The influence of external pressure is more complicated, but, as is demonstrated in the earlier papers, follows directly from our theory.[1]

In conclusion, it is probable that I have not met, in detail, all of Newbery's objections to the theory proposed by MacInnes and Adler, and I am, naturally, in the dark as to the "many other" objections that he holds in reserve. However, enough is probably outlined above to indicate the fundamental difference of point of view upon which Newbery's aggressive criticism is based.

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[CONTRIBUTION FROM THE BUREAU OF CHEMISTRY, UNITED STATES DEPARTMENT OF AGRICULTURE.]

THE QUANTITATIVE ESTIMATION OF PHOSPHATIDES.

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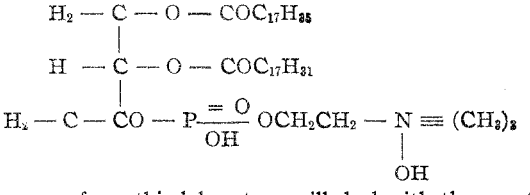
The determination of phosphatides is often required in the analysis of foods and drugs. In the case of foods, phosphatides frequently serve as an index to the quantity of egg yolk in the food under examination. In the analysis of drugs, it is often necessary to determine phosphatides in order to establish whether or not statements made concerning the lecithin content of the preparation are warranted. Experience has shown that none of the methods hitherto proposed for the separation of phosphatides from such products is wholly satisfactory, for one reason or another. The investigation herein presented was undertaken for the purpose of devising a method which would be satisfactory. Since Maclean's[2] recent book, renders the publication of a bibliography superfluous, the references given in the present work have been limited to publications that offer suggestions of value for our particular purposes.

For the estimation of phosphatides we have considered 2 different phosphatides, first, lecithin, which is generally agreed to be a combination of glycerophosphoric acid, choline, oleic acid and stearic acid, and to have the formula $C_{44}H_{86}NPO_9$ or



[1] A forthcoming paper from this laboratory will deal with the question of the relation of overvoltage to pressure. For the present purpose it is sufficient to say that the results of Goodwin and Wilson, mentioned in our first paper, have been confirmed as to the direction of the effect. The excess pressure in small bubbles has been considered and must be taken into account for higher overvoltages. However, this extra pressure is very small for the comparatively large bubbles from platinized platinum electrodes, and it does not sensibly affect the computations published.

[2] H. Maclean, "Lecithin and Allied Substances, the Lipins," Longmans, Green & Co., London, 1918.

and, second, kephalin, which is known to contain glycero-phosphoric acid, amino ethyl alcohol, stearic acid and an unsaturated acid, and to contain 4.17% of phosphorus.[1] Since choline and amino ethyl alcohol are the characteristic cleavage products which distinguish phosphatides from similar biological substances, especially the fats, a method for the estimation of phosphatides based upon the determination of these characteristic constituents should yield satisfactory results and should be successful in excluding phosphorus-containing compounds other than phosphatides from the estimation. The method may properly be applied as Maclean[2] has shown that most phosphatides in tissues consist of $^3/_4$ lecithin and $^1/_4$ kephalin, and the necessity for its use is demonstrated by Wintgen and Keller,[3] who have proved that a simple nitrogen determination is not reliable as a measure of the nitrogen content of phosphatides.

For this reason the method for the estimation of phosphatides which we propose involves several processes, namely, the separation of the phosphatides from the other substances in the material to be analyzed and the determination of phosphorus, of choline and of amino nitrogen in the mixed phosphatides after they have been obtained free from adventitious matter.

Separation of Phosphatides—Recognized Methods.

Beyer[4] first observed that seeds extracted with ether yield a certain per cent. of organic phosphorus-containing substances and that a subsequent extract obtained with warm alcohol yields to ether more of the organic phosphorus-containing substance, observations which were subsequently confirmed by Jacobson.[5] The same phenomenon was also observed when egg yolk and animal tissues were treated with ether and alcohol. Hoppe-Seyler[6] suggested that lecithin may be present in egg yolk partly in the free from and partly bound to vitellin. The combination is decomposed by alcohol but not by ether. Schulze and Steiger,[7] found this suggestion correct in the case of plant and animal tissues. Schulze[8] based a method for the quantitative estimation of phosphatides on these results. His original procedure was extraction of the finely pulverized material with anhydrous ether followed by extraction with boiling absolute alcohol, the phosphatides being determined in the combined extracts. He also suggested a modification in which the residue obtained

