THE PHOSPHOLIPINS OF BLOOD.

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CONTENTS.

Phospholipin analysis has always been a tool of importance in the hands of the worker on lipin metabolism. Attractive theories have been put forward, suggesting that phospholipins are agents active in the transport of fats, or that fatty acids are more readily oxidised in combination as phospholipins (1,2,3). All are agreed that phospholipins are probably of prime importance in the metabolism of fats, but concrete evidence both for and against the various theories is sparing and inconclusive. While this is undoubtedly due, in some measure, to the slowness of the reactions undergone by lipins -- alimentary hyperlipaemia, for example, when it does occur, disappears very much more slowly than alimentary hyperglycaemia (4) -- a contributory factor must surely be the inadequacy of the available analytical methods. For present purposes, these have been considered with special reference to blood, but the same poverty of technique applies in general to all tissue analysis.

While kephalin and sphingomyelin have both been isolated from blood (5,6,7), pure lecithin has not, and reliable micro-chemical methods for the determination of all three compounds are completely lacking. Attempts have been made to determine kephalin by estimating the amino-nitrogen of its colamine residue, but it will cause the reader no surprise to hear that this method has been shown to give highly inaccurate results, since it is not specific

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and lipoid extracts appear invariably to contain considerable amounts of unidentified nitrogenous material from which it is impossible to separate the lipins completely (8,9,10). Williams and his collaborators(11) have made indirect kephalin determinations by means of the difference between phosphorus and choline analyses. Stewart and Hendry (12) have obtained results which indicate that about one-half of the phospholipins of whole blood consists of sphingomyelin, but none of these workers have really done more than touch on the fringe of the problem.

A superficial examination of the work of Kirk (13) suggests that he has gone much further. He has published analyses for lecithin, kephalin, and sphingomyelin separately, but unfortunately a close scrutiny of his results and even of his methods shows them to be almost without value. In the first place, the determinations were carried out on a petrol-ether extract of the residue from the evaporation of an alcohol-ether extract of plasma or corpuscles. This evaporation was done by simply leaving the solution in in an open beaker in a warm place overnight. Such a procedure, in which a large surface of material is presented to the air over many hours, encourages oxidation and hence loss of phospholipins. The second stage in Kirk's analysis was the precipitation of the phospholipins by the method of Bloor(14) with acetone and alcoholic magnesium chloride. No one can take exception to/

to this, but to base results on the varying solubilities of the precipitated phospholipins in moist ether is surely most dangerous: phospholipins are so readily taken up, in either true or colloidal solution, by solutions of other phospholipins. Finally, a study of Kirk's figures is alone sufficient to condemn his technique. His values for total phospholipins vary far more among themselves (54-235mg./100 ml. in plasma, and 26 - 297 mg./100 ml. in cells) than those of any other workers, and many of them are far below the standards which are universally recognised as normal. This in itself casts grave doubt on his work, since the determination of total phospholipins, whether by quantitative oxidation of the precipitate or by estimation of its phosphorus, is a simple, standard experimental procedure, and there is no reason to suppose that the blood of the Danes should have a more variable or a lower phospholipin content than that of other healthy Europeans.

In point of fact, it was some time before the appearance of Kirk's paper that this research was undertaken. It had seemed (and, indeed, it still does) that endless possibilities for research would be opened up if it were possible to determine accurately and without overmuch tedium the relative proportions of lecithin, kephalin, and sphingomyelin in any given mixture of the three. It was evident that a separation based on differing solubilities would lead to very considerable errors when applied to small amounts of complex/

complex mixtures, and attention was therefore directed to the possibility of determining the various characteristic residues found in different phospholipin molecules, e.g.: choline, glycerol, and β-amino-ethanol or colamine.

A consideration of the distribution among the phospholipins of phosphorus and these characteristic residues (see Table 1.) shows that if it were possible to determine glycerol, choline, and phosphorus, then a little elementary algebra would give the concentrations of the three phospholipins, and further, that a determination of colamine would afford a valuable check on the other estimations, beside giving a direct estimate of the concentration of kephalin.

TABLE 1.

Substance.	Phosphorus.	Choline.	Glycerol.	Colamine.
Lecithin.	1.	1.	1.	-.
Kephalin.	1.	-	1.	1.
Sphingomyelin.	1.	1.	-	-.

Now, the determination of phosphorus in lipoid material presents little more difficulty than its determination in pure phosphate solutions, and suitable estimations of choline and glycerol, capable of application to volumes of blood of the order of 1 -2 ml., have been devised and are reported here. The attempt at the determination of β -amino-ethanol, based/

based on an apparently highly specific reaction stumbled on during the course of another part of the research, has unfortunately failed, so that the check on the other methods is lacking. They have, nevertheless, been applied to phospholipin precipitates prepared from human blood, with results which are discussed later. It has been thought fitting to report also the work on colamine, in spite of the lack of success attending it, since some of the findings were extremely interesting from a purely chemical point of view.

THE DETERMINATION OF GLYCEROL.

The determination of small amounts of glycerol has never been a matter of simplicity. Satisfactory determinations can be made on a macro- or semimicro-scale by oxidation to oxalate with alkaline permanganate, followed by titration of the oxalate with standard permanganate. The drawback to the use of this method for very small amounts of glycerol is the great difficulty attending the estimation of minute amounts of oxalate. Even if a pure precipitate of some suitable oxalate can be quantitatively separated, it is hardly convenient to titrate less than 4 - 5 micromols. of oxalate (corresponding to 0.8 - 1.0 ml. 0.01 N permanganate), and for present purposes it was hoped to find a method suitable for the determination of quantities at least as small as 1 micromol.

In 1935 Fleury and Fatome (15) published a method for the determination of 5 - 15 mg. glycerol - about 50 - 150 u-mols. - which was based on an oxidation with periodic acid described some years earlier by Malaprade (16):

$$\begin{array}{l} CH_2OH \\ | \\ CHOH \\ | \\ CH_2OH \end{array} + 2HIO_4 = 2HIO_3 + \begin{array}{l} HCHO \\ + \\ HCOOH \\ + \\ HCHO \end{array} + H_2O$$

This reaction is absolutely quantitative. Fleury and Fatome added a measured excess of sodium or potassium periodate in sulphuric acid, allowed the reaction to take place, and used the excess periodate to react with a measured excess of arsenite. Finally, the excess of arsenite was titrated with iodine:

$$HIO_4 + H_3AsO_3 = HIO_3 + H_3AsO_4.$$

$$H_3AsO_3 + I_2 + H_2O = 2HI + H_3AsO_4.$$

While this method gives excellent results in pure solutions of the concentration recommended by its authors, certain objections make themselves felt when attempts are made to apply it to more dilute solutions prepared from biological material. In the first place, it becomes necessary to deduce the result from a small difference between two comparatively large titrations, and in the second place one is simply measuring the disappearance of an oxidising agent whose specificity is by no means high.

For these reasons attention was directed to the value and possibility of estimating either the formic acid or the formaldehyde produced. Most methods for the determination of formic acid appear to depend primarily on its reduction to formaldehyde by some such agent as magnesium with dilute acid, and attention was therefore focussed entirely on the aldehyde.

