STUDIES IN MICRO-ANALYSES.

I. The Lipoids of Blood.

II. Arsenic in Keratin Tissues.

by

E. B. HENDRY, B.Sc.(Edin.)

Thesis submitted for the Degree of Doctor of Philosophy in the Faculty of Medicine, University of Edinburgh.

November 1934.

CONTENTS.

EXPERIMENTAL: Part I.

INTRODUCTION

A study of the literature on the subject of blood lipoids reveals a considerable number of methods which have been employed from time to time in determining the various fractions. Equally conspicuous is the number of different types of results. When this research work was started, the general scheme was to be an attempt to elucidate the problems of fat absorption in normal and pathological cases. It was decided, however, since there were so many differences in method and technique employed by workers in this field, that a more valuable contribution to the literature would be made, if these methods were thoroughly examined before dealing with their applications. Consequently the bulk of the experimental work so far has been devoted to methods.

For the estimation of total fat and fatty acids in blood, the more important methods are those/

those of Bloor (1) who in 1928 adapted the chromate oxidation technique to the analyses for total fatty acids. Stoddard and Drury (2) used a method involving the isolation of the free fatty acids and subsequent titration in alcoholic solution with standard sodium hydroxide. Their method is very tedious, but about the same time, a method depending upon similar principles was evolved by Stewart, Gaddie and Dunlop (3) which proved to be more practicable. Variations of the Stoddard and Drury method have been proposed by Man and Gildea (4) and by Smith and Kik (5). Older methods which have been used and finally discarded include the method of Bloor (6) wherein the fatty acids are determined nephelometrically; and although it has long since been replaced, it is worthy of mention in that it was the pioneer attempt to solve problems of fat metabolism. A titration method by Stewart and White (7) was published in 1925, and although it was later proved to be of no value, it was probably the forerunner of Stoddard and Drury's method. In addition to these seven, gravimetric methods have been used for the micro-estimation of total fat; and also there is the haemolipokrit method of Collins (8). Neither this, nor the gravimetric methods are of/

of much account, but nevertheless, the results obtained by their use, complicate the literature on the subject. Lastly there is the gasometric method employed by Backlin (9) which, however, has not been used to any extent by other workers.

With such a number of methods employed, it is to be expected that results are not uniform. With all of them the main difficulty lies in the instability of the lipoid. Fatty acids, unless they be saturated, must be handled so that any tendency towards oxidation is reduced to a minimum. Phospholipoids are so unstable that it is only recently that Levene and his co-workers have been able to isolate them in a pure state and even under optimal conditions they decompose rapidly. Soaps and glycerides are affected in the same manner as fatty acids, but cholesterol and cholesteryl esters are comparatively stable. All the lipoids,however, are decomposed at temperatures slightly over 100°C. and it is for those reasons that their isolation and analysis are so difficult. All methods employed in the determination of blood lipoids are both long and tedious, and require a considerable amount of experience before even duplicate results can/

can be obtained. This is probably the reason why there are so few workers in the field of animal fat metabolism, and this branch of biochemistry lags far behind the study of either sugars or proteins. Peters and van Slyke have described our knowledge of the factors which determine the level of the various lipoid constituents of the blood in disease as being "pitifully meagre". Our knowledge even of these levels might be described by the same phrase, and a great part of the superficial disinterest in the subject is to be found in the reasons given above.

The only general statement on the subject of fat transport by the blood stream is to be found in Bloor's original hypothesis (10, 11) concerning the transport in the form of phospholipoids by red blood cells. This theory has been condemned more frequently than it has been supported, but final proof in either direction is still lacking. Phospholipoid analyses are the most troublesome for the molecules are highly unstable. Such a property is unfortunate, as they undoubtedly play a most important part in the animal body and it is around them that most of the controversy takes place. They /

They are usually estimated through the medium of their phosphorus content and the study of this part of micro-analysis is unique in that there are probably more methods for estimation of phosphorus than for any other element or compound with the possible exception of glucose. Each has been supported by its originator but only a few have found any popularity. Bang has stated that phosphoric acid in a typical lecithin or kephalin molecule accounts for one-eighth of the whole, and that it is more logical to use the other seven-eighths in analyses. In view of the fact that phospholipoids are important more because they contain fatty acid residues than for any other reason, a determination of their fatty acid equivalent would appear to be a still more fundamental procedure. It is generally assumed that the phospholipoid present in blood is lecithin, but it may be that kephalin and sphingomyelin are also present, and the nature of phospholipoids in blood is as important as their concentration.

It must also be remembered that there is a class of substance known as the galactosides which are often classed along with the phospholipoids since they resemble them in their chemical properties, but these/

these galactosides contain no phosphorus, and other methods must be adopted for their estimation. Bang and Forsmann (11a) claim to have detected these compounds in red blood corpuscles, and hence their existence must be of some importance in fat metabolism for phrenosin and kerasin both contain one fatty acid radical in their molecule.