[1] P. A. Levene and C. J. West, *J. Biol. Chem.*, **33**, 11 (1918).
[2] H. Maclean, *Biochem. J.*, **9**, 351 (1915).
[3] Wintgen and Keller, *Archiv. Pharm.*, **244**, 3 (1906).
[4] Ad. Beyer, *Landw. Ver. Sta.*, **14**, 161 (1871).
[5] H. Jacobson, *Z. physiol. Chem.*, **13**, 38 (1889).
[6] F. Hoppe-Seyler, *Handbuch physiol. Chem.*, **1877**, p. 781.
[7] E. Schulze and E. Steiger, *Z. physiol. Chem.*, **13**, 365 (1889).
[8] E. Schulze, *ibid.*, **20**, 225 (1895); *Chem. Ztg.*, **28**, 751 (1914).

on evaporation of the combined ether and alcohol extracts is extracted with ether (secondary ether extract) in order to insure the absence of all compounds other than phosphatides. As early as 1884 Thudichum[1] proposed a complicated method, not merely for the separation of phosphatides from other components of tissues, but also from one another. In 1900, Juckenack[2] proposed the substitution of warm alcohol for ether in the extraction of phosphatides because not all the free lecithin in yolk of egg is dissolved by ether. Evaporation of the alcohol solution should be performed at a temperature not exceeding 60°, the boiling with alcohol being reduced to the time required for decomposing the protein compound of lecithin.

Fendler[3] found that some trade lecithins are soluble in alcohol and some are soluble in ether, while others require both for complete solution. He therefore extracted first with absolute alcohol and then with ether, determining the phosphorus in the combined extracts.

Erlandsen,[4] who found all phosphatides which he separated to be ether-soluble after extraction with alcohol, has shown that further purification of the phosphatide solution by precipitation with acetone or cadmium chloride is not advisable since these precipitations are not quantitative in all cases.

A special extraction apparatus was used by Koch and Woods[5] in a method which is a simplification of Thudichum's and depends upon the difference in solubility of the lead salts of the various phosphatides.

In washing the ethereal solution of phosphatide, several authors advise that the acidity be low so that hydrolysis may be avoided. Koch[6] has shown that the danger of hydrolyzing lecithin is rather slight, and his conclusions have been confirmed by Coriat.[7]

The method of Koch and Woods is objected to by Erlandsen[8] on the ground that a loss of phosphatides occurs during washing.

Bloor[9] devised a method for the determination of lecithin in small amounts of blood by running the blood into a mixture of alcohol and ether and determining the phosphorus content in an aliquot of the alcohol-ether solution. Moreover he found that inorganic phosphates added to the blood can be easily removed by shaking the alcohol-ether solution

[1] J. L. W. Thudichum, "The Chemical Constitution of the Brain," London, 1884.

[2] A. Juckenack, *Z. Nahr. Genussm.*, **3**, 12 (1900).

[3] G. Fendler, *Apoth. Ztg.*, **20**, 22 and 488 (1905).

[4] A. Erlandsen, *Z. physiol. Chem.*, **51**, 94, 151 (1907).

[5] W. Koch and H. S. Woods, *J. Biol. Chem.*, **1**, 203 (1905); W. Koch, *ibid.*, **3**, 160 (1907).

[6] W. Koch, *Am. J. Physiol.*, **11**, 318 (1904).

[7] I. H. Coriat, *ibid.*, **12**, 361 (1905).

[8] A. Erlandsen, *Z. physiol. Chem.*, **51**, 96 note (1907).

[9] W. R. Bloor, *J. Biol. Chem.*, **22**, 133, 1916 (1915); **24**, 452 (1915).

with saturated ammonium sulfate solution. It is clear, however, that the ammonium sulfate interferes with the determination of nitrogen and choline, but Bloor's use of this reagent suggested to us the use of saturated sodium chloride solution in our work.

Koch and Woods[1] washed the secondary ether extract with acid chloroform water and obtained a high percentage of extract phosphorus[2] in the wash water. Collison[3] found that by excluding moisture in the alcohol-ether extraction the acid washing of Koch and Woods is superfluous. Koch and Woods[4] themselves state that Schulze was dealing with relatively dry plant tissues and not with moist animal tissues and that, therefore, his findings as to the solubility of inorganic phosphates in absolute alcohol and anhydrous ether are not valid. Later a convenient method was devised for reducing animal tissues to finely divided dry powder and it was found that by employing this procedure the washing with acid chloroform-water which had been recommended by Koch and Woods might be omitted. The method was recommended by Leathes[5] and a modification was made and used by Robertson[6] for preparing his tethelin. Robertson's method is as follows.