Periodic acid will attack almost any compound containing two adjacent partially oxidised carbon atoms. Thus, all α-glycols are attacked, including such substances as tartaric acid. As we have already seen, the carbon chain of glycerol is split in two places, and the molecule of glucose is broken into fragments. The details of these and of several other reactions are given by the French authors (17). It is fortunately the case, however, that comparatively few of these numerous oxidations give rise to formaldehyde. Broadly speaking, only primary alcohols oxidised in the α-position -- e.g.: glycerol, glucose, ethylene glycol -- yield formaldehyde, although there is at least one interesting exception to this rule (see p.18). Hence, since glucose and certain other reducing substances can be readily removed by the copper-lime treatment used in the lactic acid determination of Friedemann and Graeser (18), and because α-oxidised primary alcohols other than sugars are not common in biological material, it seemed that an accurate formaldehyde determination might be the key to an accurate glycerol determination.

Since 1910 (19) the qualitative detection of minute/

minute amounts of formaldehyde by a highly specific reaction has been a practical possibility. Schryver himself, the originator of the reaction, claimed that the method could be used for quantitative estimations without further modification, but, as will be seen shortly, his claims were a little too ambitious. His technique was to add to 10 ccs. formaldehyde solution 2 ccs. of freshly prepared 1% phenylhydrazine hydrochloride and 1 cc. 5% potassium ferricyanide. This produced a brown colour which turned to a beautiful rose-red on the addition of 5 ccs. concentrated hydrochloric acid. This red colour, it was claimed by Schryver, was suitable for colorimetric purposes. Unfortunately, a turbidity almost invariably appears when the potassium ferricyanide is added, which does not completely dissolve on acidification. This spoils the solution for colorimetric comparisons, as does also the fact that the intensity of the colour produced under these conditions does not appear to be quite constant for a given amount of formaldehyde. While the the formation of the turbidity is prevented by slight acidification at this stage, the acid slows up or checks entirely the formation of the brown compound.

A full investigation of this colour reaction was therefore undertaken, with a view to laying down standard conditions for its employment as a quantitative method. Preliminary experiments yielded a certain amount of interesting information which it seems worth while to record, although it threw no light on/

on the nature of the reaction. For example, if potassium ferricyanide solution be mixed with phenylhydrazine hydrochloride in the proportions recommended by Schryver, the yellow colour of the ferricyanide disappears completely in a few moments, irrespective of the presence of formaldehyde. The colour of iodine is dispelled in the same way, indicating presumably its reduction to iodide. Iodate and periodate (as well as iodine) will both produce the brown colour in the presence of formaldehyde, but they both form coloured oxidation-products from phenylhydrazine itself. Perchlorate, hypochlorite, and 6% hydrogen peroxide are all ineffective as oxidising agents. One of the most interesting observations that was made is that p-bromophenylhydrazine produces very little colour with formaldehyde, ferricyanide, and concentrated hydrochloric acid.

After these preliminary experiments it was decided that no useful purpose would be served by a further search for more suitable reagents, and that modification of the original conditions was more likely to give satisfactory results. A Pulfrich photometer was used for colour determinations, but it must be emphasised that the instrument is not very well suited for experiments carried out occasionally over a long period, since the colour-producing qualities of phenylhydrazine appear to vary somewhat with the age of the specimen. The most suitable filter for use was determined by observing the extinction coefficient of the/

the coloured solution with each filter in turn and selecting the filter which gave the highest extinction coefficient. It will be seen from Table 3 that as the filters pass in colour from violet through blue to green the extinction coefficient rises to its maximum at the green filter S53, falling again until with the red filters it is very low.

TABLE 3.

Filter no.	Colour.	E.
S. 43.	Violet.	0.073.
S. 47.	Blue.	0.177.
S. 50.	Blue-green.	0.332.
S. 53.	Green.	0.354.
S. 57.	Yellow.	0.106.
S. 61.	Red.	0.04.
S. 72.	"	0.05.
S. 75.	"	0.045.

The reagents used in this investigation were B.D.H. "Analar" phenylhydrazine hydrochloride and potassium ferricyanide, and "pure" hydrochloric acid and 40% formalin from Messrs. Griffin and Tatlock. The formalin was standardised by the technique of Brochet and Cambier (20), which gives very satisfactory results on a macro-scale. A measured volume of formalin solution is mixed with a measured volume of a standard solution of hydroxylamine hydrochloride, the hydroxylamine being in excess. The oxime of formaldehyde is immediately/

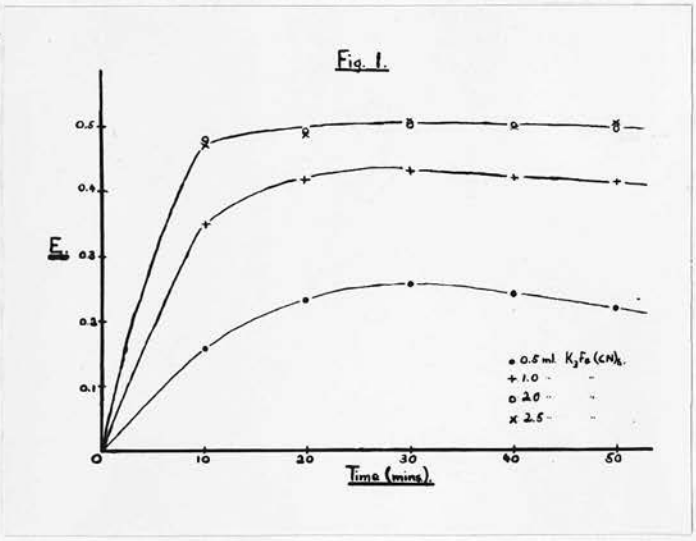
immediately formed, which has no basic properties, so that hydrochlorice acid is liberated and can be titrated with standard sodium hydroxide. Suitable indicators are bromphenol blue and methyl orange, and in order to ensure absolute sharpness of end-point it is wiser to use normal than tenth-normal solutions. Different specimens of formalin were examined repeatedly over a period of many months, and gave results between 38.5 and 40 gm. formaldehyde per 100 ml. solution. Solutions of suitable strength were prepared by dilution.

The points investigated include the following:

1. The concentration of acid necessary to prevent the formation of a precipitate or a cloudiness from the phenylhydrazine and the potassium ferricyanide.
2. The optimum relative concentrations of potassium ferricyanide and phenylhydrazine.
3. The time necessary for full development of the brown colour under the conditions determined above.
4. The concentration of acid necessary to convert the brown colour completely to red.
5. The variation in intensity of the red colour with concentration of formaldehyde.

Preliminary experiments indicated that an excess of potassium ferricyanide is one of the main factors in the production of turbidity, and that the very high concentration of both ferricyanide and phenylhydrazine employed by Schryver is also harmful. Since this discovery it has been customary to use for each determination 2 ml. 0.03 M phenylhydrazine and a suitable/

suitable volume of 0.015 M potassium ferricyanide. Under such conditions no turbidity is produced if the oxidation is carried out in a solution which is 0.02 N with respect to hydrochloric acid. In these experiments, then, 2 ml. phenylhydrazine hydrochloride 0.03 M, made up in 0.2 N hydrochloric acid and filtered, were added to the formaldehyde solution. The mixture was diluted to 18 ml. and the ferricyanide added. After definite periods of time 5 ml. concentrated hydrochloric acid were added and the whole diluted to 25 ml. The extinction coefficient was read, and the values plotted on graphs which are reproduced in Figure 1.



