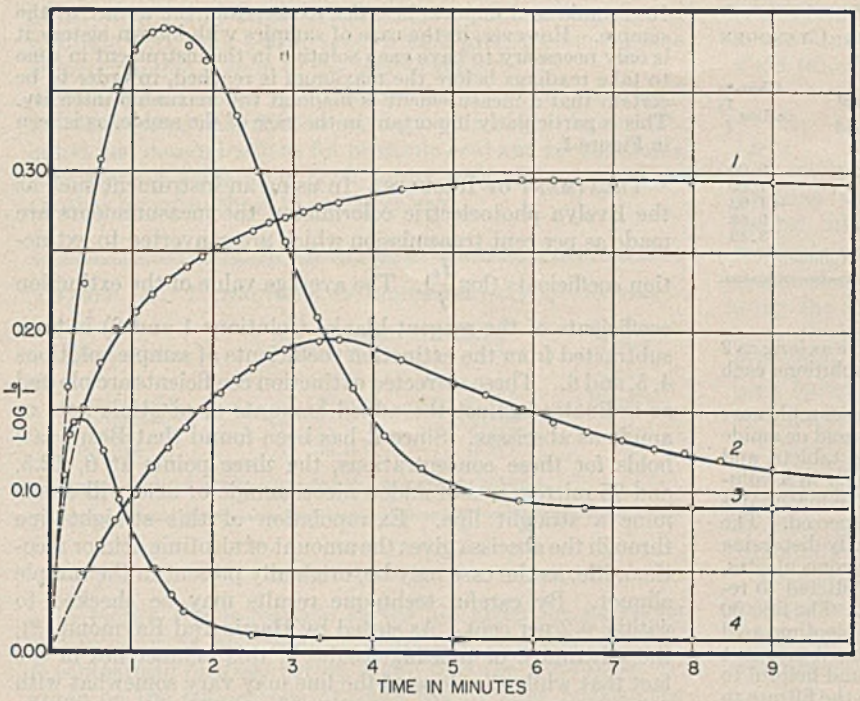


FIGURE 1. TIME-REACTION CURVES
50 micrograms of nicotinic acid (1), nicotinamide (2), pyridine (3), and β-picoline (4)

might vary considerably for a small difference in timing, particularly in the case of the amide.

The length of time for the maximum depth of color to develop varies somewhat with different cyanogen bromide preparations, and the rate of reaction increases with temperature. Various solvents have been found to change the rate of reaction—methyl alcohol, for example, retards the reaction considerably, the maximum color intensity for the amide being reached at the end of 6 to 7 minutes instead of 3 to 3.5 minutes. However, for normal laboratory conditions, maximum readings obtained in the analysis of standard nicotinic acid and nicotinamide solutions repeated from time to time during an 8-hour period were found to be reproducible to within ±2 per cent. In Figure 2 the maximum extinction coefficients for both nicotinic acid and nicotinamide as obtained by the above-described procedure are plotted against concentrations of these compounds in micrograms. This relationship was found to be linear for 0 to 75 micrograms of nicotinamide and for 0 to 100 micrograms of nicotinic acid. Since Beer's law fails to hold above these amounts, it is necessary to select sample aliquots so that the quantities of nicotinic acid and nicotinamide will fall within these ranges of concentration.

These observations form the basis of a method for the direct quantitative determination of nicotinamide which has been used successfully in the analysis of several hundred samples of synthetic vitamin mixtures. As with other chemical methods, this method is also open to criticism in that it is not entirely specific for either form. However, by making time-reaction studies it is possible to detect very quickly whether a material contains nicotinic acid, nicotinamide, or a mixture of the two, since the relationship of the extinctions of the resulting colors to time of their development is distinctly different for the acid and amide. Having determined the qualitative composition of the sample in respect to these substances, the material may then be analyzed for either nicotinic acid, its amide, or both, as described under procedure for analysis. If the time-reaction curve indicates that the sample contains a mixture of the amide and acid, it is necessary to make a time-reaction curve both before and after hydrolysis of the sample. Using Equation 1, it is then possible to calculate the amounts of amide and acid present. While the preparation of reagents, general procedure for analysis, and treatment of results are given in the literature, it is thought worth while to repeat these briefly with the inclusion of a few helpful details gained from experience in using this method during the past year.

## Experimental Procedure

PREPARATION OF REAGENTS. The cyanogen bromide reagent prepared in 4-liter quantities as described below has been found to be stable when stored at room temperatures for 2 months or more. A saturated bromine solution is prepared by dissolving 57 ml. of bromine in 4 liters of distilled water at 5° to 10° C. This solution is stirred continually while a 10 per cent sodium cyanide or potassium cyanide solution is carefully added until completely decolorized. An excess of cyanide is to be avoided. As soon as the solution becomes a pale green-yellow its per cent transmission is measured after each small addition of the cyanide solution. When the per cent transmission of the cyanogen bromide solution reaches a maximum, sufficient cyanide solution has been added. The amount of 10 per cent potassium cyanide required to decolorize 4 liters of the saturated bromine water is roughly 725 ml. The resulting 4 to 5 per cent cyanogen bromide solution is conveniently stored in a 2-liter automatic buret, from which it may be easily and safely dispensed.

A 4 per cent aniline solution is prepared from C. P. aniline in 95 per cent ethanol and stored in an amber bottle.

Standard solutions of nicotinic acid and nicotinamide are prepared containing 25 micrograms per ml. in distilled water.

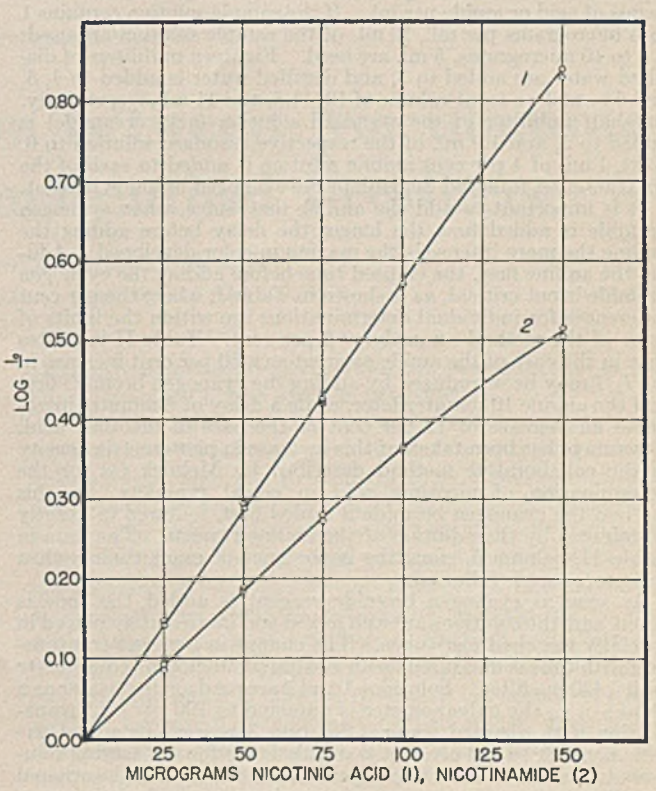


FIGURE 2

TABLE I. EFFECT OF TIME DELAY IN ADDING CYANOGEN BROMIDE FOLLOWING ADDITION OF ANILINE

| Time[a] | Log $\frac{I_0}{I}$ for 50 μg. of Nicotinamide | Change in Log $\frac{I_0}{I}$ | Log $\frac{I_0}{I}$ for 50 μg. of Nicotinic Acid | Change in Log $\frac{I_0}{I}$ |
|---|---|---|---|---|
| *Min.* | | % | | % |
| 0 | 0.195 | 0.00 | 0.295 | 0.00 |
| 2 | 0.189 | −3.08 | 0.300 | +1.69 |
| 4 | 0.192 | −1.54 | 0.298 | +1.02 |
| 8 | 0.196 | +0.51 | 0.297 | +0.68 |
| 16 | 0.196 | +0.51 | 0.305 | +3.39 |

[a] Elapsed time between addition of aniline and cyanogen bromide.

These standard solutions have been found to be stable as long as 2 weeks; however, it is advisable to prepare fresh solutions each week.

PREPARATION OF SAMPLE. Aqueous solutions of samples are prepared to contain 1 to 30 micrograms of nicotinic acid or amide per ml. In the case of synthetic vitamin mixtures, tablets, and capsules it is convenient to place the required number in a volumetric flask, heat on a steam bath, and swirl until the material is either completely in solution or uniformly dispersed. The addition of 1 ml. of ethylene dichloride aids by quickly dispersing vitamin mixtures containing waxes, fats, oils, liver concentrates, etc. The solutions are then made to volume and filtered to remove oils, fats, waxes, and other insoluble material. The first 50 to 100 ml. of filtrate are discarded to avoid loss of nicotinic acid and amide by adsorption on the filter paper. While it may not be accepted quantitative procedure, it has been found helpful to add a drop or two of a 2 per cent aerosol solution to the filtrate to aid in more accurate pipetting of the sample aliquots. Iron present in appreciable amounts forms a troublesome greenish-blue complex with cyanide. This may be avoided by treating the warm solutions with potassium hydrogen phosphate, the precipitated iron phosphate being removed in the subsequent filtration.