> Animal tissues are ground in a mortar with 3 times their weight of a mixture of equal parts by weight of anhydrous sodium and calcium sulfates and the mixture dried over a water-bath, stirring occasionally to prevent caking. Under these conditions drying is extremely rapid and involves a minimum exposure of the tissue to heated air. In an hour, a mixture of 60 g. of tissue with 180 g. of sodium and calcium sulfates becomes white and easily powdered. This dried mixture is returned to the mortar, finely pulverized and extracted with absolute alcohol at a temperature as near as possible to that of boiling alcohol.

For this purpose Robertson employed a modified Bailey-Walker extraction apparatus with alundum thimbles, the process being continued for 48 hours to complete extraction.

The method of Bordas and de Raczkowski[7] has found wide approval for the estimation of lecithin in milk. In common with other research workers in this field, they caution that it is advisable to avoid unnecessarily high temperature, lecithin being gradually destroyed by heat.

Study of the methods cited leads to the conclusion that extraction with ether and warm alcohol removes all phosphatides. The residue obtained on evaporation of the extract may be re-extracted with ether for purifica-

[1] W. Koch and H. S. Woods, *loc. cit.*

[2] Koch and Woods classify phosphorus under 3 headings: (1) Inorganic phosphorus. (2) extract phosphorus, that contained in organic phosphorus-containing substances which are not phosphatides. (3) phosphatide phosphorus.

[3] R. C. Collison, *J. Biol. Chem.*, **11**, 217 (1912).

[4] W. Koch and H. S. Woods, *idem.*, 206.

[5] J. B. Leathes, "The Fats," Longmans, Green & Co., London, **1910**, p. 53.

[6] T. B. Robertson, *J. Biol. Chem.*, **24**, 409 (1916).

[7] F. Bordas and de Raczkowski, *Compt. rend.*, **134**, 1592 (1902).

tion and to exclude inorganic phosphates. Only the results of Juckenack indicate any objection to extracting with ether the residue from extraction with warm alcohol, and the results of Erlandsen,[1] which are quoted on p. 2240 are sufficient to overcome this objection.

Proposed Method for Separating Phosphatides.

If enough material is available, an extraction yielding about 0.300 g. of phosphatides should be made. This is sufficient for the determination of phosphorus in duplicate and of choline and amino alcohol. If great accuracy is unnecessary, the amount of phosphatides may be as small as 0.200 g. If the weight of phosphatides available is less than 0.200 g. the quantitative determination of phosphorus only is made and the presence of choline is indicated by qualitative tests, indentification being effected by the periodide or the mercuric iodide salt.

Animal or vegetable tissues which cannot be reduced to powder or solutions in volatile liquids are dried with anhydrous sodium and calcium sulfates, according to the method of Robertson.[2] Solutions of non-volatile liquids such as glycerol are boiled under a reflux condenser with absolute alcohol for an hour, the excess of alcohol evaporated on the steam-bath with occasional stirring, an equal volume of saturated-sodium chloride solution added to the residue and the mixture shaken out with ether. Upon addition of the solution (about 35%) of pure phosphorus-free sodium chloride the phosphatides separate as a brown layer on top of the liquid. By adding ether and gently rotating the separatory funnel, the phosphatides are dissolved. The mixture of glycerol and sodium chloride is transferred to another separatory funnel and the process of shaking out with ether is repeated several times. Extraction is completed by allowing the glycerol-salt solution to stand overnight in contact with ether. The solubility of phosphatides in glycerol is such that the addition of sodium-chloride solution is essential for separation. The ether solution of phosphatides is collected in a dry flask and allowed to stand for a few hours to effect separation of the last small amounts of glycerol-salt solution. The solution is filtered and distilled from a weighed flask.

