6. THE ANALYSIS OF BLOOD PHOSPHOLIPINS

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WHILE the importance of phospholipins in the metabolism of fats is universally recognized, the most perfunctory examination of the relevant literature is sufficient to show that the methods of analysis at present available are inadequate. Though it is possible to determine with ease and accuracy the total phospholipin content of any biological specimen, an estimate of the partition of the lipid P among the various phospholipins is by no means as simple. Few workers have even attempted such analyses, and none of the methods proposed is completely satisfactory. Thannhauser *et al.* [1939] have determined sphingomyelin by precipitation of the whole molecule with ammonium reineckate by means of a technique published in an earlier series of papers from the same school. Hydrolysis of the lipin solution with baryta is the preliminary step in their choline estimation, and the concentration of lecithin is estimated from the difference between total choline and total lipid P. The principal drawback to this method is the amount of material required if the sphingomyelin precipitation is to be really satisfactory. On a small scale it seems that precipitation is frequently incomplete.

A method requiring only very small amounts of material has been published by Kirk [1938]. It is based fundamentally on the difference in the solubilities of the various phospholipins in different solvents. While this principle is perfectly satisfactory as long as its application is restricted to qualitative work on a large scale, it breaks down when applied to the quantitative analysis of phospholipins amounting altogether to hardly more than a milligram. This, however, is apparently not the only fault in Kirk's technique, because his total phospholipin values are far more variable than those generally accepted, and many of them are exceedingly low. In one case, for example, he reports a total phospholipin concentration in red blood corpuscles of 26 mg. per 100 ml. This corresponds to a lipid P value of about 1 mg. per 100 ml., whereas the normal limits of 10–14 mg. per 100 ml. have rarely been passed in either direction by other analysts. Finally, it may be remarked that Kirk appears to have found little difference between the concentration of phospholipins in erythrocytes and in plasma, whereas it is well known that it is generally greater in the former.

Even before the appearance of these papers we had been engaged on this problem for some time, and it had seemed to us that it was vain to hope for a solution based on solubility differences, especially when working with the small samples which we were proposing to use. Our attention, therefore, was directed towards the possibility of determining the various characteristic residues of phospholipin molecules, such as choline, glycerol and β -aminoethanol, with a view to calculating from them the concentrations of the phospholipins. It is readily seen from Table 1 that the calculation of lecithin, cephalin and sphingomyelin is possible from choline, glycerol and P values, and that β -aminoethanol determinations would afford a very useful check on the other analyses.

Table 1

Substance	Phosphorus	Choline	Glycerol	Colamine
Lecithin	1	1	1	—
Kephalin	1	—	1	1
Sphingomyelin	1	1	—	1

In this paper are described methods for the determination of choline and glycerol, and their application, as indicated above, to specimens of phospholipins prepared from 2 ml. blood; that is to say, about 5–7 mg. mixed phospholipins. Mention is also made of hitherto unsuccessful attempts to evolve a specific micro-method for the determination of β -aminoethanol, and hence of kephalin.

The determination of choline

Of the reagents generally used nowadays for the precipitation of choline we have preferred ammonium reineckate to iodine because the precipitate of choline reineckate is much easier to work with than that of choline enneaiodide. The analysis of the precipitate is also much simpler, although it must be admitted that the application of the reineckate method is limited by the fact that choline reineckate is somewhat more soluble than the enneaiodide.

The method described here is a modification of that of Beattie [1936], whose technique we have found satisfactory except in a few minor details. Our method, however, is very much more sensitive than Beattie's, and can be applied to 1 ml. of a solution containing as little as 0.25 μ mol. (0.03 mg.) choline.

Method

Reagents. Barium hydroxide, saturated solution.

H₂SO₄, approximately *N* and *N*/10.

Ammonium reineckate.

Acetone, ammonia-free, prepared by the distillation of A.R. acetone once from slightly acid solution.

Digestion mixture [Folin & Wu, 1919] diluted with 3 vol. of water.

H₂SO₄, 0.007 *N*, prepared with indicator according to Conway [1939].

NaOH, 40 % and 0.007 *N*.