TREATMENT WITH CYANOGEN BROMIDE AND ANILINE. Six 25-ml. glass-stoppered graduates are labeled 1, 2, 3, 4, 5, and 6. Graduates 1 and 2 are reagent blanks and need to be repeated only once or twice daily, 3 is a solution blank, 4 is the sample, and 5 and 6 are the sample to which known amounts of nicotinic acid or amide are added. If the sample contains nicotinic acid, known amounts of nicotinic acid are added; if the sample contains the amide, known amounts of nicotinamide are added. To graduates 1 and 2 are added 12 ml. of distilled water. Into 3, 4, 5, and 6 is pipetted 1 ml. of the sample solution containing 10 to 30 micrograms of acid or amide per ml. If the sample solution contains 1 to 5 micrograms per ml., 10 ml. of the sample solution are used; if 5 to 10 micrograms, 5 ml. are used. Eighteen milliliters of distilled water are added to 3, and distilled water is added to 4, 5, and 6 to make a total volume of 12, 11.5, and 11 ml., respectively. One-half milliliter of the standard solution (acid or amide) is added to 5, and 1.0 ml. of the respective standard solution to 6. Next, 1 ml. of 4 per cent aniline solution is added to each of the six graduates, followed by 6 ml. of the cyanogen bromide reagent.

It is important to add the aniline first, since, when cyanogen bromide is added first, the longer the delay before adding the aniline the more intense is the maximum color developed. Adding the aniline first, the elapsed time before adding the cyanogen bromide is not critical, as is shown in Table I, where the per cent differences for individual determinations are within the limits of error of the method—namely, ±2 per cent. Table II indicates that in the case of the amide as great as a 50 per cent increase in log $I_0/I$ may be introduced by adding the cyanogen bromide first and the aniline 10 minutes later, while a delay of 8 minutes introduces an increase of 12 per cent in the case of nicotinic acid. Advantage has been taken of this increase in photometric density in the collaborative method described by Melnick (*4*) for the determination of nicotinic acid in cereal products. In this method the cyanogen bromide is added first, followed in exactly 10 minutes by the addition of the aniline reagent. The data in Table II, column 5, show the importance of exact timing when this procedure is followed.

As soon as cyanogen bromide reagent is added, the time is noted and the solutions are well mixed and immediately placed in specially matched test tubes. The change in per cent transmission with time is measured on an Evelyn photoelectric colorimeter with a 420 mμ filter. Solutions 1 and 2 are read against water as a blank—i. e., the galvanometer is adjusted to 100 per cent transmission with distilled water. Solution 3 is used to adjust the galvanometer to 100 per cent transmission before measuring solutions 4, 5, and 6 in order to compensate for the color of the original sample solution. In the case of an unknown sample, it is necessary to make a complete series of transmission measurements from 15 seconds to 8 minutes in order to ascertain the nature of the sample. However, in the case of samples with known history it is only necessary to have each solution in the instrument in time to take readings before the maximum is reached, in order to be certain that a measurement is made at the maximum intensity. This is particularly important in the case of the amide, as is seen in Figure 1.

TREATMENT OF RESULTS. In using an instrument such as the Evelyn photoelectric colorimeter, the measurements are made as per cent transmission which are converted to extinction coefficients ($\log \frac{I_0}{I}$). The average value of the extinction coefficients of the reagent blanks (solutions 1 and 2) is then subtracted from the extinction coefficients of sample solutions 4, 5, and 6. These corrected extinction coefficients are plotted as ordinates against the added amounts of nicotinic acid or amide as abscissas. Since it has been found that Beer's law holds for these concentrations, the three points at 0, 12.5, and 25 micrograms of added nicotinamide or acid will determine a straight line. Extrapolation of this straight line through the abscissa gives the amount of nicotinic acid or nicotinamide, as the case may be, originally present in the sample aliquot. By careful technique results may be checked to within ±2 per cent. As stated by Harris and Raymond (*2*), the advantage of treating results in this manner lies in the fact that while the slope of the line may vary somewhat with different samples because of variations in pH, salt concentration, etc., yet the three points will still determine a straight line from which an accurate determination may be made.

TABLE II. EFFECT OF TIME DELAY IN ADDING ANILINE FOLLOWING ADDITION OF CYANOGEN BROMIDE

| Time[a] | Log $\frac{I_0}{I}$ for 50 μg. of Nicotinamide | Change in Log $\frac{I_0}{I}$ | Log $\frac{I_0}{I}$ for 50 μg. of Nicotinic Acid | Change in Log $\frac{I_0}{I}$ |
|---|---|---|---|---|
| *Min.* | | % | | % |
| 0 | 0.195 | 0.00 | 0.295 | 0.00 |
| 1 | 0.207 | + 6.02 | 0.308 | + 4.38 |
| 2 | 0.222 | +14.03 | 0.308 | + 4.38 |
| 4 | 0.238 | +22.33 | 0.315 | + 6.64 |
| 5 | 0.246 | +26.11 | ... | ... |
| 8 | ... | ... | 0.333 | +12.82 |
| 10 | 0.298 | +52.62 | ... | ... |
| 16 | ... | ... | 0.364 | +23.45 |

[a] Elapsed time between addition of cyanogen bromide and aniline.

In the analysis of samples containing both nicotinamide and nicotinic acid, the samples are first run directly as the amide and then following hydrolysis determined as total nicotinic acid. By use of Equation 1 the amounts of nicotinic acid and nicotinamide may be determined.

Let $T$ = total micrograms of nicotinic acid plus nicotinamide as determined on an aliquot of the hydrolyzed sample
$x$ = micrograms of nicotinamide originally present in an equal sample aliquot
$y$ = micrograms of nicotinic acid originally present in an equal sample aliquot
$z$ = micrograms determined as amide before hydrolysis on an equal sample aliquot
$K$ = ratio of the maximum extinction of the acid to that of an equal quantity of amide at time of maximum amide intensity as obtained from time-reaction curves of standard nicotinic acid and nicotinamide solution similar to Figure 1

Since the micrograms determined as amide before hydrolysis are equal to the actual micrograms of nicotinamide plus the micrograms of nicotinic acid times the factor $K$, the following equation may be written:

$$z = T - y + Ky$$

$$y = \frac{T - z}{1 - K} \qquad (1)$$

from which $y$ may be determined, giving the micrograms of nicotinic acid, while $y$ subtracted from $T$ gives the micrograms of nicotinamide present in the sample aliquot.

Small experimental errors in the determination of $z$ or $T$ or both become magnified in solving for $x$ and $y$. As a result, individual determinations for nicotinic acid and nicotinamide in materials containing both forms may differ from one another by as much as 10 or 15 per cent.

Table III. Extinctions of Equimolecular Quantities

| Compound | Equimolecular Weights | $\text{Log}\frac{I_0}{I}$ | Time[a] |
|---|---|---|---|
| | $\mu g.$ | | *Sec.* |
| Nicotinic acid | 50.0 | 0.295 | 360–420 |
| Nicotinamide | 49.6 | 0.195 | 210 |
| Pyridine | 32.0 | 0.248 | 87 |
| β-Picoline | 38.0 | 0.111 | 28 |

[a] Length of time for development of maximum extinction.

## Discussion

In Figure 1 time-reaction curves are given for β-picoline and pyridine as well as for nicotinic acid and nicotinamide. A comparison of these curves shows that the more polar the group on the beta-carbon atom of the pyridine nucleus the longer the time required for maximum development of color and the more stable the color produced. These results indicate that the nature of the group on the beta-carbon atom of the pyridine nucleus exerts a decided influence on the mechanism of the dianil reaction and on the intensity of the color of the resulting compound. In Figure 1 maximum extinction coefficients are plotted for equal quantities of compounds—namely, 50 micrograms. Maximum extinction coefficients for equimolecular quantities of the four compounds have been calculated from Figure 1 and tabulated in Table III. On an equimolecular basis the intensity of the color produced by these four compounds decreases in the following order: nicotinic acid, nicotinamide, pyridine, and β-picoline. This is the same order as reported by Melnick, Robinson, and Field (*6*).

## Summary

A method for the direct quantitative analysis of compounded vitamin mixtures for nicotinamide has been described, which has been used successfully in the analysis of several hundred samples during the past year. Time-reaction measurements of the color produced by the König reaction, using the reagents cyanogen bromide and aniline, form the basis of the method. The relationship between maximum extinction coefficients and time for their development has been found to be distinctly characteristic for nicotinic acid and nicotinamide as well as for pyridine and β-picoline.

A delay of a few minutes in the addition of aniline following the addition of cyanogen bromide introduces considerable error, while as much as a 15-minute delay in adding cyanogen bromide after first adding the aniline does not introduce an appreciable error.

Results obtained by this method have been found to be reproducible to within ±2 per cent for the direct quantitative determination of either nicotinic acid or nicotinamide in vitamin mixtures.