Material which can be powdered without difficulty is dried *in vacuo* at the lowest possible temperature. Extraction of phosphatides from the dry material is made with absolute alcohol in an ordinary Soxhlet apparatus or in an extraction apparatus as described by Koch and Woods or by Robertson. By insulating the extraction thimble the temperature of the extraction liquid is maintained as nearly as possible at 50–60°.[3] Depending upon the material used, the extraction is completed in 10 to 48 hours. By changing the boiling flask the progress of extraction can be

[1] A. Erlandsen, *loc. cit.*

[2] T. B. Robertson, *loc. cit.*

[3] A. Juckenack, *loc. cit.*

followed. Samples of the extract may be taken at intervals by using at the bottom of the thimble an outlet tube provided with a stopcock. In this way needless exposure of extracted phosphatide to heat is avoided and the completion of extraction observed. The incompletely exhausted residue is extracted in the same apparatus for 2 hours. The combined alcohol extracts are dried, mixed with sodium and calcium sulfates if necessary, and extracted with anhydrous ether. For small amounts it is sufficient to stir with a small pestle and to pour off the ether through a small filter, repeating the process to complete exhaustion; for larger amounts a Soxhlet apparatus should be used. The ether extract is reduced to 30–40 cc., then shaken with 15 cc. of saturated sodium chloride solution. The salt solution is drawn off and washed with 10 cc. of ether. The ether solutions are combined, evaporated, dried and weighed. This gives the weight of impure ether-soluble phosphatides free from extract phosphorus.

Weighing need be accurate to centigrams only, the object being to ascertain whether more or less than 0.20 g. of phosphatides is present, this being the smallest amount sufficient for the quantitative determination of phosphorus, choline and amino nitrogen.

Existing Methods for the Determination of Phosphorus.

Phosphorus is best determined according to the method of Neumann.[1] Koch and Woods[2] and Greenwald[3] have suggested modifications of the method for precipitating the ammonium phosphomolybdate, but all agree on the manner of destruction for organic matter. The colorimetric method, depending on the reduction of ammonium phosphomolybdate, makes possible the accurate determination of amounts of phosphorus weighing not less than 0.5 mg., requiring 0.010 g. of phosphatide. Taylor and Miller[4] used a centrifuge to separate the ammonium phosphomolybdate, made the reduction with hydrazine chloride and compared the color with a standard prepared with standard phosphate solution under similar conditions in a Duboscq colorimeter. Riegler[5] reduced with hydrazine sulfate, using a procedure similar to that of Taylor and Miller. Van Eck,[6] working under war conditions, used stannous chloride as the reducing agent and obtained satisfactory results by comparing the colored solutions in Nessler tubes. A further increase in delicacy has been obtained by Kober and Egerer[7] by using strychnine phosphomolybdate for the nephelo-

[1] Albert Neumann, *Z. physiol. Chem.*, **37**, 130 (1902).
[2] W. Koch and H. S. Woods, *loc. cit.*
[3] I. Greenwald, *J. Biol. Chem.*, **14**, 369 (1913).
[4] A. E. Taylor and C. W. Miller, *ibid.*, **18**, 220 (1914).
[5] E. Riegler, *Bull. sec. sci. acad. roumaine*, **2**, 272 (1913–14).
[6] P. N. Van Eck, *Pharm. Weekblad.*, **55**, 1037 (1918).
[7] P. A. Kober, and G. Egerer THIS JOURNAL, **37**, 2373 (1915).

metric estimation. By this method 0.005 mg. of phosphorus, requiring 0.0001 g. of phosphatide, may be accurately determined.

Method Adopted for Phosphorus Determination.

The destruction of organic matter was effected according to the method of Neumann, adopting the minor modifications proposed by Bloor; and the phosphorus determination was made by the colorimetric method with ammonium phosphomolybdate following the procedure of Van Eck. The entire procedure is described, as we have found that it is necessary to follow it closely to obtain the best results.