Procedure. A volume of lipin solution containing not more than 4.5 μ mol. (0.54 mg.) of choline is evaporated to a small volume in a 25 ml. pyrex conical flask with 1 ml. saturated aqueous baryta and the mixture is heated on the steam bath for 1 hr. A further 1 ml. of baryta is then added and the heating is continued for 1 hr. more. The mixture is neutralized to methyl red with H₂SO₄, using first *N* and then, as the end-point is approached, *N*/10 acid. The solution, with the suspended precipitate of barium sulphate and fatty acids, is evaporated to 0.3–0.5 ml. and transferred to a small filter paper with the aid of a capillary pipette and a rubber teat. The precipitate is washed with successive amounts of hot water until the total volume of filtrate amounts to not more than 1.5 ml. On no account whatever must the filtrate be alkaline, and it should not be more acid than *N*/50. 1 ml. of a cold freshly-prepared saturated aqueous solution of ammonium reineckate is then added and the tube is left in the refrigerator for 20–30 min. to ensure complete precipitation. The pale pink crystalline precipitate of choline reineckate is filtered off through a tiny pad of cotton-wool pressed into the bottom of a small filter-funnel (paper is very difficult to wash free from excess ammonium reineckate) and washed with ice-cold water and a little ether. It is dissolved by pouring a few drops of ammonia-free acetone through the

filter, the resulting pink solution being collected in a pyrex test tube. After the addition of a drop of dilute acid to prevent loss of ammonia during the subsequent heating, the acetone is removed by cautious evaporation over a free flame. 0.5 ml. of dilute digestion mixture is then added and the precipitate is ashed. The digest is made strongly alkaline and distilled with steam into 5 ml. 0.007 *N* H₂SO₄. The excess acid is titrated with 0.007 *N* NaOH. The formula of choline reineckate is HO.C₂H₄.N(CH₃)₃.[Cr(NH₃)₂(CNS)₄] and from this it can be seen that 1 mol. of choline yields ultimately 7 mol. of ammonia. Consequently, each ml. 0.007 *N* alkali corresponds to 1 μmol. choline. Table 2 shows that very satisfactory duplicate analyses can be made and that the recovery of added choline is, within the limits of experimental error, quantitative. It is theoretically necessary to do a 'blank' experiment on unhydrolysed lipin extract, but in the case of whole blood we have found the 'blank' to be so very small as to be negligible. This, of course, does not apply to the ammonia-blank of the Kjeldahl determination.

Table 2. Choline, millimol./100 ml. blood

Sample	Choline added	Choline found	Recovered	
			Amount	%
1	—	0.256, 0.257	—	—
2	—	0.217, 0.223	—	—
3	—	0.267, 0.262	—	—
4	—	0.217, 0.217	—	—
5	—	0.268, 0.275	—	—
6	—	0.220, 0.226	—	—
7	—	0.273, 0.264	—	—
8	—	0.216, 0.218	—	—
8	0.215	0.437	0.220	102
8	0.215	0.429	0.212	99

It remains to discuss the specificity of the method. Ammonium reineckate appears to be a highly specific precipitant for quaternary bases. It has been used for the isolation not only of choline and acetylcholine, but also of neurine, carnitine and sphingomyelin. Lecithin does not, on the other hand, appear to form an insoluble reineckate, and creatinine does not seem to be precipitated until its concentration reaches the quite unphysiologically high level of 5 mg. per ml. Hence, while it is in theory possible that blood might contain appreciable amounts of reineckate-precipitable bases, 'blank' estimations have shown that the amount present is not significant, and in any case it is difficult to imagine any compounds other than the choline-containing phospholipins (and, of course, acetylcholine, which has been shown by Bischoff *et al.* [1931] to be present only in very low concentration) from which reineckate-precipitable base would be liberated by hydrolysis with baryta. Furthermore, we have prepared by the method outlined above a specimen of reineckate from 600 ml. of alcohol-ether extract of blood (equivalent to 22 ml. blood) which, when analysed for nitrogen, gave a value of 23.3 %. The theoretical value for choline reineckate is 23.2 %, but this close agreement does little more than confirm the absence of gross contamination, since six-sevenths of the total nitrogen comes from the reineckate radicle. The crystalline form of the precipitate, however, seemed identical with that of the reineckate prepared from pure choline chloride solution, and it would appear that there might be no great danger in the assumption that choline is actually precipitated as a fairly pure reineckate in these experiments.