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# Chemical Differentiation between Niacinamide and Niacin in Pharmaceutical Products[1]

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THE microbiological method of Snell and Wright in its existing forms (*1*, *2*, *5*) does not distinguish between niacin and niacinamide, since both compounds exert the same microbiological stimulatory effects (*5*). By taking advantage of the relative reaction rates, the chemical method offers a means of differentiating between the free acid and the amide. This distinction is of no little importance, since niacin produces in many individuals an uncomfortable sensation of flushing and erythema of the skin; moreover, the amide is more expensive. In the chemical method (*2*, *3*) the samples are first subjected to acid hydrolysis to convert biologically active niacin derivatives to free niacin. Niacinamide, however, reacts with the reagents prior to hydrolysis to yield the same color as niacin but of lesser intensity (*3*). Niacinamide, like niacin, is quantitatively adsorbed on and eluted from Lloyd's reagent and neither is lost during the lead hydroxide clarification procedure (*2*). The color reactions due to these two compounds in one solution are additive.

When the color reaction is carried out according to the collaborative chemical procedure (*2*), the maximal color developed (the photometric density 3 to 8 minutes after the addition of the aniline reagent) is 35 to 40 per cent greater with niacin than with an equivalent amount of niacinamide. With the latter, the maximal photometric density develops in approximately 2.5 minutes but is stable for only 30 seconds. If the cyanogen bromide and aniline reagents are added in rapid sequence as in the method of Melnick and Field (*3*)—i. e., omitting the 10-minute period during which the cyanogen bromide alone is allowed to react—the sensitivity of the method for niacin is reduced by 30 to 35 per cent, but the maximal color due to reacted niacin is now practically twice that of reacted niacinamide. Figure 1 shows these differences in reaction rates. Readings were taken at 15-second intervals

[1] See Editor's note on page 352.

in an Evelyn photoelectric colorimeter (Rubicon Company, Philadelphia, Penna.) with filter 420.

In conducting a test for differentiating niacin from niacinamide, the material is tested before and after acid hydrolysis. The molecular weights of niacin and niacinamide are almost the same, 123 and 122, respectively. The details for the preparation of the reagents, the hydrolysis, subsequent clarification, and the setting up of the solutions for the color development have been described in detail elsewhere (*2*). The final color development, however, follows the earlier method of Melnick and Field (*3*). [For the first test of total niacin content the collaborative chemical procedure (*2*) may be used. However, for testing the unhydrolyzed sample the addition of the reagents in rapid sequence (*3*) is preferred, in order that the photometric densities of reacted niacin and niacinamide may show a bigger difference. The readings should be taken periodically during the color development, since maximal constant values are required for the calculation and the reaction, particularly with niacinamide, fades rapidly once the maximum is attained.]

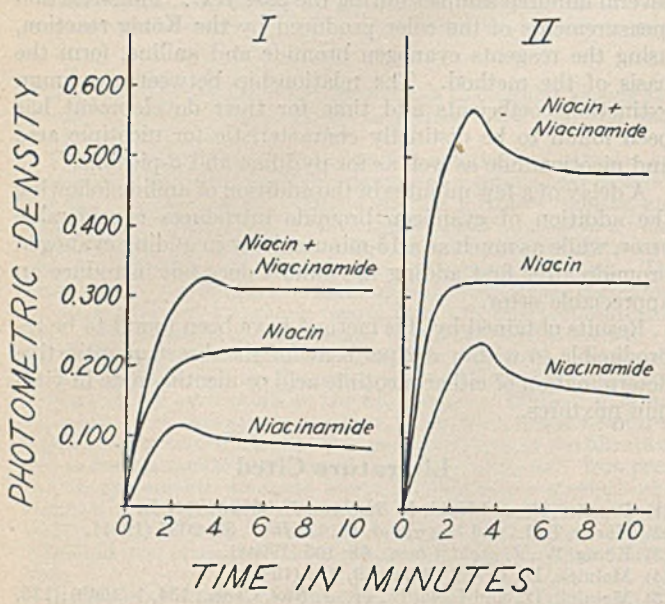


FIGURE 1. TIME-REACTION CURVES

10-microgram quantities of niacin and niacinamide with cyanogen bromide and aniline reagents.
I. Reagents added in rapid sequence (*3*)
II. Cyanogen bromide allowed to react with vitamin for 10 minutes before addition of aniline reagent (*2*)

## Typical Calculation

Test substance: vitamin capsule subjected to acid hydrolysis.

Preparation of test solution: 20 capsules dissolved in 200 ml. of water; 1-ml. aliquot carried through the collaborative chemical method (*2*) but the maximal yellow color due to reacted niacin measured after the cyanogen bromide and aniline reagents were added in rapid sequence (*3*).

Eight milliliters of the final neutralized supernatant solution are diluted to 10 ml. and 3-ml. aliquots taken for testing.

0.016 = photometric density of the blank

0.438 = photometric density of the test solution

0.699 = photometric density of the test solution containing 10 μg. of added niacin

Then

0.438 − 0.016 = 0.422 photometric density due to reacted niacin

0.699 − 0.438 = 0.261 photometric density due to 10 μg. of reacted niacin

0.422/0.261 × 10 = 16.2 μg. of niacin in 3-ml. aliquot of test solution

16.2 × 10/3 × 15/8 × 1000/1 × 1/10 × 1/1000 = 10.1 mg. of total niacin per capsule

Test for niacinamide: repeat above, omitting acid hydrolysis

0.000 = photometric density of the blank

0.216 = photometric density of test solution

0.475 = photometric density of test solution containing 10 μg. of added niacin

0.216 − 0.000 = 0.216 photometric density due to reacted niacin compounds

0.475 − 0.216 = 0.259 photometric density due to 10 μg. of added niacin

0.216/0.259 × 10 = 8.3 μg. of apparent niacin in sample

8.3 × 10/3 × 15/8 × 1000/1 × 1/10 × 1/1000 = 5.2 mg. of apparent niacin per capsule

Since this is one half of the total niacin, found after hydrolysis, the material is present in the capsule entirely as niacinamide.

Had the above value for apparent niacin been 7.0 mg. per capsule, then

$$10.1 - 7.0 = 3.1$$
$$\frac{2}{6.2}$$

6.2 mg. of niacinamide per capsule

and 10.1 − 6.2 = 3.9 mg. of niacin per capsule

Some latitude (±15 per cent) should be allowed in interpreting the results, since the accuracy of the partition of the niacin ingredient is dependent upon the precision of two assays and errors may be additive.

In testing samples such as pure niacin, niacinamide, or mixtures of the two, clear colorless solutions are obtained; the adsorption-elution and decolorization procedures are then omitted. Greater precision is also obtained. For determinations conducted on pure niacin or niacinamide preparations, direct reference to the reaction curves in Figure 1 may suffice to identify and allow rapid quantitative estimation of the compound with a good degree of precision (±2 per cent).

The nicotinamide-containing coenzymes fail to yield a yellow reaction product with the reagents unless first hydrolyzed. Nicotinuric acid reacts with the reagents to yield some color prior to hydrolysis. Strong acid hydrolysis is required to convert it quantitatively to free niacin (*4*). However, these compounds are not readily available. The test procedure described is intended for use only in assaying the niacin ingredient in vitamin capsules and tablets containing the synthetic vitamin.

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# Determination of Microquantities of Certain Proteins

## A Colorimetic Method

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IN CONNECTION with studies of serological reactions (*5*) it was necessary to have a simple and rapid method for determining quantitatively small amounts (from 10 to 1000 micrograms) of specifically precipitated antibody globulin which would permit a large number of determinations in a short time. A colorimetric method using the Folin-Ciocaltéu phenol reagent (*3*) was finally chosen. The modification of heating the protein in alkaline solution before the addition of the phenol reagent to increase the intensity of the color (*1*, *4*) was also adopted.

The phenol reagent reacts with the chromogenic reducing groups of proteins in basic solution to give a blue color. However, the phenol reagent is decomposed rapidly by alkali, and thus the color developed depends on the relative rates of reaction of the phenol reagent with protein and with alkali. In most cases the protein does not react completely, and this cannot be remedied by use of larger amounts of the reagents, since then white precipitates form rapidly. Therefore, an empirical procedure which would give reproducible results was developed, and the effect of pertinent variations was studied.

### Procedure

Add from a buret 2.5 ml. of 1.00 *N* sodium hydroxide solution to 10 to 1000 μg. of the protein sample in a calibrated 15-ml. centrifuge tube and bring to a volume of 7.5 ml. with distilled water. Specific precipitates dissolve readily in this solution. Heat in a boiling water bath from 5 to 10 minutes and cool to about 25° C. in running tap water. Add rapidly 2.5 ml. of the phenol reagent (prepared as described by Folin and Ciocaltéu, *3*, and diluted with two volumes of water) and shake well immediately. A clean rubber stopper may be used during the shaking without affecting the color. Let stand 10 minutes for color development and read the color intensity with a Klett-Summerson photoelectric colorimeter, using a red filter transmitting 640 to 700 mμ. If the reading is above 450 units, dilute the solution two- or fourfold and multiply the observed reading by the dilution factor. Make a blank run at the same time, omitting the protein, and subtract the blank reading (10 to 15 units) from the protein color reading. Read the amount of protein present from a plot, such as that in Figure 1, of the corrected color reading against the weight of protein.