The weighed residue of ether-soluble phosphatide is dissolved with alcohol, rinsed into a volumetric flask of suitable content (100 cc. for 0.300 g. of residue) and the flask filled to the mark. Five cc. of solution, representing 0.015 g. of residue, is pipetted into a large (200 mm. × 24 mm.) test-tube of resistant glass, 2 or 3 glass beads of 3 mm. diameter are added, and the solution is evaporated to dryness by immersing the tube in a water-bath. It should be watched and shaken frequently until boiling begins, after which the evaporation will generally proceed to dryness without requiring further attention. Since alcohol interferes with subsequent oxidation, the material in the tube is dried on the water-bath for 15 minutes to remove traces of this reagent. Four cc. of a 1:1 mixture of conc. nitric and sulfuric acids is added and the mixture is digested by heating with a microburner in the hood or with a Folin draught apparatus.[1] The heating is done in 2 stages with the tube inclined at an angle of about 30° to prevent spattering. During the first, the mixture is boiled gently over a low flame until red fumes cease to come off. This should require about 5 minutes. The heat is then gradually increased until the nitric acid is completely expelled, after which the sulfuric acid is boiled for 8–10 minutes. If charring occurs a small amount of strong nitric acid should be added and the process repeated. The mixture is cooled somewhat and 2 drops of a 0.25% cane sugar solution is added to remove traces of nitric acid which may prevent complete precipitation of phosphoric acid, After heating for one minute the solution is cooled and 5 cc. of water is added. The liquid is rinsed quantitatively into a porcelain dish with 5 to 10 cc. of water, 10 cc. of 30% ammonium nitrate solution added, and the whole carefully heated nearly to boiling. There is now added 4 cc. of 10% ammonium molybdate solution, made by dissolving the salt in cold water and filtering. The solution and precipitate should be heated for a short time. If care is taken not to heat the dish above the level of the liquid, the precipitate settles without adhering to the sides of the dish. Filtering is best done through a plug of asbestos fiber in a small funnel and the precipitate is washed several times with ammonium nitrate solution. The funnel is inverted over a beaker and

[1] O. Folin and W. Denis, *J. Biol. Chem.*, **9**, 503 (1911).

dil. sulfuric acid (1 to 10) is poured into the stem of the funnel to rinse the plug and precipitate into the beaker. 0.5 g. of stannous chloride is added and the solution allowed to stand for half an hour. If any precipitate adheres to the sides of the dish, it may be rinsed into the beaker with the dil acid. The blue liquid is filtered through a small plug of asbestos into a 100-cc. volumetric flask and made up to volume with the dil. sulfuric acid. The color is best washed from the plug by treating it in a dish with excess of sulfuric acid and filtering through another plug.

The blue color is compared with a standard made by adding to 10 cc. of a solution containing 0.05 mg. of phosphorus per cc. in a porcelain dish 2 cc. of conc. sulfuric acid and cc. of 30% ammonium nitrate solution, proceeding thereafter as directed. The standard is sufficiently stable to allow of making the comparisons.

Ten cc. of the standard blue liquid is put in a Nessler tube. So much of the blue liquid to be investigated is placed in another Nessler tube that the colors are roughly estimated to be the same. Usually 10 cc. is required if a 0.010 g. sample of phosphatide is used. Both tubes are filled to 100 cc. with the dil. sulfuric acid. Sulfuric acid must be used for the reason that the turbidity caused by separation of the tin salt on addition of water interferes with the determination. If the colors are not exactly the same, enough liquid is poured from one tube into a graduate to make the 2 colors identical in shade. Since the colors are not comparable if the dilutions differ by more than a very small amount, the more dilute being more green, this procedure must be strictly followed. If the liquid becomes turbid upon dilution it will clear after standing for an hour.

Consideration of Existing Methods for Determining Nitrogen and Amino Nitrogen.

For general work, the determination of nitrogen according to Dumas requires less substance than do other methods. Moreover, as the residue remaining from a Dumas nitrogen analysis contains the phosphorus as phosphoric acid, one sample would be sufficient for both determinations. However, the difficulty of transferring the residue from the tube quantitatively is so great that it is more advantageous to use a second sample for phosphorus.

Distearyl lecithin contains 1.74% of nitrogen and 3.84% of phosphorus. Other lecithins show a variation from these figures of only about 0.1% of their nitrogen and phosphorus content. 0.200 g. of phosphatide yields 3.04 cc. (0.0035 g.) of nitrogen at 20° and 760 mm., a quantity sufficient for accurate estimation. By the application of the micro-Dumas method as described by Fischer,[1] the weight of phosphatide required may be reduced to 0.010–0.020 g., yielding 0.0003 to 0.0007 g. of nitrogen.