This figure indicates that maximum colour development is attained in 30 - 40 minutes, using 2 - 2.5 ml. 0.015 M potassium ferricyanide. Consequently, in all subsequent experiments 2 ml. 0.015 M potassium ferricyanide were used, and the oxidation was allowed to/

to proceed for thirty minutes.

Figure Two shows the extinction coefficient plotted against the concentration of hydrochloric acid in the final solution. It is readily seen from this that 5 ml. concentrated hydrochloric acid per 25 ml. solution is indeed a very suitable amount.

Fig. 2.

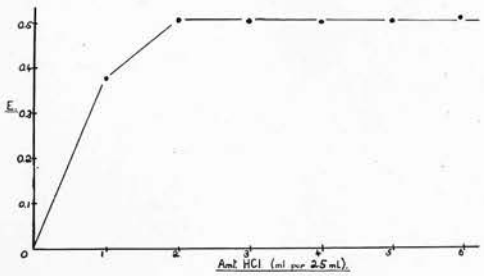
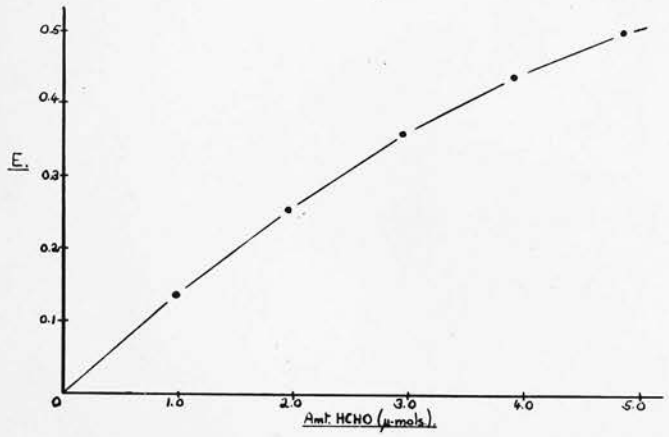


Figure 3 shows that the intensity of the colour is not proportional to the concentration of formaldehyde, and that if it is desired to use a colorimeter for the estimation, then the standard must approximate fairly closely to the unknown in concentration. In order that the "blank" due to the phenylhydrazine might be entirely eliminated, these figures were obtained by performing the oxidation in 20 ml. solution as described before and then diluting to nearly 100 ml., adding a suitable volume of concentrated sulphuric acid (4 ml.) before completing the dilution. Care must be taken that the solution does not get too hot during the addition of the acid, since this leads to a perceptible fading of the colour.

Fig. 3.



On the basis of all these preliminary experiments the following technique is recommended for the estimation of formaldehyde in the absence of interfering substances :-

A volume of formaldehyde solution not exceeding 15 ml., and preferably containing not more than 3 u-mols. (0.10 mg.) formaldehyde, is treated with 2 ml. 0.03 M phenylhydrazine hydrochloride in 0.2 N hydrochloric acid. After making up to 18 ml. the mixture is treated with 2 ml. 0.015 M potassium ferricyanide (neither the phenylhydrazine nor the ferricyanide should be more than a week old, and they must both be kept in dark bottles) and the tube left to stand for half an hour. During this time a colour develops which varies from pale yellow to a deep purplish/

purplish brown, depending on the concentration of formaldehyde present. The solution is diluted in a 50 or 100 ml. volumetric flask and treated with either 2 or 4 ml. concentrated sulphuric acid, or, in order to avoid overheating, 10 or 20 ml. of a 20% v/v solution of sulphuric acid. After diluting to the mark and mixing, the intensity of the colour is compared with that of a suitable standard similarly prepared.

Application of method to pure glycerol solutions.

If a solution containing glycerol is treated with periodic acid, then before it can be estimated the formaldehyde produced must be separated from the other substances in the solution, which interfere with the development of the colour. Under certain special circumstances, the formaldehyde can be distilled off in a special apparatus (see Fig. 4) which consists essentially of a 10 ml. r.b. flask attached to a sawn-off micro-Kjeldahl head and a water-condenser. Cork is inadmissible, and rubber should be reduced to a minimum in the apparatus.

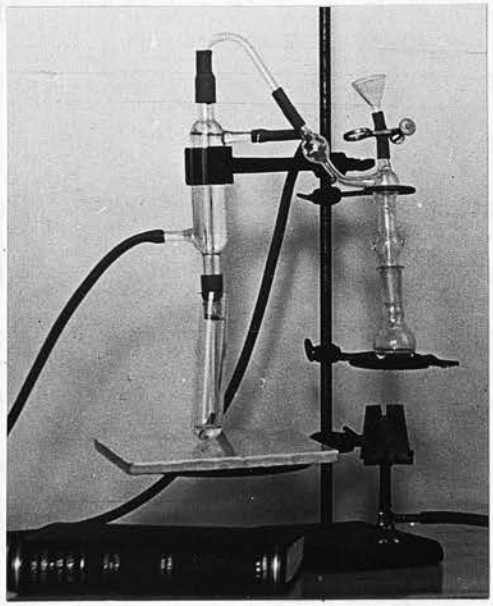


Fig. 4.

Since periodic acid at high temperatures unfortunately destroys formaldehyde, it is necessary before distillation to destroy excess periodate by reduction. Moreover, it is necessary to carry the reduction completely to the stage of iodide. Sodium thiosulphate is a suitable reducing agent. It is further necessary to render the solution alkaline before distillation, otherwise no formaldehyde is recovered. The estimation of glycerol in pure solution can be satisfactorily carried out as follows:-

To 1 - 3 ml. solution containing not less than 0.2 u-mols. (0.02 mg.) glycerol is added 1 ml. 0.05 M sodium periodate in 3 N sulphuric acid (Note: Different manufacturers supply periodates of different formula. That supplied by the British Drug Houses, Ltd., has the formula $Na_5H(IO_5)_2$, and appears to be a product of high purity.). After five minutes the excess of periodate is destroyed by the dropwise addition of 50% sodium thiosulphate. Iodine is first liberated, but after the addition of 3 - 4 drops thiosulphate it disappears again. Its disappearance indicates that sufficient sodium thiosulphate has been added. The contents of the flask are then neutralised with 20% caustic soda, and an excess of 2 - 3 drops added. The formaldehyde can now be distilled off in the apparatus illustrated in Fig. 4. The distillation is carried on until the bottom of the flask is almost dry. 2 - 3 ml. water is then added through the funnel and the evaporation repeated. The receiving tube contains 2 ml./

2 ml. 0.03 M phenylhydrazine hydrochloride, and formaldehyde is determined in the distillate as described on pp. 14 – 15. Some typical results are given in Table 5.

TABLE 5.

Amt. glycerol taken.	Amt. glycerol found.	% recovered.
1.32 u-mols.	1.18 u-mols.	97
1.32	1.26	103
1.33	1.24	101
0.51	0.51	100
0.49	0.50	102
0.332.	0.325	98
0.288	0.288	100
0.242	0.239	99
0.206	0.20	97
0.22	0.233	105.