The author has found it convenient to run the determinations in sets of twelve. By staggering the sets, one man can average 40 analyses per hour or two men can average 100 analyses per hour with a single instrument.

Using simple substances as antigens (*5*), the author found that the antigen usually has a negligible chromogenic value. When colored proteins are used as antigens, the amount of antigen in the precipitate can be determined colorimetrically before the addition of the phenol reagent and a correction can be made for the chromogenic value of the antigen.

Although the same amounts of different proteins have different chromogenic values, the plots are similar. By changing the protein scale (abscissa), the plots can be made the same for rabbit globulin, bovine globulin, crystalline ovalbumin, and whole rabbit serum.

The plot of Figure 1 was made for rabbit serum globulin (rabbit anticrystalline ovalbumin) by the following procedure:

A solution of ovalbumin of known concentration as determined by Kjeldahl analysis (*6*) was added to an excess of rabbit anti-ovalbumin serum and the mixture was permitted to stand overnight in the refrigerator. The precipitate (*ca.* 12 mg.) was washed three times with 20-ml. portions of 0.9 per cent saline, dissolved in 1.00 *N* sodium hydroxide solution, and made up to a definite volume as a stock solution. Aliquots of this solution were analyzed by the Kjeldahl method for nitrogen and the amount of protein was calculated by multiplying the weight of nitrogen by the factor 6.25. Dilutions of the stock protein solution were made and analyzed colorimetrically by the method outlined above. The amounts of protein and the color readings were corrected for the small amount of ovalbumin present. All the ovalbumin added was assumed to be in the precipitate, since the precipitate was obtained in the region of antibody excess. The experimental points used in Figure 1 were run in duplicate with average deviation of ±1.5 per cent.

The phenol reagent was checked from time to time against a standard solution of tyrosine in hydrochloric acid stabilized with formalin, as described by Anson (*2*), and was found to give the same color intensity over a period of 4 months and for three different preparations of the reagent. This indicates the stability and the reproducibility of the reagent.

In order to check the effect of variations from the chosen procedure, experiments were carried out to study the effect of varying the amount of sodium hydroxide used; the amount of phenol reagent used; the length of time of exposure to sodium hydroxide before, during, and after heating; the

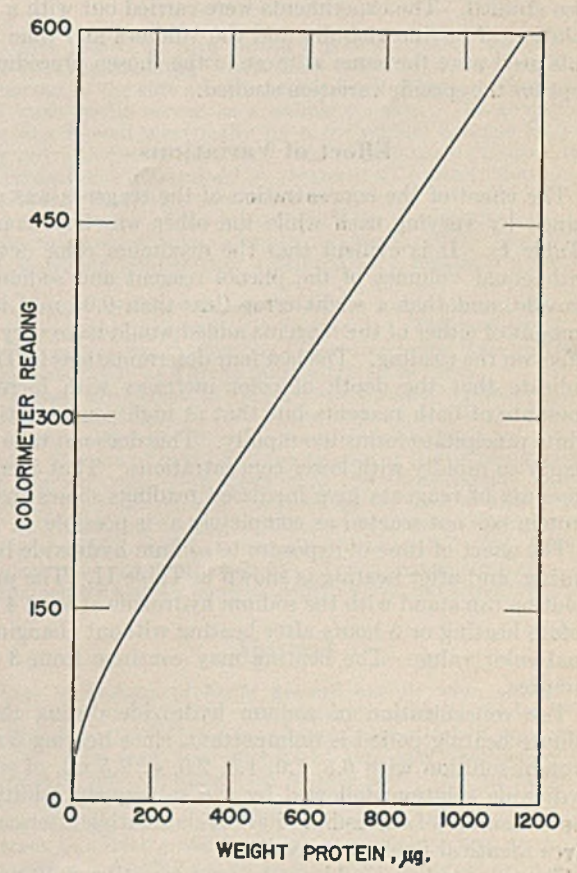


FIGURE 1. PLOT OF COLORIMETER READING AGAINST WEIGHT OF RABBIT ANTIBODY GLOBULIN

TABLE I. EFFECT OF CONCENTRATION OF REAGENTS

(4 ml. of stock globulin solution)

| NaOH | Phenol Reagent | Color[a] Reading |
|---|---|---|
| *Ml.* | *Ml.* | |
| 2.5 | 1.8 | 305 |
| 2.5 | 1.9 | 300 |
| 2.5 | 2.0 | 323 |
| 2.5 | 2.1 | 340 |
| 2.5 | 2.2 | 350 |
| 2.5 | 2.3 | 355 |
| 2.5 | 2.4 | 370 |
| 2.5 | 2.5 | 398 |
| 2.5 | 2.6 | 378 |
| 2.5 | 2.7 | 380 |
| 2.5 | 2.8 | 365 |
| 2.5 | 2.9 | 320 |
| 2.5 | 3.0 | 285 |
| 2.5 | 3.1 | 240 |
| 2.5 | 3.2 | 234 |
| 1.8 | 2.5 | 188 |
| 1.9 | 2.5 | 218 |
| 2.0 | 2.5 | 242 |
| 2.1 | 2.5 | 308 |
| 2.2 | 2.5 | 355 |
| 2.3 | 2.5 | 390 |
| 2.4 | 2.5 | 397 |
| 2.5 | 2.5 | 400 |
| 2.6 | 2.5 | 390 |
| 2.7 | 2.5 | 373 |
| 2.8 | 2.5 | 358 |
| 2.9 | 2.5 | 350 |
| 3.0 | 2.5 | 345 |
| 3.1 | 2.5 | 330 |
| 3.2 | 2.5 | 327 |
| 1.0 | 1.0 | 197[b] |
| 2.0 | 2.0 | 225[b] |
| 3.0 | 3.0 | 244[b] |
| 4.0 | 4.0 | Ppt. formed[b] |

[a] Average of two determinations with average deviation of 2.0%.
[b] One-half amount of protein used.

length of time of reaction with the phenol reagent; the temperature of the reactants; and the mode of addition of the phenol reagent. The effect of omitting the heating step was also studied. The experiments were carried out with a stock solution of bovine globulin and the volumes and time intervals used were the same as those in the chosen procedure except for the specific variation studied.

## Effect of Variations

The effect of the concentration of the reagents was determined by varying each while the other was kept constant (Table I). It is evident that the maximum color develops with equal volumes of the phenol reagent and sodium hydroxide, and that a slight error (less than 0.05 ml.) in the amount of either of the reagents added would have very little effect on the reading. The last four determinations in Table I indicate that the depth of color increases with increasing amounts of both reagents but that at high concentrations a white precipitate forms too rapidly. This does not take place nearly so rapidly with lower concentrations. That increased amounts of reagents give increased readings shows that the protein has not reacted as completely as is possible.

The effect of time of exposure to sodium hydroxide before, during, and after heating is shown in Table II. The protein solution can stand with the sodium hydroxide at least 4 hours before heating or 5 hours after heating without changing the final color value. The heating may continue from 3 to 14 minutes.

The concentration of sodium hydroxide during the 10-minute heating period is unimportant, since heating 5 ml. of protein solution with 0.5, 1.0, 1.5, 2.0, or 2.5 ml. of sodium hydroxide solution, followed by the subsequent addition of the remainder of the sodium hydroxide solution after cooling, gives identical final color readings.

The color value should not be read for at least 10 minutes after the addition of the phenol reagent, as is shown in Table III. The reading is then constant until a white precipitate begins to form. The time of formation of this precipitate is variable. It probably is a supersaturation phenomenon, since traces of the precipitate greatly increase the rate of precipitate formation.

The temperature at which the mixing takes place was varied (Table IV). Apparently the optimum temperature is room temperature, 20° to 25° C. The rate of addition of the phenol reagent is important, since adding the reagent with stirring in small portions over a period of 1 minute increased the color intensity by over 10 per cent. However, adding the reagent rapidly, all in one portion, followed by shaking immediately or even 1 minute later gave constant results.

If the heating step is omitted, the optimum volume ratio of phenol reagent to sodium hydroxide solution remains the same and the rate of color formation is similar to that when the solution is heated, but the maximum color is 20 per cent less. The main difficulty incurred when the heating is omitted is

TABLE II. EFFECT OF EXPOSURE TO SODIUM HYDROXIDE BEFORE, DURING, AND AFTER HEATING

(5 ml. of stock globulin solution)

| Time before Heating | Time during Heating | Time after Heating | Color[a] Reading |
|---|---|---|---|
| *Min.* | *Min.* | *Min.* | |
| 1 | 10 | ... | 463 |
| 3 | 10 | ... | 452 |
| 6 | 10 | ... | 452 |
| 15 | 10 | ... | 455 |
| 30 | 10 | ... | 452 |
| 60 | 10 | ... | 457 |
| 120 | 10 | ... | 463 |
| 240 | 10 | ... | 463 |
| 10 | 0 | ... | 360 |
| 10 | 1 | ... | 430 |
| 10 | 2 | ... | 442 |
| 10 | 3 | ... | 450 |
| 10 | 5 | ... | 447 |
| 10 | 10 | ... | 440 |
| 10 | 14 | ... | 435 |
| 10 | 30 | ... | 430 |
| 10 | 10 | 5 | 438 |
| 10 | 10 | 10 | 433 |
| 10 | 10 | 15 | 428 |
| 10 | 10 | 30 | 442 |
| 10 | 10 | 60 | 448 |
| 10 | 10 | 160 | 443 |
| 10 | 10 | 300 | 452 |

[a] Duplicate analysis with mean deviation ±1%.