The Dumas method is not so well adapted to making a series of deter-

[1] H. Fischer, *Ber.*, **51**, 1322 (1918).

minations as is that of Kjeldahl. In working with phosphatides it has been shown that the Gunning modification of the Kjeldahl procedure is the most satisfactory. A study of the relative accuracy of colorimetric and titrimetric procedures for determining nitrogen as ammonia has been made by Allen and Davisson,[1] who have shown that the use of 0.02 *N* solutions in conjunction with methyl red as indicator, as proposed by Mitscherlich and Herz,[2] yields an accuracy even greater than that of the colorimetric micro-Kjeldahl method as developed by Folin and Denis.[3] We use the latter method on account of the advantage of its brevity.

Amino nitrogen is determined by the method of Van Slyke.[4]

Methods Adopted for Determination of Choline and Amino Nitrogen.

We have adopted the method of acid hydrolysis of the phosphatides, because of its recognized superiority to alkaline hydrolysis and especially because Maclean[5] and Levene and Rolfe[6] have proved this beyond doubt, the latter investigators having found that on hydrolysis with barium hydroxide, barium salts of the fatty acids are obtained which can be freed from nitrogen only with the greatest difficulty.

A weighed amount (about 0.300 g.) of the ether-soluble phosphatides or an aliquot of the alcoholic solutions from which the alcohol is evaporated is boiled with about 30 cc. of 0.2 *N* sulfuric acid for 48 hours under a reflux condenser. A piece of tile and a small piece of paraffin may be added to prevent bumping and foaming. After cooling, the solution is filtered to separate the fatty acids. The filter with the fatty acids is boiled with 4 to 6 small portions of water and the cooled wash water added to the filtrate. The solution is then carefully neutralized with barium hydroxide solution, the precipitate collected on a filter and washed thoroughly with boiling water. The filtrate is acidified and evaporated to dryness. The residue is extracted with alcohol, the solution filtered through a small filter and the volume reduced to 10 cc. The alcoholic solution of choline must not be too highly concentrated since some of the amino ethyl alcohol platinum chloride, though soluble in alcohol, is carried down from concentrated solution by the precipitate of choline platinum chloride. Alcoholic solution of platinum chloride is added and the mixture allowed to stand until the precipitate settles. The precipitate is collected on a weighed filter, dried to constant weight at 105°, cooled and weighed. This is the weight of choline platinum chloride containing also some amino-ethyl alcohol platinum chloride. Upon multiplying the weight of the pure choline platinum chloride by 2.608, the weight of lecithin is obtained.

[1] E. R. Allen and B. S. Davisson, *J. Biol. Chem.*, **40**, 183 (1919).

[2] Mitscherlich and Herz, *Landw. Jahrb.*, **38**, 279 (1909).

[3] O. Folin and W. Denis, *J. Biol. Chem.*, **11**, 502 (1911).

[4] D. D. Van Slyke, *ibid.*, **12**, 275 (1912).

[5] H. Maclean, *loc. cit.*

[6] P. A. Levene and Ida P. Rolfe, *J. Biol. Chem.*, **40**, 1 (1919).

The filtrate, which is kept for the determination of amino nitrogen, is acidified with hydrochloric acid and carefully evaporated to dryness in a small evaporating dish. It is made slightly alkaline with sodium hydroxide, acidified with acetic acid and made up with water to a volume of 2.5 cc. in a small graduate. A Van Slyke's micro-amine nitrogen apparatus having been prepared, 2 cc. of the solution or suspension—corresponding to 0.240 g. of substance—is used for the determination of amino nitrogen.

A portion of the precipitate is used for the determination of amino nitrogen. The weight of amino-ethyl alcohol platinum chloride is obtained upon multiplying the weight of nitrogen by 19. The weight of the precipitate minus weight of amino-ethyl alcohol platinum chloride equals the weight of pure choline platinum chloride.

Amino nitrogen in the precipitate is added to that found in the filtrate and the total weight of nitrogen multiplied by 53.20 to obtain the total weight of kephalin.

Experimental Testing of the Combination of Methods Proposed.

For the purpose of testing the proposed combination of methods, a supply of lecithin was prepared by regenerating it from the cadmium compound of egg lecithin by treatment with ammonium carbonate as described by Bergell.[1] Although kept in a dark closet in a vacuum desiccator over calcium chloride, it was yellowish and gradually became dark brown.

For checking the Neumann method, about 4 g. of disodium phosphate was dissolved in about one liter of watr, 50 cc. of this solution was evaporated in a weighed porcelain dish and ignited, yielding 0.0987 g. of sodium pyrophosphate. This is equivalent to 0.0265 g. of phosphorus pentoxide per 25 cc. of solution. 25 cc. of the same solution treated according to Neumann's method required 20.2 cc. of 0.5 *N* alkali, corresponding to 0.0256 g. of phosphorus pentoxide, which is in fairly good agreement with the amount found on ignition.

