CCI. THE PHOSPHOLIPINS OF BLOOD.

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THE phospholipins account for a very considerable fraction of the total fatty acids in blood. This, with the fact that theories of fat transport and utilisation have been based on variations in the phospholipin content of the blood, makes it very desirable to determine whether the available methods are adequate for the estimation of blood phospholipins, and also what phospholipins are present.

For the estimation of phospholipins, two main methods are available, the first elaborated by Bloor [1929], and modified by Boyd [1931], depends on the oxidation of material precipitated by acetone and magnesium chloride from an ethereal extract; the result is expressed in terms of lecithin. The second method is that of estimating the "lipoid phosphorus" by one of the various methods proposed for determining small amounts of phosphorus; this method suffers from the defects that (*a*) the "lipoid phosphorus"—*i.e.* the phosphorus in an alcohol-ether extract of blood—has been stated to contain phosphorus not derived from phospholipins, and (*b*) it has generally been tacitly assumed that a method suitable for the micro-estimation of inorganic phosphate will also, after incineration of the organic material, satisfactorily measure the "lipoid phosphorus", and this is by no means always the case.

In several respects a method based on the "lipoid phosphorus" is to be preferred, provided it can be shown to be reliable. One important advantage is that it makes no assumption as to the exact phospholipin present; a second is that it is both easier and quicker to carry out than the oxidative procedure. We have therefore examined in detail the steps in the estimation of the "lipoid phosphorus", and after defining the conditions for accurate estimation of this fraction of the blood phosphorus, have shown that no serious error is involved in regarding it as the phosphorus derived from phospholipins.

The convenient colorimetric methods for the estimation of phosphorus depend upon the reduction, to a blue substance, of phosphomolybdic acid, without molybdic acid being similarly affected. Of the various reducing agents suggested, that of Fiske and Subbarow [1925], 1:2:4-aminonaphtholsulphonic acid, has proved by far the most satisfactory in our hands. The production and reduction of phosphomolybdic acid depend upon a number of other factors, of which the most important seems to be the acidity of the solution during reduction. Absence of acid results in reduction of molybdate itself whilst excess of acid prevents the reduction of phosphomolybdate; and it must be remembered that removal of organic matter from the alcohol-ether extract is accomplished in an acid medium.

It is evident that in the destruction of organic matter prior to phosphorus estimation, sufficient acid must be used to keep the foot of the test-tube ($6 \times \frac{5}{8}$ in., pyrex) covered during the process. Moreover, during that process, some acid is invariably lost by evaporation and by reduction, the amount depending upon the amount of organic matter present and the length of time of heating. The minimum of sulphuric acid which can be used without complete

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evaporation to dryness during the ashing is 1 ml. of $10N$ acid, and this amount diluted to 10 ml. completely inhibits the reduction of phosphomolybdic acid in the cold, although not at 100°—*i.e.* in a boiling water-bath. From Fig. 1,

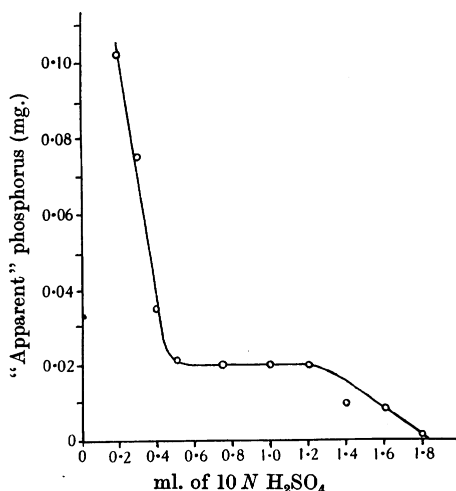

Fig. 1.

which shows the effect of varying the final concentration of acid on the apparent amount of phosphorus present, it appears that the permissible final concentration lies between 0·75 and $1{\cdot}20N$. This, since the final volume is 10 ml., corresponds to 0·75 ml. of $10N$ acid as the minimum which must remain after incineration. Actually, if 1·0 ml. of $10N$ acid is used initially, the loss only approaches 25 % if the amount of organic matter present is very exceptionally large, and this quantity of acid is therefore suitable. It can be increased to 1·2 ml. with a rather greater margin of safety but not beyond that amount. To conserve acid for the incineration, it is our practice to use an aqueous solution of ammonium molybdate in place of the more usual molybdic acid-sulphuric acid mixture, and these figures for the permissible amount of acid are based on this practice.

The destruction of organic matter by heating with sulphuric acid and perhydrol has been criticised by Baumann [1924] and others on the ground that phosphoric acid may be lost by volatilisation and by conversion into metaphosphoric acid. In actual practice these criticisms seem to be unwarranted, for standard amounts of phosphate have been heated for several times the normal period and with addition of several times the normal amount of perhydrol (to destroy excessive amounts of added organic matter) without any appreciable loss of phosphorus.