As can be seen from these results, the method is perfectly satisfactory for pure solutions. What of possible interfering substances ? Glucose is the most striking possibility, but fortunately it can be removed by treatment with copper sulphate and calcium hydroxide, as is done in the lactic acid determination of Friedemann and Graeser (18). A number of other substances have been tested, particularly substances which might not be expected to yield formaldehyde but do occur in blood. Creatinine, creatine, uric acid, lactic acid, urea, and various amino-acids have been been examined in concentrations far in excess of that expected. From none of these has formaldehyde been obtained./

Glycerophosphate has not been tested, since the method was designed for application to lipin-containing extracts, and Stewart and Hendry (12) have shown that that added glycerophosphate is not extracted from blood by the alcohol-ether mixture employed by Bloor (14). Of the simple compounds which are built into the complex phospholipin molecules, both choline and colamine have been examined. Choline is hydrolysed by caustic alkalies to trimethylamine and ethylene glycol. Ethylene glycol is quantitatively oxidised by periodic acid to two molecules of formaldehyde, and it was therefore thought extremely probable that choline would interfere with the accuracy of the method. It does not, however, even after heating for 45 - 60 minutes with alkali, the reason presumably being that ethylene glycol is either lost by evaporation or converted to some inert substance. If such dilute solutions of ethylene glycol alone are heated, two-thirds of the initial quantity disappear within 45 minutes.

β-amino-ethanol has been found to be attacked very slowly in acid and rapidly in alkaline solution by periodate with the formation of two molecules of formaldehyde and one of ammonia, in accordance with the following equation:

$$\begin{array}{l} CH_2OH \\ | \\ CH_2NH_2 \end{array} + 2\ IO_4^- = 2\ IO_3^- + \begin{array}{l} HCHO \\ + \\ HCHO \end{array} + NH_3.$$

Unsuccessful attempts, which are described later, have been made to base upon this reaction a determination of/

of colamine and kephalin, but in the meantime it will be sufficient to observe that if the oxidation of glycerol is not permitted to last longer than 5 - 8 minutes, then the error resulting from the concomitant oxidation of as much as three molecular proportions of colamine is negligible. The oxidation of glycerol itself is complete in three minutes, so that five minutes is ample time to allow.

Application of the method to pure fat solutions.

The hydrolysis of pure fat solutions, prepared from triolein, tripalmitin, or tristearin, always presents a more difficult problem than the hydrolysis of the complex mixtures of fats and lipins derived from natural sources. The saponification of pure tristearin may require many hours for completion, and even the hydrolysis of olive oil (which can for all practical purposes be regarded as 100% triolein) usually takes two or three times as long as the hydrolysis of the lipins in a Bloor extract. Consequently, there is danger that during prolonged heating glycerol may be lost by volatilisation, but as Table 4 will show, this can be avoided.

A solution was prepared by dissolving 0.2024 gm. olive oil in 100 ml. alcohol. This was calculated to contain 0.229 millimols. triolein per 100 ml. solution. 1 ml. portions were heated on the hot-plate with 1 ml. 0.2 N caustic soda under different conditions, and at the end of the hydrolysis the mixture was neutralised and/

and glycerol was determined. The results obtained were used to calculate the concentration of fat in the original solution.

TABLE 4.

Hydrolysis time.	Experimental conditions.	Fat found.	%
30 mins.	Evaporation to 0.3 ml.	0.078 mmols.	33
60 "	Repeated addition of alcohol.	0.209 "	91
60 "	"	0.207 "	90
90 "	Repeated addition of alcohol and water.	0.233 "	102
120 "	"	0.223 "	97.
120 "	Repeated addition of alcohol and water, but flask permitted to go dry once or twice.	0.198 "	86.

This table shows that the hydrolysis of olive oil is complete under the above conditions in an hour and a half, and that there is no appreciable loss of glycerol unless some part of the bottom of the flask is allowed to go dry. Similar experiments with a solution of tristearin yielded very poor results, and fatty acid determinations indicated that four to five hours might be necessary for the completion of the hydrolysis. It would indeed be surprising if it were possible to maintain a solution of glycerol in an open vessel at nearly 100 deg. C. without at least some slight loss.

Application of the method to blood lipins.

It is evident from the foregoing pages that a method is now available for the estimation of glycerol either/

either in pure solution or in fats, and that substances likely to interfere can be removed or their interference avoided. Attempts were made to determine glycerol in blood lipin hydrolysates by the following procedure:

10 ml. Bloor extract, equivalent to 0.4 ml. blood, were pipetted into a 25 ml. Pyrex conical flask. After the addition of 0.2 ml. 5 N caustic soda the mixture was evaporated down to about 1 ml., the rate of heating being so adjusted that the evaporation took about 45 minutes. The residual fluid was transferred with a capillary pipette and teat to a narrow centrifuge tube graduated at 3.0 ml. The flask and pipette were rinsed several times with small amounts of hot water, and the contents of the tube, after the addition of 50 mg. solid calcium hydroxide and 0.3 ml. 10% copper sulphate, were made up to 3.0 ml. The tube was allowed to stand for an hour during which it was repeatedly stirred. It was then centrifuged, and an aliquot of the supernatant fluid (usually 2.5 ml.) was used for the glycerol estimation. As a rule two standards, containing respectively 0.4 and 0.7 u-mols. glycerol, were used. Some typical results, including a few recoveries of added glycerol, are shown in Table 5.

TABLE 5.

Sample.	Added.	Glycerol Found.	Recovered.	Percentage.
1.	-	0.088, 0.090	-	-
2.	-	0.127, 0.134	-	-
3.	-	0.076, 0.0735	-	-
4.	-	0.152, 0.147	-	-
5.	-	0.176, 0.176	-	-
1.	0.1025	0.197	0.108	105
2.	0.1025	0.221	0.096	94
3.	0.1025	0.224	0.099	97.

Note: all values expressed as millimols./100 ml. blood.

THE DETERMINATION OF CHOLINE.

Modern methods for the chemical determination of choline all depend on one or other, if not both, of two principles: precipitation with ammonium reineckate or reinecke acid and precipitation as the enneaiodide with an approximately normal solution of iodine in potassium iodide. Neither of these reactions is itself of recent date. The iodine precipitation seems to have been discovered by Stanek in 1906 (21), and the most recent methods embodying it all borrow extensively from the technique described in 1930 by Roman (22). This worker precipitated choline periodide in a centrifuge tube from solutions containing as little as 0.01 mg. choline per ml. The precipitate was washed with ice-cold water, dissolved in chloroform, and titrated with 0.002 N or 0.01 N sodium thiosulphate. The procedure is capable of yielding excellent results, but the washing of the precipitate requires very great care, and it is by no means easy to titrate an exceedingly dilute solution of iodine in chloroform with an equally dilute aqueous thiosulphate solution. Moreover, the method has the disadvantage that the reagent precipitates other bases, notably purines, as well as choline. (23). It has, nevertheless, been used for the determination of "lecithin" in blood (13).

The ammonium chromammine thiocyanate -- $NH_4(Cr(NH_3)_2(CNS)_4).H_2O$ -- which now bears his name was discovered by Reinecke in 1863 (24), but the discovery/

discovery that it could be used to precipitate such quaternary bases as choline and neurine and acetyl choline does not appear to have been made until 1930 by Kapfhammer and Bischoff (25). Since that time the salt has been used for the qualitative or semi-quantitative separation of choline and acetyl choline from blood and tissue extracts (25), and in 1936 Beattie (23) published a quantitative micro-method depending on the pink colour produced when the reineckates of choline or acetyl choline are dissolved in acetone. The intensity of this colour is, in fact, the limiting factor in the method of Beattie, which cannot be used, even with a micro-colorimeter, for the determination of less than 0.3 mg. choline, although the low solubility of choline reineckate permits its quantitative separation from solutions containing as little as 0.03 mg. per ml.