The determination of glycerol

The determination of glycerol has never been a simple procedure. On an industrial scale the purity of glycerol is sometimes checked by means of a quantitative chromic acid oxidation. Such a technique is obviously quite unsuitable for the present purpose. More hope is offered by permanganate oxidation. Alkaline permanganate oxidizes glycerol with fair regularity to oxalate, which can then be determined in the usual way by precipitation as the calcium salt. This method has been applied to lipid glycerol [Dickson, 1935], but has been shown to give somewhat erratic results, largely because of the difficulty of determining oxalate accurately in very low concentration. Fleury & Fatome [1935] have recently published a method which depends on the oxidation of glycerol with an acid solution of sodium or potassium periodate and oxidimetric determination of the excess periodate, in accordance with the following equation:

The method as published proved unsuitable for two main reasons: in the first place, it deals with amounts of glycerol which are far too large (5–15 mg.), and in the second, it simply measures the disappearance of an oxidizing agent whose specificity is by no means high. It appeared to us, however, that a solution to the problem might be found in the determination of formaldehyde, since each molecule of glycerol yields two molecules of that compound in this oxidation. While many substances are attacked by periodic acid, comparatively few of these (in general, only primary alcohols in which the primary alcoholic group is directly linked to another partially oxidized carbon atom) yield formaldehyde. The number of these which would be liable to interfere with the present experiments is limited, and the most likely one, glucose, can be readily removed by treatment with copper sulphate and calcium hydroxide. Glycerophosphates would also presumably interfere, but these have been shown by Stewart & Hendry [1935] not to be extracted from blood by the usual alcohol-ether mixture.

Formaldehyde, however, is by no means easy to estimate accurately on a micro-scale, although Schryver [1910] has described an exceedingly delicate and highly specific colour reaction for its detection in minute concentration. Schryver himself claimed that his own technique was satisfactory for quantitative use, but in our hands it has given inconsistent and inaccurate results, owing apparently to side reactions among the various reagents which Schryver employed in rather high concentration. We have consequently found it necessary to modify his technique very considerably, and the glycerol method incorporating the modifications is described below.

Reagents. NaOH, 20 % solution.

Copper sulphate cryst., A.R., 10 % solution.

Calcium hydroxide, as pure as possible.

Sodium periodate, 0.05 *M* in 3 *N* H₂SO₄ (formula of salt supplied by B.D.H. Na₅H(IO₅)₂).

Sodium thiosulphate, 50 % solution.

Phenylhydrazine hydrochloride, 'Analar', 0.03 *M* in 0.2 *N* HCl.

Potassium ferricyanide, 'Analar', 0.015 *M*. Neither of these last two solutions should be more than a week old, and both should be kept in the dark.

H₂SO₄, 20 % by volume.

Procedure. To a volume of lipin solution expected to contain not less than 0.2 μmol. (about 0.02 mg.) glycerol is added 0.2 ml. 20 % NaOH. The solution is

made up to about 10 ml. with alcohol and evaporated on the hot-plate until its volume does not exceed 1 ml. This evaporation should take about 45 min., and great care must be taken that at no time does any part of the bottom of the flask become dry, otherwise appreciable quantities of glycerol will be volatilized and lost. The residual fluid is transferred with the aid of a capillary pipette to a narrow centrifuge tube graduated at 3 ml., and the flask is rinsed out with several small volumes of hot water. When the solution has cooled to room temperature, 0.3 ml. 10 % copper sulphate, a knife-point of slaked lime and sufficient water to make up the volume to 3 ml. are added. The tube is left to stand for 1 hr., during which it is frequently shaken, and it is then centrifuged. An aliquot of the supernatant fluid, usually 2.5 ml., is pipetted into a 10 ml. round-bottomed pyrex flask with a neck ground to fit a micro-Kjeldahl head with the long tube removed (see Fig. 1). 1 ml. acid sodium periodate solution is added and the flask left to stand for 4-5 min. (not longer). The excess periodate is reduced to iodide by the dropwise addition of 50 % sodium thiosulphate, of which excess should be avoided. The contents of the flask, after neutralization with 20 % NaOH (3 or 4 drops extra being added), are distilled into 2 ml. acid phenylhydrazine solution. When the flask is almost dry a little water is added and the distillation is repeated to ensure that all the formaldehyde passes over. It is important that the solution should not become acid during the distillation. Methyl red and phenol red are suitable indicators for observing this precaution. The distillate is diluted to about 20 ml. and 2 ml. potassium ferricyanide are added. After 25-30 min., during which a colour varying from pale yellow to a rich dark sherry develops, the solution is diluted in a 50 ml. volumetric flask to about 30 ml., and 10 ml. 20 % H_2SO_4 are added. This changes the colour to a rose-red. The solution is made up to the mark with water and compared with a standard made by oxidizing a measured volume of standard glycerol solution. Since the intensity of the final pink colour is not quite proportional to the concentration of formaldehyde, and hence to the concentration of glycerol, it is important to employ a standard the concentration of which approaches that of the unknown fairly closely. Table 3 gives a few typical results, including not

Fig. 1.