TABLE III. COLOR READINGS AFTER ADDITION OF PHENOL REAGENT

(5 ml. of stock globulin solution)

| Time | Color[a] Reading |
|---|---|
| *Min.* | |
| 1 | 327 |
| 2 | 365 |
| 4 | 410 |
| 6 | 432 |
| 8 | 445 |
| 10 | 457 |
| 12 | 467 |
| 14 | 472 |
| 16 | 477 |
| 18 | 472 |
| 20 | Ppt. forms |

[a] Average of duplicate analyses with mean deviation of 1%.

TABLE IV. EFFECT OF TEMPERATURE OF MIXING

(5 ml. of stock globulin solution)

| Temperature of Mixing ° C. | Color[a] Reading |
|---|---|
| 15 | 422 |
| 20 | 445 |
| 25 | 450 |
| 30 | 430 |
| 35 | 412 |

[a] Average of duplicate analyses with mean deviation of 1%.

TABLE V. EFFECT OF STANDING WITH SODIUM HYDROXIDE WITHOUT HEATING

(5 ml. of stock globulin solution)

| Time of Exposure *Min.* | Color[a] Reading |
|---|---|
| 1 | 353 |
| 3 | 365 |
| 5 | 365 |
| 10 | 378 |
| 15 | 390 |
| 30 | 405 |
| 60 | 423 |
| 120 | 435 |
| 1440 | 437 |

[a] Duplicate analyses with mean deviation of 1.5%.

that the color reading depends on the time of exposure to sodium hydroxide for any relatively short time, as shown in Table V. This would require that subsequent steps in the analyses be delayed inconveniently for at least 2 hours, since in the author's work the protein samples are precipitates which are often slowly soluble in alkali, and thus no standard short time could be chosen. Heating with alkali presumably denatures the protein, so that all chromogenic groups are exposed, and thus any slight denaturation in the handling or aging of the protein (*4*) can be neglected.


## Acknowledgment

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# Microdetermination of Volatile Matter in Coal and Coal Products


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THE need for microdetermination of volatile matter arose in this laboratory in connection with a study of the combustion of pulverized coal (*6*). Volatile matter had to be determined on small amounts of physically similar particles which had been separated by microscopic examination. Since only small amounts of products were available from the combustion studies, the standard A. S. T. M. (*1*) procedure for determining volatile matter was impractical.

The method decided upon consists, essentially, of carbonization of a 3-mg. sample in a stream of dry, oxygen-free nitrogen. The sample is contained in a platinum microboat placed in a quartz combustion tube. Similar tube methods for macrodetermination of volatile matter have been described in the literature (*2, 3, 4*). This micromethod offers the advantages of introduction of the boat into a cold part of the tube, carbonization at a high heating rate, and cooling in an oxygen-free atmosphere. Oxidation of the sample is thereby minimized. No attempt has been made to obtain agreement with the standard A. S. T. M. method. Statistical analysis of the results obtained by the micromethod on 43 samples showed that this procedure gives results as reproducible as those obtained by the A. S. T. M. method. The statistical analysis was similar to one made on macro results for volatile matter (*5*). The limited data available indicate that there is a linear correlation between the standard differences of duplicate analyses and the absolute level of volatile matter. The results obtained by the micromethod are somewhat higher, owing to a higher rate of heating. Similar results have been observed previously (*7*).

## Apparatus

The apparatus consisted of a quartz combustion microtube with a side arm. Nitrogen was freed of oxygen by passage through an electrically heated tube containing metallic copper and then passed in turn through a concentrated sulfuric acid wash bottle and a large U-tube filled with Anhydrone, which was connected to the side arm of the combustion tube. The sulfuric acid wash bottle served as a bubble counter. The combustion tube was heated electrically by a cylindrical furnace 22.5 cm. long and 11.5 cm. in diameter. The temperature gradient along the furnace was determined by means of a Chromel-Alumel thermocouple. Readings were taken at 1.5-cm. intervals on both sides of the center of the furnace with the thermocouple inserted in the combustion tube, nitrogen flowing at 10 cc. per minute. The maximum temperature difference for a length of 6 cm. was 5° C.

To permit introduction of the boat in a cold part of the tube, the furnace was mounted on a steel track. This permitted it to slide laterally along the length of the combustion tube and be replaced accurately at a given place around the tube.

The boat was placed in the tube and pushed to a fixed spot by means of a marked glass rod. This position was at the center of the furnace when the furnace was moved into position. To determine the rate of heating of the sample as it was being carbonized, "blank" runs were made with the thermocouple junction placed in the same position occupied by the boat in a determination with nitrogen flowing at the standard rate. The temperature reached 865 ± 5° C. in one minute and 950 ± 5° C. between the sixth and the tenth minute. Temperature constancy was maintained by manual control using a Varitran voltage regulator.

## Method

Three milligrams of finely ground sample were spread uniformly along the bottom of a platinum microboat, dried in a Pregl block at 115° C. for 15 minutes, and weighed on a microbalance. The boat was introduced into the combustion tube and pushed into position with a glass rod. Dry nitrogen was passed through the combustion tube for 15 minutes at 10 cc. per minute to displace the air. The furnace was then moved along the track to a fixed position so that the boat was now at the center position. At the end of 10 minutes, measured with a stop watch, the furnace was moved away from the boat. The boat was permitted to cool for 7 minutes, preliminary tests with a thermocouple having shown that the temperature dropped to

TABLE I. MICRODETERMINATION OF VOLATILE MATTER

| Sample | Volatile Matter Dry basis | Volatile Matter Dry basis, A. S. T. M. | Sample | Volatile Matter Dry basis | Volatile Matter Dry basis, A. S. T. M. | Sample | Volatile Matter Dry basis | Volatile Matter Dry basis, A. S. T. M. | Sample | Volatile Matter Dry basis | Volatile Matter Dry basis, A. S. T. M. |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | % | % | | % | % | | % | % | | % | % |
| Coke 1 | 1.02 | | Coal residue 5 | 10.17 | | Coal 1 | 17.34 | | Coal 6 | 38.15 | |
| | 1.13 | | | 9.91 | | | 17.49 | | | 38.35 | |
| | 1.17 | | Av. | 10.04 | .. | | 17.61 | | Av. | 38.25 | .. |
| | 1.04 | | Coal residue 6 | 10.67 | | Av. | 17.48 | 15.4 | Coal 7 | 36.22 | |
| | 1.09 | | | 10.61 | | Coal 2 | 36.76 | | | 36.06 | |
| | 1.17 | | Av. | 10.64 | .. | | 36.93 | | Av. | 36.14 | .. |
| Av. | 1.10 | 0.99 | | | | | 36.60 | | | | |
| Coke 2 | 0.84 | | | | | | 37.03 | | Coal 8 | 30.15 | |
| | 0.94 | | | | | | 36.53 | | | 30.43 | |
| Av. | 0.89 | 0.39 | | | | | 36.91 | | | 30.19 | |
| Coal residue 1 | 1.23 | | | | | Av. | 36.79 | 34.3 | Av. | 30.26 | .. |
| | 1.35 | | | | | Coal 3 | 38.88 | | | | |
| Av. | 1.29 | .. | | | | | 38.13 | | Coal 9 | 28.61 | |
| Coal residue 2 | 1.19 | | | | | | 38.26 | | | 28.63 | |
| | 1.28 | | | | | | 38.54 | | Av. | 28.62 | .. |
| Av. | 1.24 | .. | | | | Av. | 38.45 | 37.5 | Coal 10 | 36.53 | |
| Coal residue 3 | 1.60 | | | | | Coal 4 | 38.29 | | | 36.69 | |
| | 1.44 | | | | | | 38.49 | | | 36.38 | |
| Av. | 1.52 | .. | | | | Av. | 38.39 | .. | Av. | 36.53 | .. |
| Coal residue 4 | 2.77 | | | | | Coal 5 | 38.22 | | Coal resin | 89.12 | |
| | 2.80 | | | | | | 38.18 | | | 89.04 | |
| Av. | 2.79 | .. | | | | Av. | 38.20 | .. | Av. | 89.08 | .. |

40° C. in this period. The boat and residue were removed and placed on a cooling block for 5 minutes and weighed. In cases of low volatile samples more accurate results were obtained by weighing the boat and residue in a glass-stoppered pig. The results on various samples are indicated in Table I.

## Summary

A micromethod has been described which has the same reproducibility as the A. S. T. M. macroprocedure, but gives somewhat higher results. Carbonization, sample introduction, and sample removal occur under conditions which minimize air oxidation. The method can be applied to samples varying over a wide range of volatile matter without modifying the technique.

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# New Reactions of 2,2′-Dichlorodiethyl Sulfide

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THE reaction of 2,2′-dichlorodiethyl sulfide with the chlorides of palladium, gold, and other heavy metals—especially with palladium chloride—has been used for the identification of this compound. Reactions with salts of copper are not very characteristic under ordinary circumstances, but they can be modified to yield sensitive, rapid, and simple methods for the identification of 2,2′-dichlorodiethyl sulfide.