The reineckate precipitation is somewhat less delicate than that with iodine, but it has been chosen for the present work largely because it is more clean-working; the precipitate is crystalline and can be easily filtered, even through cotton-wool. It has been necessary, however, to exchange the colorimetric procedure of Beattie for a more sensitive one, in order that it might be possible to apply the method to reasonably small amounts of blood-lipin hydrolysates. Experiments in two directions have met with some success: if the precipitate is heated with caustic soda and bromine water the chromium is converted to chromate, which will then oxidise diphenylcarbazide (0.2% in 1:9 acetic-acid-alcohol)/

acetic-acid-alcohol) to an intensely purple-red compound (26). This is an exceedingly delicate reaction for chromate, but it had unfortunately to be abandoned because of occasional departures from the theoretical result even after the optimum conditions had been carefully established and strictly observed. A more successful method of estimating the reineckate precipitate has been the simpler one which consists in evaporating the acetone solution, heating the residue with Folin's digestion mixture(27), and determining the ammonia formed by steam-distillation and titration. Since each molecule of choline reineckate yields seven molecules of ammonia, each ml. 0.007 N caustic soda is equivalent to 1 u-mol. choline.

The following procedure has been found satisfactory for pure choline solutions:

Saturated ammonium reineckate solution is prepared fresh by shaking the solid with water for a few minutes and filtering. It may be necessary to repeat the filtration, using the same filter paper. 1 ml. of this solution is added to 1 - 2 ml. choline solution, which may be neutral or very slightly acid, but should not be alkaline (alkali hastens the decomposition of the reineckate). There is an immediate separation of fine pink crystals, and the tube is left in the refrigerator for 20 miutes. The mixture is then filtered. For the filtration it is possible to use a Jena G4 filter-stick, but if the original solution has contained more than 0.15 - 0.20 mg. choline filtration

by/

by this method is apt to become tedious, and it has been found preferable to filter through a tiny plug of cotton wool rolled between the fingers and pressed gently into the bottom of a small filter funnel. Paper is exceedingly difficult to wash free from excess ammonium reineckate. Washing is done with cold water and ether (not with alcohol, as recommended by Beattie, since choline reineckate seems to be appreciably soluble especially in certain mixtures of alcohol and water). The pink residue in the filter is now treated with a few drops of acetone, in which it readily dissolves, and the acetone is rinsed into a hard glass test-tube. A drop of dilute acid is added in case any ammonia should be liberated, and the acetone is removed by evaporation. 0.5 ml. digestion mixture is added, and the tube carefully heated until digestion is complete. The ammonia formed is steam-distilled from alkaline solution into 5 ml. 0.007 N sulphuric acid (made up with indicator according to Conway (29)), and the excess of acid is titrated with 0.007 N alkali.

Some typical results are given in Table 6.

TABLE 6.

Choline taken.	Choline found.	Percentage.
0.48 u-mols.	0.515 u-mols.	105%
0.49	0.504	103
0.98	0.93	95
0.98	1.00	102
0.98	1.00	102
1.40	1.35	96
1.40	1.33	95
2.21	2.11	95
2.21	2.10	95
2.21	2.15	97.

Application of the method to blood lipins.

That choline can be liberated from phospholipins by hydrolysis with either acid or baryta has been known for many years (28), but no worker appears hitherto to have taken the trouble to ascertain the stability of choline towards his reagents. The first applications of the method were therefore directed to this end, and the results in Table 7 show clearly that neither 2 N hydrochloric acid nor saturated baryta (approximately 0.2 N) has the slightest effect on choline at 90 - 100 deg. C.

The treatment with acid was carried out in a 10 ml. Pyrex flask under reflux on a sand-bath. Bumping was prevented by silver sand and a layer of benzene, which is an efficient anti-bumping agent in conjunction with sand or beads, and does not lower the temperature more than 5 - 10 deg. C. The treatment with baryta was done in open conical flasks on a steam-bath, water being added when necessary. At the end of the experiment the mixture was neutralised and the barium sulphate filtered off and washed. Choline was determined in the filtrate.

TABLE 7.

Note: 1.13 u-mols. choline used for each experiment.

Reagent.	Time heated.	Choline found.	Percentage.
2 ml. 2N HCl	3 hrs.	1.18 u-mols.	105%
"	7	1.07	96
"	11	1.09	97
2 ml. 0.2N $Ba(OH)_2$.	3	1.09	97
"	4	1.08	96
"	6	1.11	99.

For the investigation of the hydrolysis of the phospholipins in blood, a litre of alcohol-ether extract was prepared according to the method of Bloor as described by Stewart and Hendry (12). 20 ml. portions were evaporated just to dryness on a steam-bath or hot plate. "Blank" estimations were done by adding to the residue 1 ml. cold water, filtering, washing, and precipitating choline as described on p. 24. Hydrolyses with acid and baryta were carried out as on p. 26, and choline was determined in the hydrolysed mixture. The results are summarised in Table 8, from which it will be seen that baryta is a very much more efficient hydrolysing agent than hydrochloric acid. It has been assumed that the hydrolysis with baryta is complete, since such varying conditions have given such similar results.

TABLE 8.

Reagent.	Hydrolysis time.	Choline/100 ml. blood.
-	-	4 u-mols.
-	-	3
1 ml. 2N HCl.	2 hrs.	83
"	5	104
"	17	105
3 ml. 2N HCl.	3	126
"	22	148
1 ml. 5N HCl.	3	133
"	21	152
"	22	156
2 ml. 0.2N $Ba(OH)_2$.	2	156
3 ml. "	3	155
4 ml. "	4	158
5 ml. "	5	158.

On the basis of these preliminary experiments the following procedure has been devised for the determination of "lipoid choline" (i.e., choline liberated by baryta hydrolysis from an alcohol-ether extract.): 15 - 20 ml. alcohol-ether extract are evaporated to dryness in a conical flask. 2 ml. saturated aqueous barium hydroxide are added, and the mixture is heated on a steam-bath. At the end of one hour 1 ml. baryta is again added (as a precautionary measure, since barium carbonate is very rapidly formed) and the heating continued for a second hour. The mixture is then neutralised to methyl orange with approximately normal sulphuric acid, and evaporated down to about 0.5 ml. The fluid is transferred to a small filter with a dropping pipette which is used to rinse the flask with successive small portions of hot water. Choline is determined in the filtrate as previously described. That duplicate analyses agree well is shown by Table 9, while Table 10 shows that the recovery of added choline is quantitative:

TABLE 9.

Sample.	Choline found (millimols./100 ml.).
1	0.356, 0.357
2	0.317, 0.333
3	0.367, 0.362
4	0.217, 0.217
5	0.368, 0.375
6	0.330, 0.336
7	0.373, 0.364.

TABLE 10.

Choline added.	Choline found.	Choline recovered.	%.
-	0.316	-	-
-	0.318	-	-
0.215	0.437	0.220	103
0.215	0.439	0.213	99.

Note: values expressed as millimols./100 ml.