The amount of molybdate is of some importance. There must be sufficient to combine with the largest amount of phosphorus likely to be encountered, but excess tends to cause development of the blue colour even in absence of phosphate. It is true that "standard" and "unknown" are similarly treated, but a colour due to the reagent seriously diminishes the range of colour variation over which proportionality of colour and phosphorus concentration exists. Under our conditions, 0·5 ml. of 2·5 % ammonium molybdate is satisfactory, and the maximum allowable is 0·75 ml.

Excess of reducing agent (aminonaphtholsulphonic acid) up to five times the normal amount introduces no error but produces no advantage.

The colour under our conditions of acidity is not developed in the cold, but in the boiling water-bath it reaches 96 % of its maximum depth in 5 minutes, is fully developed in 10 minutes and is not altered by further heating up to a total of 25 minutes. Thereafter it changes to a brown which is useless for colorimetric purposes.

The method as finally adopted differs only in details from those already published, but the details are so important that a full description is warranted here. Only by attention to them can accurate and reproducible results be obtained, though in practice the method adds the virtues of ease and rapidity.

Determination of "lipoid phosphorus".

Solutions required:

10*N* sulphuric acid, 90 ml. conc. acid added to 260 ml. water.

Merck's Perhydrol.

Standard phosphate solution, 1 ml. equivalent to 0·01 mg. P.

2·5 % aqueous solution of ammonium molybdate.

1:2:4-Aminonaphtholsulphonic acid solution, made up according to the directions of Fiske and Subbarow.

Details of method:

2 ml. of blood or plasma are run into 3:1 alcohol-ether mixture according to the method of Bloor, extracted, cooled, made up to 50 ml. and thoroughly mixed. 5 ml. of the extract are pipetted into a 6 × ⅝ in. pyrex tube graduated at 10 ml., a glass bead is added and the solvent evaporated off. After cooling, 1 ml. of 10*N* sulphuric acid is added and the heating continued until the mixture is thoroughly charred. It is then allowed to cool and the carbon is removed by the addition of one drop of perhydrol. The acid is heated until it has distilled up as far as the 10 ml. graduation mark, care being taken to avoid excessive loss by fuming. Further charring invariably occurs at this stage, and a second drop of perhydrol is required. The acid should be again distilled up as far as the mark to decompose the excess of peroxide completely.

The tube is allowed to cool and the sides are washed down with water so that the final volume is approximately 9 ml. The contents of a second tube containing 0·02 mg. phosphorus are also diluted to about the same volume. To both tubes are added 0·5 ml. of the molybdate, and 0·4 ml. of the reducing agent, and after mixing, the tubes are placed in a boiling water-bath for 10 minutes. They are then cooled in running water and the contents made up to the mark and compared.

With a method capable of estimating accurately a small amount of phosphorus in the presence of a considerable amount of organic matter, it became possible to attack the second part of the problem, and to consider whether the phosphorus present in the alcohol-ether extract of blood really affords a true measure of the phospholipins of blood. The evidence of this is necessarily indirect.

In the first place it is significant that, as in the case of total fatty acids [Stewart and Hendry, 1935], the same amount of phosphorus is extracted from blood by alcohol-ether, alcohol-chloroform and alcohol-light petroleum. Naturally less is extracted by acetone. It is further significant that refluxing the mixture of blood and solvent does not increase the amount of phosphorus extracted. These two facts suggest very definitely that the alcohol-ether mixture extracts all the phospholipins but do not reply to the criticism that other phosphorus-containing compounds may be extracted as well.

Two sets of experiments have been made to decide this latter point. In the first, various phosphorus compounds, organic and inorganic, were added in the solid state to blood in amounts vastly greater than the normal, and the alcohol-ether extractable phosphorus was then estimated in the usual way. Only in

Table I.

Results expressed in mg. "lipoid phosphorus" per 100 ml. whole blood.

	3:1 alcohol-ether	3:1 alcohol-chloroform	3:1 alcohol-light petroleum	Acetone
Case 1	10·6	10·7	11·3	7·90
Case 2	11·5	11·5	11·6	—

"Lipoid phosphorus"...	1	2	3	4	5	6	7
Ordinary extraction	11·9	11·7	11·6	9·6	12·0	10·4	12·0
After refluxing in 3:1 alcohol-ether	11·4	11·4	11·8	9·8	12·6	9·6	11·9

the case of sodium glycerophosphate was there any suggestion of an increase in the apparent amount of "lipoid phosphorus", and even here the increases were so small as to warrant the conclusion that the estimation of "lipoid phosphorus" is justifiable.

Table II.