A sheet of filter paper saturated with a 2 per cent solution of copper sulfate in water, or a saturated alcoholic solution of copper nitrate, containing 1 to 2 per cent of glycol or glycerol, is laid horizontally on a few layers of clean filter paper, and dried at room temperature. The reactions are more distinct if the copper nitrate solution is used. Finally, the test paper is cut to size and stored in an airtight container.

The test paper is not affected by pure glycol. Commercial glycol containing triethanolamine phosphate slowly produces a blue ring. Thiodiglycol, the parent compound of 2,2′-dichlorodiethyl sulfide, produces almost immediately a bright green spot with a very marked green border; 0.1 to 0.4 mg. of thiodiglycol can easily be detected in this way. The sensitivity of the reaction is greater if the thiodiglycol is applied free of solvent.

Hydrochloric acid produces a very pale yellow spot, easily distinguished from the dark brown spot caused by a mixture of hydrochloric acid and 2,2′-dichlorodiethyl sulfide; the center of the brown spot gradually changes to violet. Less than 0.2 mg. of 2,2′-dichlorodiethyl sulfide can be recognized by this reaction.

The direct reaction of 2,2′-dichlorodiethyl sulfide is not entirely satisfactory at lower concentrations, but the following indirect reaction is satisfactory in every respect.

The sample suspected of containing 2,2′-dichlorodiethyl sulfide is extracted with ether, and the ether solution is refluxed with normal alkali on a water bath kept at about 60° C. for 30 minutes. If the amount of 2,2′-dichlorodiethyl sulfide is known to be very small, a few minutes' refluxing will suffice. A green color will appear on the test paper if more than 1 to 2 mg. of 2,2′-dichlorodiethyl sulfide is present.

To detect smaller quantities, the alkaline solution is acidified with dilute sulfuric acid in the presence of phenolphthalein, and the slightly acid solution is diluted with 5 volumes of alcohol. The precipitated sulfate is filtered and washed with absolute alcohol, and a few corns of anhydrous sodium carbonate are added to the filtrate to remove traces of acid. The test is positive if more than 0.1 to 0.2 mg. of 2,2′-dichlorodiethyl sulfide was originally present.

The test can be made still more sensitive by removing some or all of the solvent, but it is less sensitive than the sodium iodoplatinate test. Its practical advantage lies in the fact that the necessary reagents are easily procured and the test can be carried out in poorly equipped or emergency laboratories, or by A. R. P. workers who are not professional chemists.

# SARGENT *Fluorescent* LABORATORY LAMPS

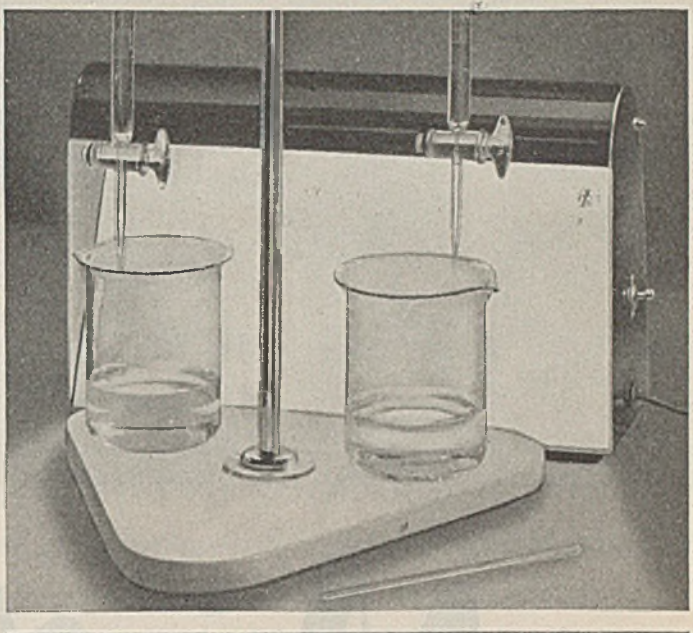
## For Titrations

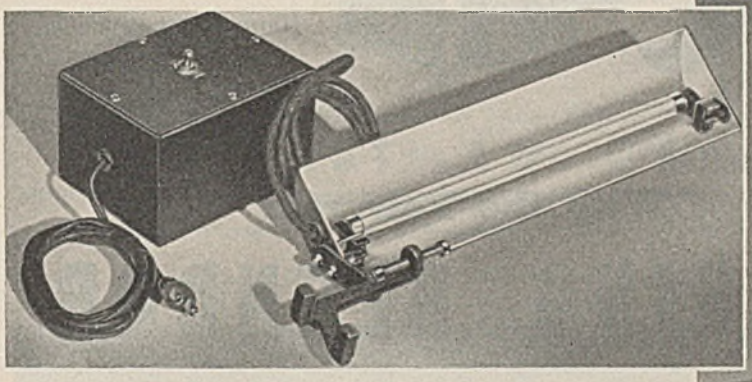
Supplies soft, glareless fluorescent illumination of daylight quality, correct intensity and color characteristics to beakers in which titrations are being carried out.

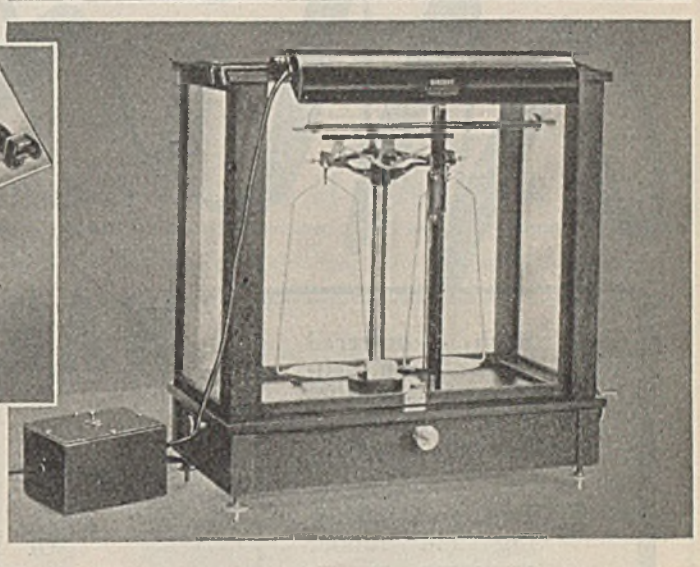
A *parabolic* reflector directs light against a flat, white, acid and alkali resistant vitreous enameled inclined background, giving protection against discoloration, eliminating direct illumination and so preventing glare and undesirable illumination intensities.

The outer surface of the lamp is also finished in a dark blue vitreous enamel. Fitted with snapswitch, connecting cord and plug. Length, 12½ inches; height, 8 inches; depth, 4½ inches.

**S-44290 Sargent Fluorescent Titration Lamp.** Complete with fluorescent tube, type T-5. For operation from 115 volt 60 cycle circuits..... $13.00







## For Directed Frontal Illumination of Balances

The lamp can be attached to all makes and styles of balances. Mounted at the front of a balance case, the *parabolic* reflector directs all light through the front door over the entire working area of a balance to uniformly illuminate beam, chain scale, pointer index and pans. Objectionable shadows and poorly lighted areas are entirely eliminated.

The lamp is supported by a simple clamp and permits free movement of the sliding balance door. Bulk and weight of the reflector are greatly reduced by making a separate unit of the auxiliary.

Both inside and outside of the reflector are finished in vitreous enamel which gives excellent protection against corrosion and discoloration thus assuring maintenance of the high illumination efficiency of the lamp.

**S-3820 Sargent Fluorescent Balance Lamp.** Complete with fluorescent tube, type T-5. For operation from 115 volt 60 cycle circuits..... $11.50