It remains to discuss the specificity of the method. Ammonium reineckate seems to be a highly specific precipitant for quaternary bases. It has been used for the isolation of choline, acetyl choline, neurine, carnitine, and sphingomyelin. Lecithin does not appear to form an insoluble reineckate, a point of some interest when comparing it with its companion phospholipin sphingomyelin. Creatinine is not precipitated by reineckate until the quite unphysiologically high concentration of 5 mg. per ml. is reached.

While blood might contain unknown reineckate-precipitable bases, "blank" estimations can be done, and it is difficult to imagine any compounds other than the choline-containing phospholipins (and, of course, acetyl choline, which is never abundant) from which reineckate precipitable base would be liberated by hydrolysis with baryta.

A specimen of choline reineckate was prepared by the method outlined above from 600 ml. alcohol-ether extract, and, analysed for nitrogen by the micro-Kjeldahl technique, gave a nitrogen-content 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. Old facts and new findings, however, do suggest that no great risk attaches to the assumption that the crystals isolated in the course of these estimations are actually pure choline reineckate.

THE ANALYSIS OF BLOOD PHOSPHOLIPINS.

The methods described in the preceding pages have been applied to the analysis of specimens of mixed phospholipins prepared from blood in the following way:

50 ml. alcohol-ether extract (12) were evaporated just to dryness on a hot plate and finally in the steam oven. The residue was immediately taken up in petroleum ether (b.p. 40 - 60 deg. C.) and the turbid solution was transferred to a centrifuge tube where it was concentrated to about 0.3 ml. by immersion in a beaker of warm water. 5 ml. acetone were then added from a pipette, very slowly and with continuous shaking, so that the phospholipins separated as a flocculent white to pale buff precipitate and not as a tarry mess. Precipitation was completed by adding three drops of a saturated alcoholic solution of magnesium chloride and allowing the tube to stand for two hours, preferably in the refrigerator. At the end of that time any precipitate adhering to the walls of the centrifuge tube was 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 three parts of ether, two of alcohol, and one of water, in which it gave a perfectly clear, very pale yellow solution. The entire procedure can be carried out in about/

about three hours, of which the phospholipins spend two in the refrigerator. This should minimise the oxidation by atmospheric oxygen to which these compounds are so very susceptible. A preliminary series of analyses by the lipoid phosphorus method of Stewart and Hendry (12) showed that the procedure extracted 92 -97% of the phosphorus in the original alcohol-ether extract. These figures show that not only is the phosphorus of the alcohol-ether extract virtually all phospholipin phosphorus, but the extraction and precipitation procedure with acetone and magnesium chloride is also satisfactory and complete.

2 ml. portions of the ether-alcohol-water solution were analysed for glycerol and choline by the methods described in the foregoing pages, and 1 ml. portions for phosphorus by the method of Stewart and Hendry (12). The results of twelve analyses are given in Table 11. The blood specimens were taken from the superficial arm veins of apparently healthy young men (the majority were veterinary students), and potassium oxalate was used as an anti-coagulant. In all cases the alcohol-ether extract was prepared within an hour or so of the withdrawal of the blood. As a general rule the subjects were not in a truly post-absorptive state (this in any case is very difficult to ensure where fats are concerned) so that it would be unwise to regard the results as much more than a simple exercise in technique. Values are expressed as millimols. per 100 ml. blood, except in the case of phosphorus, where the unit is the milliatom.

TABLE 11.

Sample.	Phosphorus.	Choline.	Glycerol.
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	0.134
5	0.340	0.255	0.119
6	0.366	0.271	0.138
7	0.316	0.256	0.111
8	0.319	0.237	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.

Reference to Table 1, on page 4, will show that from these values it is possible to calculate the concentrations of lecithin, kephalin, and sphingomyelin, provided that these be the only phospholipins or choline- or glycerol-containing lipins in the final extract:

If L, K, and S be the concentrations of lecithin, kephalin, and sphingomyelin, and p, c, and g the experimentally determined concentrations of phosphorus, choline, and glycerol, then

$$L + K + S = p \quad . \quad . \quad . \quad (1)$$

$$L + S = c \quad . \quad . \quad . \quad (2)$$

$$L + K = g \quad . \quad . \quad . \quad (3)$$

From/

From (1) and (2), $K = p - c$ . . . (4)

From (1) and (3), $S = p - g$ . . . (5)

From (2) and (5), $L = c - p + g$ . . . (6).

The application of these equations to the results in Table 11 is shown in Table 12, all values being expressed, of course, as millimols. per 100 ml. blood.

TABLE 12.

Sample.	Lecithin.	Kephalin.	Sphingomyelin.
1	0.028	0.148	0.250
2	0.110	0.039	0.184
3	0.054	0.055	0.248
4	0.071	0.053	0.212
5	0.034	0.085	0.211
6	0.043	0.035	0.238
7	0.052	0.059	0.204
8	0.035	0.093	0.192
9	0.031	0.077	0.244
10	0.000	0.104	0.245
11	0.017	0.088	0.244
12	0.025	0.078	0.267.

Examination of this table brings to light several interesting facts. The first of these is that while the phospholipins of blood have often been loosely described as "lecithin", lecithin itself accounts for only a small proportion of the total. In one case, in fact (no. 10), no lecithin at all was found. It seems unlikely, moreover, that twelve consecutive blood specimens would show this same phenomenon by chance. In the/

the second place, sphingomyelin is quantitatively by far the most important of the phospholipins present, and thirdly, in nearly all cases the concentration of kephalin has been considerably greater than that of lecithin. These things are more clearly seen in Table 13, in which the concentrations of the phospholipins have been expressed as percentages of the total.

TABLE 13.

Sample.	Lecithin.	Kephalin.	Sphingomyelin.
1	0%	35%	59%
2	33	11	56
3	15.5	15.5	69
4	23	14	63
5	13	25	62
6	11	26	63
7	16	19	65
8	14	26	60
9	9	22	69
10	0	30	70
11	5	25	70
12	9.5	20.5	70
Mean.	13%	22%	64%

Since the series is so short, it has not been thought that any statistical analysis of these results would have any significance, but it is quite evident from even these few cases that the proportion of sphingomyelin is fairly constant. It seems possible that this might be emphasised by a series of analyses on plasma and corpuscles separately from subjects known to be in a truly post-absorptive state.

As for a comparison with the results of former workers, little can be said. There is no basis at all for comparison with the results of Kirk (13), which in any case can have but little significance, in spite of the length of his series. He speaks of a "total phosphatide" concentration which may be as low as 26 mg. per 100 ml. corpuscles. This corresponds to a phosphorus concentration of something like 1 mg. per 100 ml., and even his average "total phosphatide" concentration works out at something like 8 mg. P per 100 ml., which is 50 - 70% lower than the usual values, obtained repeatedly by many workers using many different methods in many different laboratories. Another aspect of Kirk's figures which throws suspicion on them is the fact that there is little difference between the plasma and corpuscles figures, whereas it is generally recognised that corpuscles contain a good deal more phospholipims than plasma.

The kephalin concentrations published by Williams(11) are rather higher than those found in this work, possibly because his choline determinations may be slightly low. He carried out the reineckate precipitation in a solution in which one ml. saturated baryta had been neutralised with 0.6 ml. N hydrochloric acid. 1 ml. saturated baryta contains about 0.2 milli-equivalents of alkali, so that the final solution contains an excess of about 0.4 milliequivalents of acid per ml. It has been found in this work that such high concentrations of acid interfere somewhat with the precipitation/

precipitation of choline by reineckate.