Nature of the added phosphorus	Original "lipoid phosphorus" mg. per 100 ml.	Conc. of added P mg. per 100 ml.	"Lipoid P" of the mixture mg. per 100 ml.	Percentage of the added P recovered %
Disodium hydrogen phosphate	11·0	12·0	11·0	Nil
	11·0	30·3	11·0	Nil
Potassium dihydrogen phosphate	12·14	15·4	12·0	Nil
Sodium glycerophosphate	12·61	14·5	12·21	Nil
	11·05	14·5	11·73	4·6
	11·05	34·0	11·97	5·6
Caseinogen	11·05	11·3	11·05	Nil

In the second series of experiments, the phospholipins were precipitated by the method used in Bloor's [1929] oxidimetric estimation. The alcohol-ether extract was evaporated to dryness (with a current of hydrogen in the final stages), the residue was extracted with anhydrous ether (free from peroxide), the centrifuged extract was concentrated by evaporation, and the phospholipins were precipitated by acetone and an alcoholic solution of magnesium chloride. The precipitate, washed with acetone, was extracted with moist ether to dissolve the phospholipins. The residues and washings at all stages were analysed for phosphorus with the results shown in Table III.

Table III.

"Lipoid P" of the original blood mg. per 100 ml.	P left after ether extraction mg.	P in the acetone washings mg.	P left in the $MgCl_2$ residue mg.	P in the final ether extract mg.	Percentage of the original P recovered
9·66	0·05	0·00	0·27	9·06 (94 %)	97·1
12·71	0·07	0·00	0·32	12·06 (95 %)	98·0
11·56	0·05	0·00	0·42	10·55 (91 %)	95·5
12·48	0·06	0·00	0·22	11·91 (95 %)	97·6
10·45	0·08	0·00	0·28	9·92 (95 %)	98·3
12·77	0·05	Trace	0·23	12·22 (96 %)	98·0

The original alcohol-ether phosphorus is adequately accounted for in the various residues and extracts. In all but one case, at least 94 % of the original phosphorus was recovered in the final ether extract. No appreciable loss occurs except at the stage where the purified phospholipins are extracted with moist

ether. This cannot be manipulative error, for it would not all be concentrated at this point, and 99·5 % of the total phosphorus was initially extractable with ether. The most obvious, and probably the correct, explanation lies in the assumption that decomposition has occurred to a slight extent. It is noteworthy that the precipitate, pure white at first, invariably darkens slightly on standing even when the ether used is completely peroxide-free. The 1–3 % of phosphorus which remains with the magnesium chloride cannot be recovered by increasing the number of extractions with moist ether; nor is there any insoluble phospholipin-magnesium chloride complex, since acidification does not increase the quantity of phosphorus extracted. It is noteworthy too, that repeated purification by reprecipitation with acetone and magnesium chloride involves, each time, the loss of 1–3 % of phosphorus in the residue of magnesium chloride (with a corresponding loss of fatty acids)—a fact which also points to decomposition.

Having regard to the notorious instability of the phospholipins, especially when they are in the pure state, it is almost certain that after precipitation there is slight decomposition to inorganic phosphorus, or some other ether-insoluble fraction, with loss of the corresponding amount of fatty acids in the acetone washings. Even without such an assumption, 95 % of the original "lipoid phosphorus" has turned out to be true lipin phosphorus, and there are indications, as noted above, that the percentage is even greater. It will be seen in the above table that a small loss (2–3 %) arises from the difficulties of manipulation, and this also is probably true lipin phosphorus.

It seems justifiable to deduce from these experiments that the use of "lipoid phosphorus" estimations as a means of determining the phospholipins of blood involves a maximum error of 5 %, and that in all probability the error is much less than that, and is, in fact, within the limits to be expected of a microchemical procedure.

The method of precipitation by acetone and magnesium chloride provided a means of investigating the nature of the blood phospholipins, since it was possible to estimate not only the phosphorus but also the fatty acids in the ether-soluble matter of the precipitate. The presence of magnesium introduced a certain difficulty, however, since the ethereal extract of the precipitate apparently contained a magnesium complex, which, after hydrolysis, yielded a small but appreciable amount of magnesium soaps. As these are not attacked by the addition of dilute hydrochloric acid, the results of fatty acid estimations may thus be too low. After unsuccessful attempts to remove the magnesium by ammoniacal sodium phosphate or by 8-hydroxyquinoline, it was found that a white gelatinous precipitate which formed early during the hydrolysis of the fats consisted of magnesium hydroxide and could be removed by centrifuging without loss of fatty acid. After the completion of the hydrolysis, acidification and extraction of fatty acids, there was no remaining precipitate of magnesium soaps. Attempts to dispense with magnesium chloride resulted in a considerable loss of material as did replacement of that salt by alcoholic solutions of lithium chloride or cadmium chloride.