Table 3. *Glycerol, millimol./100 ml. blood*

Sample	Added	Found	Recovered	
			Amount	%
1	—	0.088, 0.090	—	—
2	—	0.127, 0.124	—	—
3	—	0.076, 0.0735	—	—
4	—	0.152, 0.147	—	—
5	—	0.178, 0.176	—	—
1	0.1025	0.197	0.108	105
2	0.1025	0.221	0.096	94
2	0.1025	0.224	0.099	97

only duplicate analyses of alcohol-ether extracts of whole blood but also recoveries of added glycerol.

These two methods have been applied, in conjunction with a standard lipid P technique [Stewart & Hendry, 1935], to specimens of phospholipins prepared from whole blood in the following way.

50 ml. alcohol-ether extract of blood [Bloor, 1928] were evaporated just to dryness on the hot-plate and finally in the steam-oven. The residue was immediately taken up in light petroleum and the turbid solution transferred to a centrifuge tube where it was concentrated to about 0.3 ml. by immersion in a beaker of hot water. Acetone was then added from a 5 ml. pipette, very slowly and with continuous shaking so that the phospholipins separated as a flocculent white precipitate and not as a tarry mass. Precipitation was completed by adding 3 drops of a saturated alcoholic solution of magnesium chloride and allowing the tube to stand for 2 hr., preferably in the refrigerator. At the end of that time any precipitate adhering to the walls of the centrifuge tube was carefully removed by scratching and the precipitate was thrown down in the centrifuge. It invariably settled rapidly and completely and was twice washed at the centrifuge with 5 ml. cold acetone before being taken up in 10 ml. of a mixture of 3 parts of ether, 2 of alcohol and 1 of water, in which it gave a perfectly clear, almost colourless solution. The entire procedure could be carried out in about 3 hr., and during this time the phospholipins spent 2 hr. in the refrigerator, which should have minimized the oxidation by atmospheric oxygen to which these compounds are so very susceptible. Analyses showed that the procedure extracted 92-97 % of the P in the alcohol-ether extract, so that not only is this P virtually all phospholipin-P, but the extraction and precipitation with acetone and alcoholic magnesium chloride are also satisfactory and complete.

Aliquot portions of the ether-alcohol-water solution were analysed for glycerol (2 ml.) and choline (2 ml.) by the methods just described, and for phosphorus (1 ml.) by the method of Stewart & Hendry [1935]. The results of 12 analyses on specimens of whole blood from healthy men in their early twenties are given in Table 4. Table 5 shows the concentrations of lecithin, kephalin and sphingomyelin calculated from the figures in Table 4, the calculations being based on the assumption that these are the only choline-, phosphorus- and glycerol-containing compounds present in the material analysed; this Table also shows the same results calculated as percentages of the total phospholipins.

The figures in Table 5 show one or two rather interesting facts. In the first place, it is clear that although the phospholipins of blood have often been loosely described as 'lecithin', lecithin itself actually accounts for only a small proportion

Table 4

No. of sample	Phosphorus milliatoms/100 ml. blood	Choline millimol./100 ml. blood	Glycerol millimol./100 ml. blood
1	0.432, 0.420	0.274, 0.283	0.178, 0.176
2	0.333	0.290, 0.298	0.147, 0.151
3	0.355	0.300	0.109
4	0.336	0.283	0.124
5	0.340	0.255	0.119
6	0.366	0.271	0.138
7	0.316	0.256	0.111
8	0.319	0.227	0.127
9	0.352	0.275	0.108
10	0.351	0.245	0.104
11	0.347	0.261	0.103
12	0.380	0.292	0.113

Table 5. *Concentrations of phospholipins in blood samples*

No. of sample	Lecithin		Kephalin		Sphingomyelin	
	millimol./100 ml.	% of total	millimol./100 ml.	% of total	millimol./100 ml.	% of total
1	0.028	6	0.148	35	0.250	59
2	0.110	33	0.039	11	0.184	56
3	0.054	15.5	0.055	15.5	0.246	69
4	0.071	23	0.053	14	0.212	63
5	0.034	13	0.085	25	0.211	62
6	0.043	11	0.095	26	0.228	63
7	0.052	16	0.059	19	0.204	65
8	0.035	14	0.092	26	0.192	60
9	0.031	9	0.077	22	0.244	69
10	0.000	0	0.104	30	0.245	70
11	0.017	5	0.086	25	0.244	70
12	0.025	9.5	0.078	20.5	0.267	70
Mean		13		22		64

of the total. In one case (No. 10), indeed, no lecithin at all was found. While errors in estimations will have been magnified in the calculations, it seems highly improbable that this low concentration of lecithin would be either artificial or fortuitous in every one of 12 consecutive analyses. A second point of interest in these tables is that sphingomyelin is quantitatively by far the most important of the phospholipins present. Thirdly, in nearly all cases the concentration of kephalin is considerably greater than that of lecithin. Even so short a series, moreover, has demonstrated clearly that the proportion of sphingomyelin is fairly constant. It seems possible that this constancy might be emphasized by a series of analyses on plasma and corpuscles separately.