Schmitz and Kock (10) determined "kephalin" in the blood of guinea-pigs. Their method, which is known to give markedly high results, consisted in hydrolysing the lipin solution with 5% sulphuric acid at 140 deg. C. for six hours and determining amino-nitrogen in the residual fluid. They found kephalin to be normally 30 - 50% of the phospholipins of the blood of guinea-pigs. Owing to the inaccuracy of the method employed, it is really impossible to compare these results with those obtained in the present work.

The sphingomyelin isolations of Thannhauser (6), being large-scale semi-quantitative preparations, cannot be compared with these results either. They show much more variation, and sphingomyelin rarely amounted to as much as half of the total, whereas reference to Table 13 shows that this phospholipin has never, in the present series of twelve cases, formed less than 56% of the total.

The phosphorus-fatty-acid analyses of Stewart and Hendry (12) 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 fortuitous. On the other hand, Stewart and Hendry based their conclusion on the ratio of phosphorus atoms to fatty acid molecules in the phospholipin precipitate, and a very slight oxidation of the fatty acids prior to estimation/

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% sphingomyelin the same ratio 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 results of Stewart and Hendry agree with these reported just now, 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 during the preparation and storage for a few hours of the phospholipin solution may be very much greater than this. These ideas are supported by the work of Paal (29), who studied the oxidation of alcoholic solutions of lecithin, and found the number of free carboxyl groups to increase very rapidly.

A few months ago Thannhauser and some colleagues (30) published some analytical figures for the content of lecithin, kephalin, and sphingomyelin not only in serum, but also in many other tissues such as brain, heart, liver, kidney, spleen, etc. The most interesting thing about these results is that they also show a remarkable constancy as regards concentration of sphingomyelin, although it must be admitted that as a rule the serum concentrations of that phospholipin are rather lower than might be expected from the whole blood values reported here.

It cannot be too strongly stressed that this is in no way regarded as a complete piece of work; rather is it considered to be only the prelude to researches on lipin metabolism which, if conditions were to permit, might occupy many years and might give interesting and important results. The methods, which are the most "micro" ever published in this field (with the possible exception of the inaccurate methods of Kirk) must be applied not only to the separate analysis of blood cells and plasma, but also to the analysis of the lipins of other tissues in both normal and pathological states. Efforts must be made to answer decisively such questions as whether or not the phospholipins of blood do increase after the ingestion of fat, and if so, which fractions are involved; and, in general, whether in any alteration of the total phospholipin concentration in a tissue, any one phospholipin is more affected than the others. No one has any real idea of whether any of the phospholipins has a greater metabolic significance than the others, and it is surely high time that some light was thrown on this problem. For its complete solution some such analytical scheme as that described here is surely a sine qua non. A direct micro-determination of kephalin would be of such value that the partially successful experiments to that end are recorded in the following pages, in case they should prove of some future value.

SOME STUDIES IN THE CHEMISTRY OF COLAMINE.

Few who have contemplated the commonplace structure of the colamine molecule can ever have hoped for a simple and accurate micro-method for its determination. It is a strongly basic liquid, somewhat hygroscopic, all of its salts are at least fairly soluble, and its characteristic groups, the primary alcohol and the primary amine, are of such widespread occurence throughout the world of organic chemistry that they hold out not the faintest hope of a specific determination. Consequently, the discovery of the reaction described on p.18 was thought to be too good an opportunity to be allowed to slip, and efforts were made to build a quantitative estimation upon it.

The reaction, it will be recalled, consists in the oxidation of β-amino-ethanol in aqueous solution with an alkaline periodate solution (the sodium salt is somewhat less insoluble in alkaline solution than its potassium analogue.). In this oxidation two molecules of formaldehyde are produced and one of ammonia is, as it were, left behind:

$$\begin{matrix} CH_2NH_2 \\ | \\ CH_2OH \end{matrix} + IO_4^- = IO_3^- + \begin{matrix} HCHO \\ + \\ HCHO \end{matrix} + NH_3.$$

That the reaction takes exactly this course has been shown decisively in the following ways:
(1.) The disappearance of periodate has been measured by the arsenite-iodine method of Fleury and Fatome (15).
(2.) The formation of formaldehyde has been measured by the/

the method described earlier in the present work, after rendering the solution acid and destroying the excess periodate and iodate.

(3.) The ammonia formed has been estimated by a number of methods, including distillation and Nesslerisation, aeration and titration, and the diffusion method of Conway (31). For the minute amounts of ammonia which it was hoped to estimate, the method of Conway has been found remarkably accurate. The distillation or aeration of 0. 3 u-mols. ammonia (about 4 gamma) is a process unfortunately subject to occasional error, and in any case the apparatus is so large, ungainly, and complex in structure when compared with a Conway unit that its "blank" is appreciable, whereas in the Conway estimations the apparatus itself appears to have no "blank", and the reagent "blank" (due mostly to the B.D.H. sodium periodate) amounts to only about 0.004 ml. 0.006 N alkali, or 0.024 u-mols. ammonia, per estimation.

These estimations have been carried out as follows: into the outer chamber of the Conway unit is pipetted not more than 2.5 ml. of an alcoholic solution, or not more than 1 ml. of an aqueous solution containing not more than about 1 u-mol. (61 gamma) colamine. The solution is made alkaline with a drop or two of strong caustic soda and incubated at 37 - 40 deg.C. for an hour to remove any preformed ammonia. It is advisable to run a greased finger round the ridge between the chambers of the unit, as this checks any tendency for traces of the alkaline solution to creep into the inner chamber.

During/

During this preliminary incubation the aqueous solution decreases perceptibly in volume, and the alcoholic solution is evaporated almost, if not quite completely, to dryness. After the unit has been removed from the incubator, 1 ml. 0.001 N H_2SO_4, with Tashiro's indicator incorporated (made up according to the instructions of Conway - loc.cit.) is pipetted into the inner chamber, and to the outer chamber is added 0.5 ml. 0.05 M sodium periodate, dissolved by suspension in the calculated volume of water and dropwise addition of the minimum amount of concentrated sulphuric acid. Immediately after the further addition to the outer chamber of 1 ml. saturated aqueous potassium carbonate, the unit is sealed and replaced in the incubator. After a minimum of 1½ hours the acid in the inner chamber is titrated with 0.005 N NaOH from a suitable burette. In the present work a 0.2 ml. Rehberg burette has been used. By varying the concentrations of acid and alkali any desired range of concentrations of colamine can be covered. A few typical results are given in Table 14.

TABLE 14.

Colamine taken (u-mols.).	Colamine found.	Percentage.
0.333	0.333	100%
0.333	0.330	99
0.466	0.447	96
0.466	0.452	97
0.700	0.661	94
0.700	0.696	99
0.740	0.741	100
0.740	0.722	98
0.965	0.917	95
0.965	0.950	98
1.930	1.930	100
1.930	1.950	101.