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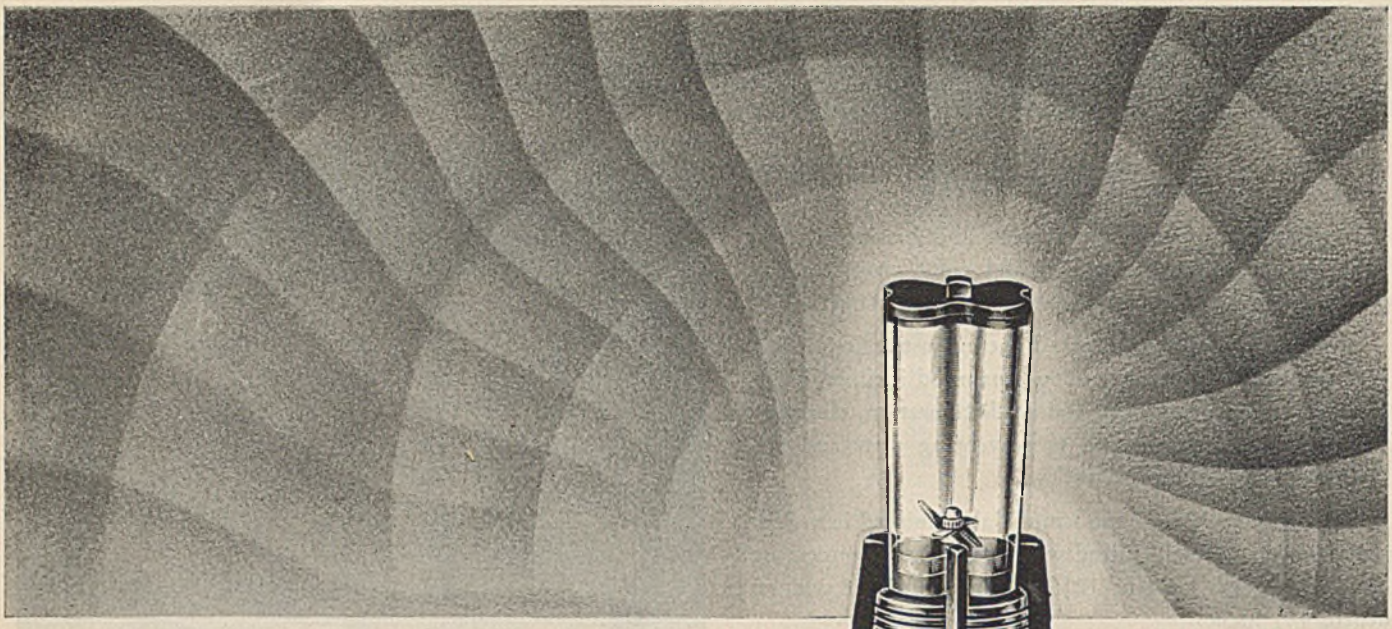
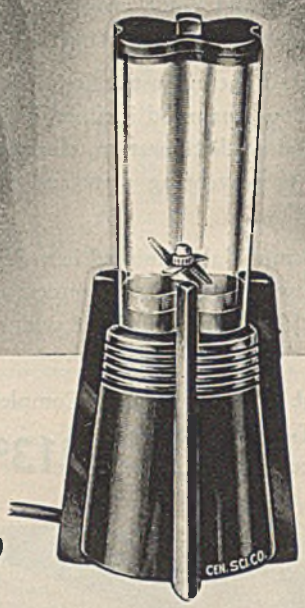


Mix

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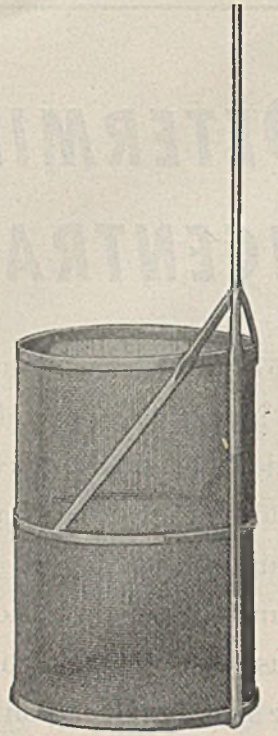

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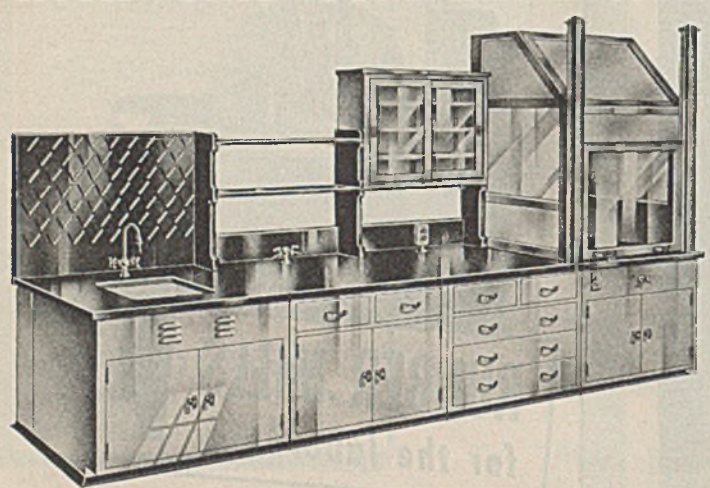

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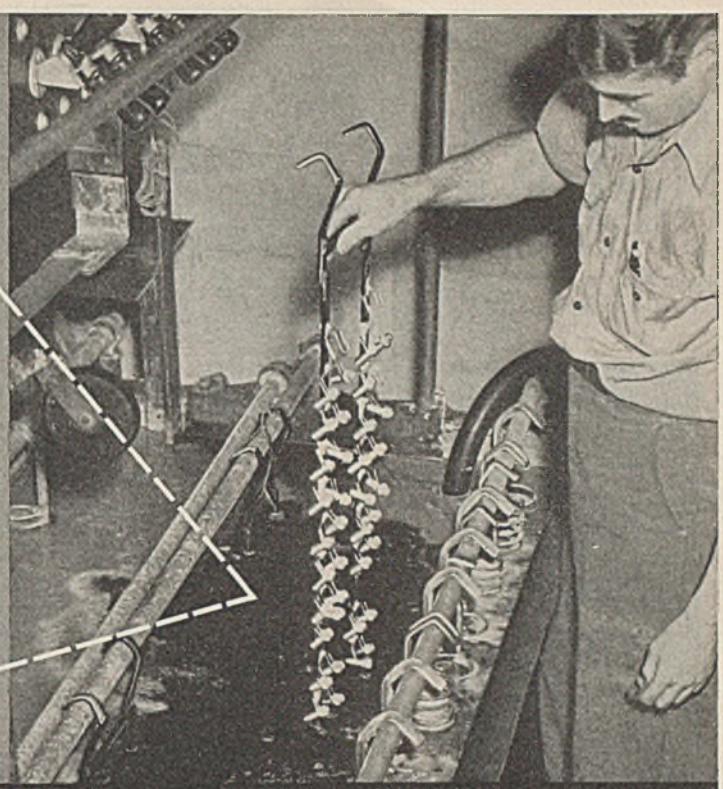

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***THE POINT:*** This is only one example of how Baker & Adamson is furnishing "special" chemicals to industry in *finished, ready-to-use* form. If *your* industrial processes require such chemicals in commercial quantities, call a conference, and let us discuss your problem in terms of the end chemical required.

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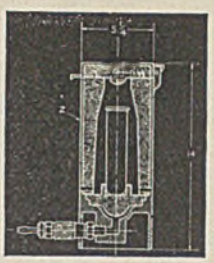
*For Total Immersion of Candle*



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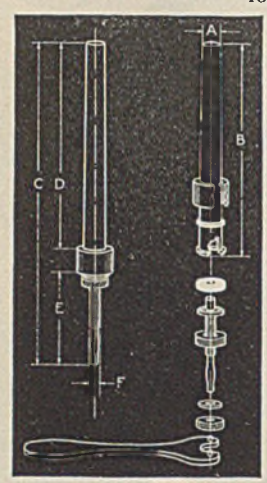


| Catalog Number | Available Porosities* XF | 10 | 01 | 02 | 03 | Type of Connection | Important Dimensions: Diameter (A) | Candle L'gth (B) | Overall L'gth (C) | Filtering L'gth (D) | (E) | (F) | Candle Price | Connector Price** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| FD-52 | | • | • | • | • | Detachable Metal Connector | 5/8" | 2 3/16" | 4 5/8" | 2" | 2 1/16" | 1/4" | $1.00 | $3.25 |
| FD-54 | | • | • | • | • | | 5/8" | 4 3/16" | 6 5/8" | 4" | 2 1/16" | 1/4" | 1.00 | 3.25 |
| FD-56 | | • | • | • | • | | 5/8" | 6 3/16" | 8 5/8" | 6" | 2 1/16" | 1/4" | 1.10 | 3.25 |
| FD-86 | • | • | • | • | • | | 1" | 6 3/16" | 9 5/8" | 6" | 3 1/16" | 1/2" | 1.75 | 4.00 |
| FD-88 | | • | • | • | • | | 1" | 8 3/16" | 11 5/8" | 8" | 3 1/16" | 1/2" | 1.75 | 4.00 |
| FD-126 | | • | • | • | • | | 1 1/2" | 6 1/4" | 9 11/16" | 6" | 3 1/16" | 1/2" | 4.50 | 4.50 |
| FD-128 | | • | • | • | • | | 1 1/2" | 8 1/4" | 11 11/16" | 8" | 3 1/16" | 1/2" | 5.00 | 4.50 |
| FP-52 | | • | • | • | • | Permanent Glazed Porcelain Nipple (can be repeatedly heated to incandescence with candle) | 5/8" | 3" | 3" | 2" | | | 1.00 | For rubber hose or rubber stopper connection |
| FP-54 | | • | • | • | • | | 5/8" | 5" | 5" | 4" | | | 1.00 | |
| FP-56 | | • | • | • | • | | 5/8" | 7" | 7" | 6" | | | 1.10 | |
| FP-86 | • | • | • | • | • | | 1" | 7 1/4" | 7 1/4" | 6" | | | 1.75 | |
| FP-88 | | • | • | • | • | | 1" | 9 1/4" | 9 1/4" | 8" | | | 1.75 | |
| FP-126 | | • | • | • | • | | 1 1/2" | 7 3/8" | 7 3/8" | 6" | | | 4.50 | |
| FP-128 | | • | • | • | • | | 1 1/2" | 9 3/8" | 9 3/8" | 8" | | | 5.00 | |