Cholesterol in blood has been put upon a sounder basis than any of the other lipoid fractions, and the chief credit among recent workers must be accorded to Okey (12) and to Yasuda (13); the former for having arrived at a sound micro method, and the latter for having introduced one or two excellent modifications. Although Okey has done a considerable amount of work on the problem of cholesterol and cholesteryl esters in blood, there is still a great deal to be done. The relationship of cholesteryl esters to fat absorption, for example, is still obscure.

Soaps have yet to be thoroughly investigated and although a method for their estimation in blood has been worked out by Stewart and White(14) no applications of it are found in the literature. There/

There are no methods for estimation of free fatty acid in blood, nor is there any direct method for estimating triglycerides. Triglycerides are in the unfortunate position of being represented by the difference between the total fatty acids and the fatty acids derived from various known sources. As far as has been discovered, there is little evidence even to justify the hypothesis of their existence in blood. It is presumed that such expressions in the literature as "fat", "neutral fat", "residual fat" etc. are meant to represent triglycerides.

Difficulties in the analyses for total fatty acids are caused by the molecules being no more than a string of carbon atoms with no peculiar groupings by which they may be distinguished other than a carboxyl group. They give no colour reactions, and have no reducing properties; they cannot be converted to other known substances, nor have they any nitrogen, phosphorus, halogen etc. upon which an analysis may be based. Their iodine number varies considerably, but they are almost insoluble in a 5% saline solution. Since gravimetric analyses are of no value, one must either

make/

make use of the carboxyl group, or isolate the acids quantitatively and complete the estimation either by chromate oxidation or by the gasometric technique.

Soaps, free fatty acids, and triglycerides have similar properties to render their analyses as difficult, but phospholipoids are distinguished by their phosphorus content and their insolubility in acetone. Cholesterol is characterised by its reaction in chloroform with concentrated sulphuric acid and acetic anhydride and also by the peculiar solubility properties of its digitonide.

Experimental work on the problem of methods has been devoted largely to a correlation of the existing methods rather than an attempt to elaborate new ones. However, in attempting to differentiate between the various fractions of phosphorus which occur during the separation of phospholipoids, what might be described as a new method for estimating total and lipoid phosphorus has been arrived at. In view of the large number of existing methods for the determination of phosphorus, and the multiplicity of their modifications, the description of still another has been undertaken with due regard/

regard to the further complication of this particular section of the work. However, the results obtained by it are more interesting than is the method itself.

The difference in the lipoid concentration of plasma and corpuscles has been observed by several workers. In plasma, about 40-60% of the cholesterol occurs in the form of esters; in the cells, esters are completely absent. Soaps are found only in plasma according to Stewart and White (14),but the corpuscles are relatively far richer in both fatty acids and phospholipoids. No reason has yet been given for such distributions and even Bloor's theories of the function of red blood cells in fat transport cannot be accepted in face of all the evidence to the contrary. The present work does not attempt to solve the problem of lipoid distribution in blood, but the analytical methods discussed have been applied to analyses of the blood fractions with interesting results. All analyses have been carried out on either whole blood, plasma or cells, coagulation having been prevented by the addition of the minimum amount of finely powdered potassium/

potassium oxalate. The use of serum is to be avoided since it does not correspond to any fluid actually found in the body. In addition, Howell (14a) has shown that phospholipoids play an important part in the coagulation of blood and this may involve an alteration in the distribution between cells and serum as compared with the normal distribution between cells and plasma.

Estimation of Fatty Acids in Blood

The fatty acids in blood are known to consist of oleic, palmitic, stearic, linolic, linolenic and arachidonic acids, with possible some unknown hydroxy acids, phrenosinic acid, and lignoceric acid, the latter, provided that sphingomyelin and galactosides be present. If they are estimated by means of their carboxyl groups, then the best method of expressing their concentration is in milli-equivalents per 100 c.c. Otherwise an assumption must be made regarding their average composition. The average molecular weight of the first six acids mentioned above is 281.1. But since those present in/

in the largest amounts are oleic and palmitic acids, and since the former is generally considerably in excess of all the others, the molecular weight of oleic acid (282.5) is sufficiently close to the truth for all practical purposes. It is more convenient to express results in mgms. of oleic acid than in any other way and this mode of expression has been adopted here.

In estimations where the chromate oxidation technique is used, a similar assumption must be made and a uniform method of expressing results has its advantages. Stoddard and Drury (2) took the average molecular weight to be 277.2, while Channon and Collinson (15) have found the average molecular weight of the fatty acids in ox blood to be 301. It is curious that the average molecular weight of the eight fatty acids mentioned above comes to 306.7 and therefore agrees very well with Channon and Collinson's figure. But since we have very little idea of the relative proportions of these fatty acids, such an agreement must be regarded as a mere coincidence. Since the figure adopted does not alter the actual estimation, the assumption that the average is 281.1 will affect only the final/

final result and is capable of being corrected at some future date should new light be thrown upon the subject.

It is necessary at this stage, to give a résumé of the methods at present employed for the estimation of total fatty acid. Two of these have been selected as being the most modern and probably the most correct.