It is difficult to compare these findings with those of Kirk [1938], since, as we have already mentioned, there appear to be grounds for doubting the accuracy of his results. Other workers have, however, recently published comparable figures. Williams *et al.* [1938] have given figures for kephalin concentrations in blood plasma and corpuscles obtained in much the same way as our own by difference between lipid P and choline. Their results are somewhat higher than we should have expected from our own analyses of whole blood, but this may be explained by the fact that they carried out the precipitation of choline reineckate in a solution sufficiently acid to interfere with the precipitation. This may have led to low results for choline and consequently high kephalin figures. The latter, nevertheless, are of the same order as our own.

Other interesting figures are those of Thannhauser *et al.* [1939] for blood serum and various tissues. These again show a remarkable constancy in sphingomyelin concentration, although it must be admitted that as a rule their results for serum sphingomyelin are rather lower than we should expect from our results with whole blood.

The phosphorus-fatty acid analyses of Stewart & Hendry [1935] indicated that approximately one-half of the total phospholipins of whole blood consisted of sphingomyelin, a proportion somewhat lower than that observed in the present work. Neither series is long, so that the difference may be a chance one. On the other hand, Stewart & Hendry based their admittedly rough conclusion on the ratio of P atoms to fatty acid molecules in the phospholipin precipitate, and a very slight oxidation of the fatty acids before estimation would result in a shift of this ratio sufficient to account for the observed discrepancy. For a mixture containing 50 % sphingomyelin the ratio P:—COOH is 1:1.5; for a

mixture containing 64% it is 1:1.36. Hence, a 10% increase in the number of carboxyl groups is all that it is necessary to postulate in order to make the two sets of figures agree, and it seems not unreasonable to suggest that such a small amount of oxidation may have taken place. A few preliminary analyses have suggested that on occasion the increase of carboxyl groups may be very much greater than this, and this conclusion is supported by the work of Paal [1929] who studied the oxidation of alcoholic solutions of lecithin and found the number of free carboxyl groups to increase very rapidly.

The attempted estimation of β -aminoethanol and kephalin

During the investigation of substances which might have interfered with the determination of glycerol, it was observed that β -aminoethanol was oxidized slowly in acid and rapidly in alkaline solutions of periodate in the following way:

This equation has been verified, not only by the estimation of the periodate used up and the formaldehyde produced, but also by the determination of the ammonia. Several methods were used, depending on the quantities of β -aminoethanol under consideration, and much the most satisfactory proved to be the method of Conway [1939], with which it was practicable to determine accurately the ammonia from as little as $0.2 \mu\text{mol}$. β -aminoethanol. The reaction, which appeared to be highly specific, was carried out simply by mixing in the outer chamber of a Conway unit 0.5–1.5 ml. β -aminoethanol solution, 0.5 ml. 0.05 *M* sodium periodate (dissolved in water with the minimum amount of H_2SO_4) and 1 ml. saturated aqueous K_2CO_3 . The ammonia formed was allowed to diffuse into standard H_2SO_4 in the inner chamber, the excess acid being titrated after the completion of the diffusion. When the method was applied to hydrolysed lipoid extracts figures which were certainly of the right order were obtained, but it did not seem possible, except on rare occasions, to recover more than 65–75% of added β -aminoethanol. Various methods were tried for the purification and separation of the base before estimation; these did not alter materially the results of the analysis, but they did not enhance the recovery of added β -aminoethanol to any great extent. A certain amount of success, but not sufficient to justify the use of the method, attended the adsorption of the β -aminoethanol, after hydrolysis of the lipins, from slightly alkaline solution on to frankonit KL, followed by periodate oxidation of the base *in situ* on the adsorbent.

SUMMARY

Methods for the determination of choline and glycerol in lipin solutions are described, with their application to the separate determination of the various phospholipins in whole blood.

In 12 blood samples the concentration of sphingomyelin ranged from 56 to 70% of the total phospholipin, with a mean of 64%. In most of the cases the concentration of kephalin appeared to be considerably greater than that of lecithin.

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