* Porosity #XF has bubbling pressure of 0.44 lbs. per sq. in. (maximum pore diameter, 100 microns).
" #10 " " " " 4 " " " " ( " " " 11 microns).
" #01 " " " " 6 " " " " ( " " " 7.5 microns).
" #02 " " " " 20 " " " " ( " " " 2 microns).
" #03 " " " " 35 " " " " ( " " " 1.3 microns).
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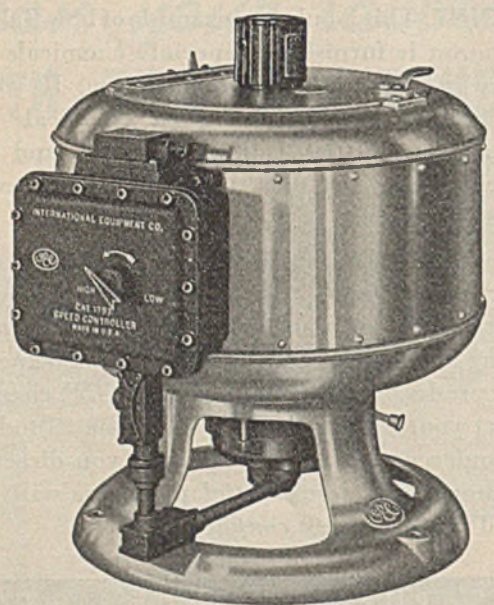


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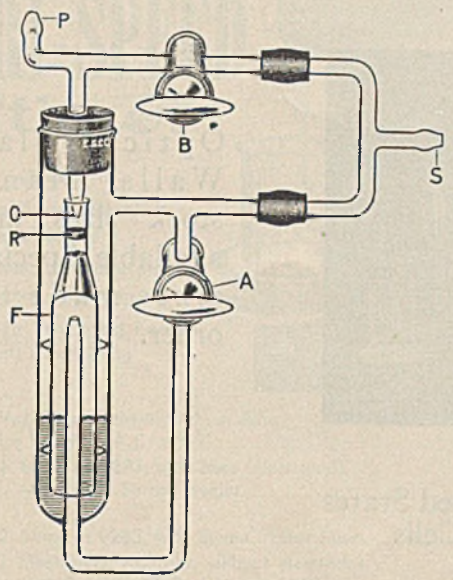


We recommend this device as a most convenient, effective means for obtaining reduced pressure control in vacuum distillations, etc. This device presents a simple, positive means for setting and maintaining any desired vacuum in a system. It eliminates all electrical appliances and no additional accessories are required other than shown in illustration.

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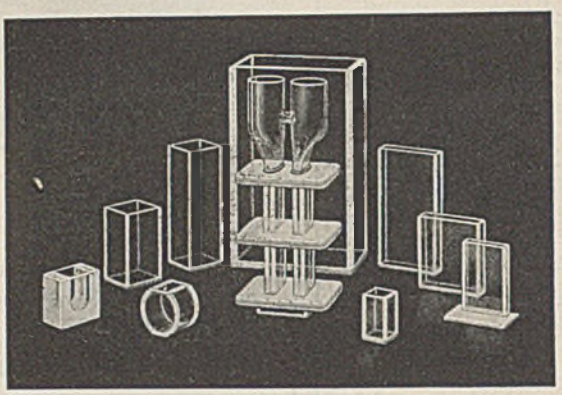

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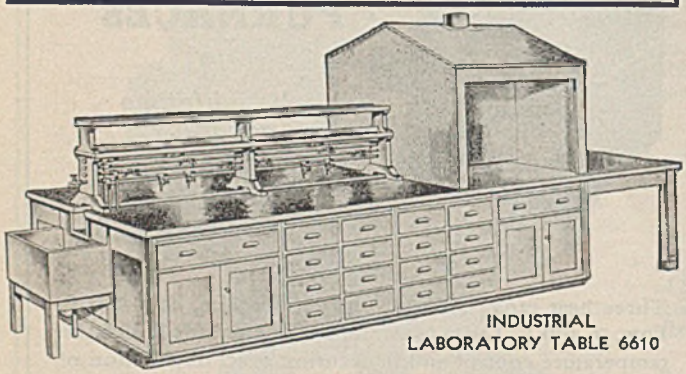

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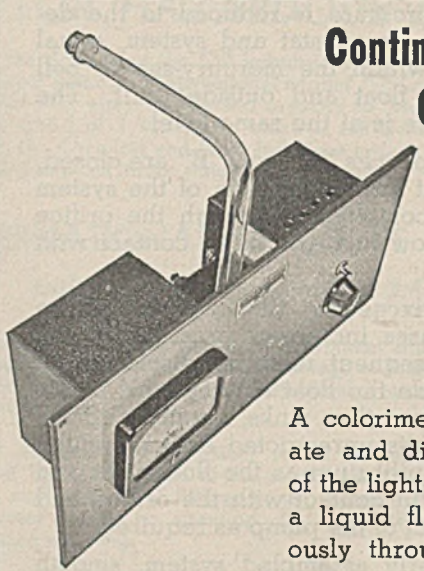

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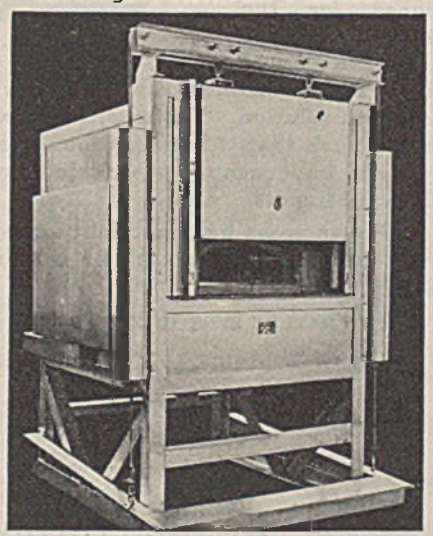

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The new revised edition of this outstandingly successful monograph has been eagerly awaited by industry for many months. In general the basic outline of the first edition has been adhered to, but the latest and most authoritative developments have been included and are described in detail. As in the previous book the gases discussed are those which occur predominantly in industrial processes; and one of the most useful features of this volume is the extensive and accurate classification of these gases according to their actions; for example, irritants, asphyxiants, volatile drugs, inorganic and organo-metallic gases.

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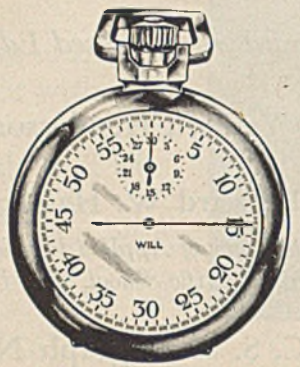
**STOPWATCH**



**16062 A**

**7 Jewel Movement** **⅕ Second Interval**

A new, American-made stopwatch with rugged, reliable 7-jewel continuous running movement.

The large sweep hand travels once around the dial in 60 seconds. The dial is white enamel with black numerals and is graduated in ⅕ seconds. The small hand registers up to 30 minutes in 1 minute intervals.

Through successive depressions of the winding crown, the sweep-second hand is controlled for start, stop, and flyback to zero. The case is nickel, chromium-plated, with double back to make the action dust-proof. Fitted with permanently clear unbreakable crystal.

Conforms to Federal Specification GG-W-111a, Type B—Class 7.

**No. 16062A . . . . . $30.00**

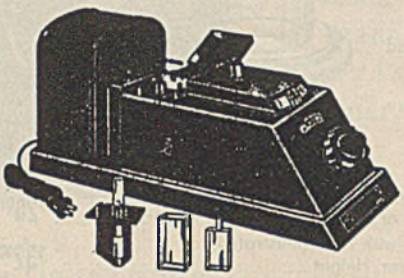
**WILL** CORPORATION
ROCHESTER, NEW YORK



*Klett* . . . .

Photometers

*Klett-Summerson*

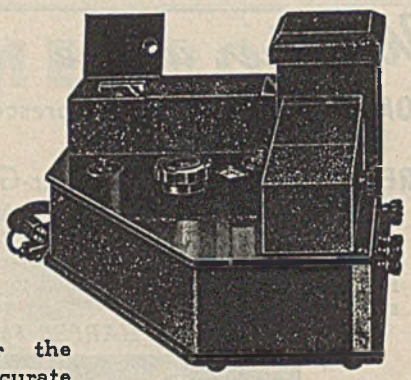


*Photoelectric Glass Cell Colorimeter*

No. 900-3

**For the varied needs of industrial, clinical and agricultural laboratories. Complete and self contained—no accessory equipment necessary. Fused cells for readings on solution depths of 2.5, 10, 20, and 40 mm. Selective light filters available.**

*The Klett Fluorimeter*



No. 2071

**Designed for the rapid and accurate determination of thiamin, riboflavin, and other substances which fluoresce in solution. The sensitivity and stability are such that it has been found particularly useful in determining very small amounts of these substances.**

**To further increase its adaptability a separate circuit and scale is provided for colorimetric determinations.**

LITERATURE SENT UPON REQUEST

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