Experiments on these lines gave the rather surprising result that the ratio of fatty acid molecules to phosphorus atoms is approximately 1·5 : 1·0. For lecithin and kephalin the ratio is 2 : 1, and for sphingomyelin, 1 : 1. The same ratio was obtained when magnesium chloride was omitted and the phospholipins were precipitated by acetone alone, although here there was an appreciable loss of material. Purification of the material by reprecipitation with acetone and magnesium chloride does not affect the ratio.

Table IV. *Table showing the equivalent of 1 mg. of "lipoid phosphorus" in terms of mg. of fatty acids in the phospholipins of blood.*

	Equivalent found	Notes
Magnesium not removed	13·24	Average of 10 analyses
Magnesium not used during precipitation	13·07	Average of 2 analyses
Magnesium removed as phosphate	13·07	Average of 4 analyses
Magnesium removed as hydroxide	12·88	Average of 3 analyses
	13·12	Average of 19 analyses

It seems, therefore, that the phospholipins of blood must consist approximately of 50 % sphingomyelin and 50 % of a mixture of lecithins and kephalins. The presence of some kephalin is indicated by the fact that the purified material is only partly soluble in dry ether.

This deduction would be modified if the presence of galactosides were demonstrated since they contain fatty acids but no phosphorus. Their presence in the purified material would mean that sphingomyelin formed more than 50 % of the mixture, while lecithin and kephalin accounted for correspondingly less. We believe, however, that galactosides are not present in the material precipitated by acetone and magnesium chloride. It is true that this material, extracted by water, gives a positive Molisch reaction (of intensity varying in different samples) and shows the presence of "sugar" which can be estimated by the Hagedorn-Jensen method. Purification by repeated reprecipitation and elution with ether, however, completely abolishes the Molisch reaction without appreciable alteration of the fatty acid : phosphorus ratio. Elimination of galactosides by purification would result in a change in this ratio. Moreover, consideration of the delicacy of the Molisch reaction and the amounts of material concerned indicate that the repurified phospholipin fraction contains not more than 5 % of fatty acid, at most, which could be combined with carbohydrate.

Theoretically, it should be possible to estimate the relative amounts of lecithin (and/or kephalin), sphingomyelin and galactosides by determination of the nitrogen, phosphorus and fatty acid contents of the purified material. Taking as a basis the recognised composition:

	Phosphorus atoms	Nitrogen atoms	Fatty acid residues
Lecithin and kephalin	1	1	2
Sphingomyelin	1	2	1
Galactosides	0	1	1

this procedure should give three simultaneous equations. Previous workers have found excessively high values for the nitrogen content of partly purified preparations [Channon and Collinson, 1929; MacLean, 1912] and the presence of foreign nitrogenous substances has of necessity been postulated. In our analyses, material purified by a single acetone-magnesium chloride precipitation gave values for the N:P ratio varying from 2·35 to 3·67. Calculation from the observed fatty acid:phosphorus ratio of 1·5:1 shows that the maximum value for the N:P ratio is 2·5 which occurs when lecithin (and kephalin) is absent, and sphingomyelin accounts for 66 % of the material, the remainder being galactosides. The figures for nitrogen thus point, in our analyses also, to the presence of foreign matter. Purification (which, as already stated, does not alter the relative amounts of phosphorus and fatty acids) certainly reduces the N:P ratio, but we have never found a value below 2·0 which would correspond to a mixture of 1 part of lecithin (and/or kephalin), 2 parts of sphingomyelin, and

1 part of galactosides. With the amount of material at our disposal, the carbohydrate of such a mixture should be readily detectable.

Since we failed to detect any carbohydrate in the purified material, and since the N:P ratio was very variable even in the purified specimens, it seems that the evidence from the nitrogen estimations must be regarded as unreliable, that the absence of galactosides from the purified phospholipins is most probable, and that therefore the phospholipins of blood consist essentially of a mixture of equal parts of sphingomyelin and lecithin (and/or kephalin).

Summary.

Conditions are described for the accurate measurement of organic phosphorus by the Fiske and Subbarow method, and in particular for the determination of "lipoid phosphorus".

Evidence is advanced that the "lipoid phosphorus"—*i.e.* the phosphorus present in the alcohol-ether extract of blood—affords an accurate measure of the phospholipins.

In preparations of phospholipins, purified by repeated precipitation by acetone and alcoholic magnesium chloride, the ratio of fatty acid molecules to phosphorus atoms is about 1·5:1. Since the balance of evidence is against the presence of galactosides, this is interpreted as indicating that about half of the phospholipin consists of sphingomyelin and half of a mixture of lecithin and kephalin.

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