Attempts were made to apply the method without further modification to blood lipin hydrolysates, since none of the commoner bases found in blood could be shown to yield appreciable amounts of ammonia under the prescribed experimental conditions. 10 ml. portions of Bloor extract were hydrolysed by slow evaporation with 0.2 ml. 5 N caustic soda, such treatment having been previously shown not to affect pure solutions of colamine. The residue was transferred by rinsing with alcohol to a Conway unit, and the estimation of colamine carried out precisely as in the preceding description. Duplicate analyses were on the whole found to agree excellently, although in about 5% of some hundreds of estimations a difference of 20 – 30% between duplicates would suddenly appear. Unfortunately, the recovery of β-amino-ethanol added to the extract was not quantitative, although it was fairly regular. This is shown in Table 15, where all values are expressed as millimols./100 ml. blood.

TABLE 15.

Experiment.	A.E.A. added.	A.E.A. found.	A.E.A. recovered.	%.
Blood hydrolysate.	-	0.086	-	-
"	-	0.088	-	-
A.E.A. added before hydrolysis.	0.053	0.126	0.039	74
"	0.053	0.123	0.036	68
"	0.053	0.127	0.040	75
"	0.053	0.122	0.035	67.

Note: all values expressed as millimols./100 ml. blood.

Two possible explanations suggested themselves: firstly, that the oxidation might be for some reason or other not complete, and secondly, that the diffusion of ammonia might be inhibited. That ammonia/

ammonia is free to diffuse was shown by the addition of ammonium sulphate to the hydrolysate prior to oxidation. Its recovery was quantitative (see Table 16.).

TABLE 16.

Ammonia added.	Ammonia recovered.	Percentage.
0.69u-mols.	0.67 u-mols.	97%
0.69	0.66	96
0.69	0.675	97
0.69	0.655	95.

Consideration of the alternative explanation of the poor recovery of amino-ethanol added to blood extracts led to the idea that the difficulty might be overcome by a purification of the phospholipins prior to hydrolysis and oxidation. Phospholipins were precipitated from 10 ml. Bloor extract by the method described on p.30, and the precipitate while still in the centrifuge tube was hydrolysed by heating with aqueous-alcoholic caustic soda. In some cases known amounts of colamine were added to the centrifuge tube before the hydrolysis. β-amino-ethanol was determined in the hydrolysate by the method already described. The figures in Table 17 illustrate the finding that no improvement in recovery was effected by this refinement of technique.

TABLE 17.

A.E.A. added.	A.E.A. found.	A.E.A. recovered.	Percentage.
-	0.328 u-mols.	-	-
-	0.334	-	-
0.24 u-mols.	0.506	0.180 u-mols.	75
0.36	0.573	0.247	69
0.36	0.591	0.255	71
0.48	0.806	0.336	75
0.48	0.683	0.356	74

It then seemed possible that some interfering substance might be carried down with the phospholipins, and that better results might be obtained by separating the amino-ethanol from the other products of hydrolysis of the lipin mixture. With this end in view, attempts were made to extract colamine from aqueous solution with other solvents. The apparatus used was a continuous extraction apparatus of the design employed by Griffith (32) for the extraction of hippuric acid from urine, and was made from a 6" 5/8" Pyrex tube and an 8" 1¼" boiling tube of the same glass. In two experiments, in which 5 ml. water containing 0.925 u-mols. colamine and a few drops of 0.1 N NaOH were extracted with ether for one hour, the recoveries were only 16.5% and 18%, so ether as a solvent was abandoned. It was then found by chance that a half-saturated solution of potassium carbonate was almost immiscible with either ethyl alcohol or isopropyl alcohol, so experiments were carried out with these solvents and a solution of colamine/

colamine to which an equal volume of saturated potassium carbonate solution had been added. The results of these experiments are given in Table 18. In each experiment the amount of colamine used was 0.925 u-mols.

TABLE 18.

Extraction time.	Recovery of colamine with Ethyl alcohol.	Isopropyl alcohol.
40 mins.	80%	-
60	95	82%
90	99	92
120	-	95.

Hence, either ethyl or isopropyl alcohol can be used to extract colamine from potassium carbonate solution, but ethyl alcohol appears to to be a more suitable solvent, since isopropyl alcohol undergoes a variety of chemical changes under the influence of the mild alkali. The application of this technique to Bloor extract hydrolysates was unsuccessful. Two recoveries, for example, of added colamine were only 43% and 56%.

As a last resort attempts were made to recover amino-ethanol by adsorption from either aqueous or alcoholic solution. The most suitable adsorbent was found to be Frankonit KL, an adsorbent employed for the adsorption of bases from acid solution by Fuchs (33). Frankonit KL is a silicate preparation which gives a markedly acid reaction to water in which it has been suspended./

suspended. Fuchs used it with great ease and success for the adsorption of amino-acids from normal sulphuric acid, but any acid at all appears to inhibit the adsorption of colamine. The adsorption from solutions of varying degrees of alkalinity was tested in the following way: 2 ml. portions of a solution of colamine containing 2.7 u-mols./ml. were treated with different volumes of standard alkali and water was added to make a total of 5 ml. About 0.5 gm. Frankonit KL was added and the mixture either filtered or centrifuged. 1 ml. aliquots of the clear fluid were used for the estimation of β-amino-ethanol. The results are given in Table 19.

TABLE 19.

Normality of NaOH.	A.E.A. found in s.n.f.	Percentage.
0.12 N	0.460 u-mols.	44%
0.06	0.033	3
0.04	0.076	7
0.03	0.043	4
0.02	0.047	4.5
0.01	0.038	4.

It should be pointed out that the addition of Frankonit KL makes the solutions a good deal less alkaline, and in some cases frankly acid, than they would seem from the figures in the first column of the table.

Attempts to elute the amino-ethanol with acid, more concentrated alkali, alcohol, pyridine, and other solvents were all completely unsuccessful. In fact, alcohol/

alcohol was found to be quite as suitable a solvent as water from which to adsorb the base. As Table 20 shows, however, it was found quite possible to recover adsorbed colamine by rinsing the Frankonit KL into a Conway unit, evaporating off the alcohol and ether used for the rinsing, and oxidising the amino-ethanol in situ on the adsorbent.

TABLE 20.

A.E.A. taken.	A.E.A. found.	Percentage.
0.000 m-mols.	0.000 m-mols.	100%
0.000	0.000	100
0.000	0.000	98
0.000	0.000	97
0.000	0.000	98
0.000	0.000	98
0.000	0.000	97
0.000	0.000	98
0.000	0.000	97.

Unfortunately, even this method proved inapplicable to the amino-ethanol of a hydrolysed Bloor extract. Recoveries were better than those using the extraction technique, but were far from quantitative and showed no improvement at all over the simpler direct method. Some results are given in Table 21.

TABLE 21.

A.E.A. added.	A.E.A. recovered.	Percentage.
0.000 m-mols.	0.000 m-mols.	93%
0.000	0.000	95
0.000	0.000	74
0.000	0.000	66
0.000	0.000	66
0.000	0.000	72.

No explanation at all can be offered at present for the persistent failure to recover colamine added to a Bloor extract. It should be added that recoveries of amino-ethanol added after the adsorption were absolutely quantitative, so that it cannot be argued that some interfering substance is being adsorbed.

The experiments have been described because it seems advisable to place them on record, in case the future may bring to light some fact which may enable advantage to be taken of this undeniably neat reaction.

SUMMARY.

Quantitative micro-chemical analyses are described for the determination of glycerol and choline in biological material, particularly in lipin mixtures. The methods have been applied, with a phosphorus determination, to specimens of phospholipins prepared from human blood. Attempts to evolve a direct kephalin determination are also described.

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