The phosphorus and nitrogen determinations on lecithin gave the following results.

Substance. G.	Alkali required.	P. %.	N (by Kjeldahl). %.	Calc. for stearyl oleyl lecithin. % P.	% N.
0.2738	18.90 cc. 0.5 *N*	3.82	....	....	....
0.2568	17.77 cc. 0.5 *N*	3.83	....	3.86	....
0.7273	10.81 cc. 0.1 *N*	....	2.08	....	1.74

Although our results for phosphorus agree very well with the theoretical it will be noted that the nitrogen is high. This is often the case with lecithin preparations.

On account of the changes that take place on aging, the ordinary leci-

[1] P. Bergell, *Ber.*, **33**, 2584 (1900).

thins are not satisfactory as standards; and for this reason "hydrolecithin"[1] was used. A few grams were prepared from egg lecithin according to the method of Ritter.[2] Analysis did not yield very concordant results, but this may have been due to the small amount of substance, as the method of recrystallization recommended was not entirely satisfactory. Although the product was white when dry, it always produced colored solutions.

Subs., 0.1903, 0.1996: CO_2, 0.4527, 0.4746; H_2O, 0.1736, 0.1898.

Calc. for $C_{44}H_{90}NPO_9$: C, 65.37; H, 11.23. Found: C, 64.89, 64.80; H, 10.20, 10.60.

A standard solution for the colorimetric estimation was made by dissolving 0.200 g. of sodium phosphate in 200 cc. of water. 50 cc. of this solution was evaporated in a weighed porcelain dish, ignited, and weighed, yielding 0.0967 g. of sodium pyrophosphate, or 0.0113 g. of phosphorus per 25 cc. of solution.

Twenty-five cc. of the same solution treated according to Neumann's method required 21.17 cc. of 0.5 *N* alkali, equivalent to 0.0117 g. of phosphorus. For the standard, 50 cc. of the former solution was diluted to 500 cc., so that 10 cc. of this solution contained 0.00046 g. of phosphorus.

Colorimetric determination yielded results as follows:

Hydrolecithin. G.	Poured out from hydrolecithin tube to match standard. Cc.	P. Found. %	P. Calc. %
0.0015	17.0	3.70	3.84
0.0025	53.5	3.86	

The choline and amino nitrogen determinations were made with 0.260 g. of hydrolecithin and gave the following results:

Choline Platinum Chlorid 0.0751 g.

Precipitate used, 0.040 g. Calc. for whole precipitate: N, 0.000338 g. Found: 0.31 cc. or 0.00018 g.

0.000338 × 19 = 0.0064 g. of amino-ethyl alcohol platinum chloride.

0.0751 g. — 0.0064 g. = 0.0687 g. of pure choline platinum chloride.

0.0687 g. × 2.608 = 0.1792 g. of lecithin.

Filtrate.

4/5 of filtrate taken: 1.90 cc. of amino nitrogen obtained; whole filtrate would yield 2.37 cc. or 0.00138 g. of N; filtrate + ppts. 0.00138 g. + 0.000338 g. = 0.00172 g.

0.00172 × 53.20 = 0.0915 g. of kephalin. Therefore, 0.260 g. of phosphatides yielded

Lecithin		Kephalin		Phosphatides
0.1792 g.	+	0.0915 g.	=	0.271 g.

	M. P.	
ppts.	240°	with foaming.
Pure choline platinum chloride	224°	

[1] Hydrolecithin, a crystalline phosphatide, was first prepared by Ritter, using Paal's colloidal palladium as a catalyzer for the reduction of egg lecithin by hydrogen. Ritter pointed out that this hydrolecithin may occur in addition to other lecithins in egg yolk. It contains 2 stearyl groups in its molecule instead of one stearyl and one oleyl group.

[2] R. Ritter, *Ber.*, 47, 530 (1914).

The water-alcohol solution of the platinum chloride precipitate yielded on evaporation the yellow octahedrons which were also produced by the platinum double salt made from pure choline hydrochloride, closely resembling platinum chloride crystals except that the choline double salt is easily soluble in water.

The proposed method was further tested on a sample of lecithin prepared from vitellin lecithin.

2.400 g. of this lecithin was dissolved in absolute alcohol and filtered into a 200-cc. volumetric flask. Only traces of insoluble matter were left on the filter. 25 cc. of the solution was made up to 100 cc. in a volumetric flask and 5 cc. of the latter solution was used for a phosphorus determination according to the colorimetric method. The water-alcohol solution of the choline precipitate produced the characteristic crystals whose melting-point was 230°. Colorimetric determination:

Phosphatides: 0.015 g. Calc.: P, 3.86; Found: 3.70.

Precipitation of choline platinum chloride and determination of amino nitrogen showed,

Phosphatides: 0.285 g. Found: Lecithin, 0.264 g.; kephalin, 0.0579; phosphatides (total), 0.3219.

Another test was made with a commercial preparation, "Glycerole Lecithin." It was stated on the label of the bottle that each teaspoonful contains one grain of pure lecithin. If we figure that one teaspoonful—4 cc.— of the liquid, consisting mainly of glycerol, weighs about 5 g. (200 cc. was found to weigh 245 g.), then 100 g. should contain 20 grains of lecithin or 0.052 g. of phosphorus = 0.05%. A colorimetric phosphorus determination showed 0.05% phosphorus to be present.

Two and a half g. of the preparation was mixed with an equal amount of saturated sodium chloride solution, shaken with ether, and treated as directed in the proposed method for the separation of phosphatides. The weight of the separated impure phosphatide was about 8 g., and contained, according to the phosphorus determination, about 3. g. of lecithin. The whole residue was dissolved in absolute alcohol, filtered into a 200-cc. volumetric flask and made up to volume. 25 cc. was diluted to 100 cc. with absolute alcohol and 5 cc. of the latter solution was taken for the phosphorus determination, which gave 0.045% of phosphorus. No phosphorus could be detected in the liquid which had been exhausted with ether, and only traces were found in the residue left on the filter when the alcoholic solution was filtered. Therefore, all of the phosphorus was present as phosphatides. The washing of the ether solution with saturated sodium chloride solution is here performed at the same time as the extraction. Upon multiplying the per cent. of phosphorus by the factor 25.4, based upon Maclean's[1] findings that phosphatides consist of $^3/_4$ lecithin and $^1/_4$

[1] H. Maclean, *loc. cit.*

kephalin and the figures for nitrogen content of these substances as determined by Levene and West,[1] the phosphatide content is obtained; thus, 0.045 × 25.4 = 1.1%. According to the statement on the label, one teaspoonful—5 g.—contains 1 grain or 0.065 g. of lecithin, or 1.3%. In the 95 cc. remaining from the phosphorus determination, choline and amino nitrogen were determined. These 95 cc. correspond to $^{2\frac{1}{2}5}/_{8}$ × $^{95}/_{100}$ = 29.1 g. of "Glycerole Lecithin" and this weight contains, according to the phosphorus determination, 0.011 × 29.1 = 0.3201 g. of phosphatide. According to the statement on the label, this would be 0.013 × 29.1 = 0.3783 g. of phosphatide. Analysis showed 0.3038 g. of lecithin + 0.0636 g. of kephalin = 0.3674 g. of phosphatide.

The choline platinum chloride precipitate showed a melting point of 240°, and the characteristic crystals were observed when the water-alcohol solution of the precipitate was evaporated.

Summary.

1. A general method for the separation of phosphatides to be used in the quantitative estimation of these substances has been described and tested and has been found suitable for the analysis of proprietary pharmaceutical preparations as well as for quantitative estimation of phosphatides occurring in food products.

2. It has been shown that the method of drying with mixed sodium and calcium sulfates is very satisfactory for volatile liquids which contain galenical extracts that are hygroscopic. Further, it has been demonstrated that the proposed method of washing the ether extract with saturated sodium chloride solution to separate inorganic phosphates and extract phosphorus is effective.

3. It has been shown that the colorimetric method for the determination of phosphorus in the separated phosphatides is adapted to purposes of food and drug analysis. About 0.010 g. of material is required and from the phosphorus content the per cent. of phosphatide is calculated.

4. The determinations of choline and amino nitrogen serve as a check on that of phosphorus in the phosphatides, to exclude other phosphorus compounds from the estimation and establish the ratio of lecithin to kephalin.

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[1] P. A. Levene and C. J. West, *loc. cit.*