In 1928, Bloor (1) published a method in which the fatty acids were extracted from blood with 3:1 alcohol-ether mixture. An aliquot portion of the extract was then hydrolysed by means of sodium ethylate and the soaps taken down to dryness. The free fatty acids were liberated by acidification with sulphuric acid, and the mixture thoroughly extracted with petroleum ether (B.P. 40-60°C.). This petroleum ether extract contains all the fatty acid and cholesterol. It is then evaporated to dryness, the solvent being completely removed, and the total lipoid (i.e. fatty acid and cholesterol) estimated by heating with silver dichromate, the excess of which is measured by titrating with thio-sulphate the iodine which it liberates from potassium/

potassium iodide. Cholesterol is estimated separately and after the amount of oxidising agent which it requires is subtracted from the total volume used, the remainder is attributed to fatty acid.

Later, in 1931, Stewart, Gaddie and Dunlop published a method in which saponification of the extracted lipoids was carried out by alcoholic sodium hydroxide. When hydrolysis is complete, the soaps are dried in the steam oven and the mixture made just acid with N/10 hydrochloric acid. After standing 12-24 hours,the fatty acids are filtered off and both the precipitate and the flask which had contained it, are washed three times with 2 c.c. of 5% sodium chloride solution. The filter paper is then returned to the flask and thoroughly extracted by boiling alcohol. The extract is cooled, made up to 10 c.c. and an aliquot portion is titrated with N/10 sodium hydroxide from a Rehberg burette using either phenolphthalein or thymol blue as indicator.

At the beginning of his wonderful series of researches on fat metabolism, Bloor adopted the alcohol-ether method for the extraction of lipoids from/

from blood. 4-5 c.c. of blood or plasma are slowly dropped into about 75 c.c. of 3:1 alcohol-ether mixture, the solvent being continuously rotated to ensure a fine division of the protein precipitate. The mixture is then boiled for about 30 seconds in hot water, cooled, made up to the mark and thoroughly mixed. Aliquot portions are then removed for analysis. It is generally agreed that this process is very nearly complete but as the following table shows, alcohol-ether is not the only solvent which will give similar results. Experiments were carried out in which 4 c.c. portions of the same blood were extracted with 3:1 alcohol-ether, 1:1 alcohol chloroform, 1:1 alcohol-petroleum-ether and pure acetone. As expected, acetone gave low results owing to the insolubility of phospholipoids in it, but the figures for the other three solvents are remarkably close together.

Table I /

Table I.

All results are expressed in mgms. per 100 c.c. whole blood.
Total fatty acids by the method of Stewart, Gaddie and Dunlop.
Lipoid phosphorus by a method to be described later.
Free and total cholesterol by oxidation of the digitonide.

	Alcohol ether	Alcohol-chloroform	Alcohol petrol ether	Acetone
1. Total fatty acids	300	303	309	232
Lipoid P.	10.6	10.7	11.3	7.9
Total cholesterol	132	136	138	-
Free do.	83	85	-	-
2. Total fatty acids	298	298	286	-
Lipoid P.	11.5	11.5	11.6	-
Total cholesterol	180	175	176	-
Free do.	93	106	101	-

The most important figures in Table I are those for fatty acids. Being a composite mixture derived from a variety of sources, they show that all three mixed solvents extract the same amount of fatty acid-containing material. Conversely, they also/

also afford strong evidence that extraction by alcohol-ether is as complete as possible, there being no component which is insoluble in this, and soluble in the other two mixtures. 3:1 Alcohol-ether was therefore used for extraction in all the following work.

Man and Gildea (4) have shown that when plasma is refluxed in alcohol-ether for one hour, a further 5-31% of fatty acid may be obtained, presumably by the breakdown of some protein-fat complex. This result has been confirmed in the present series of experiments on whole blood (Table II) and the increase appeared to be proportional to the length of time of refluxing, a maximum being reached after about 5 hours. In both cases, estimation of the fatty acids was carried out by determining the concentration of carboxyl groups with N/10 sodium hydroxide. It is possible, and even probable, that the increase is due, not to increased extraction of fatty acid, but to oxidation of double bonds to carboxyl groups. In order to avoid this, samples of blood were refluxed in alcohol-ether and a current of purified hydrogen slowly bubbled through the mixture. Control analyses

on/

on the same blood were carried out by Stewart, Gaddie and Dunlop's method in which the extract was raised to boiling for only 30 seconds. These results are also shown in Table II.

Table II.

All results are expressed in mgms. per 100 c.c. whole blood.
Figures in columns (1) are the controls in which the extract was boiled for 30 seconds; columns (2) contain the analyses of the refluxed specimens.

Blood sample	Atmosphere	Length of time of refluxing in hours	Total fatty acids		Lipoid P.	
			(1)	(2)	(1)	(2)
1	air	1	282	331	-	-
2	"	2	285	351	11.9	11.4
3	"	3	299	421	11.7	11.4
4	"	4½	370	451	-	-
5	"	5	311	405	11.6	11.8
6	"	12	261	392	9.6	9.8
7	"	18	279	404	12.0	12.6
8	hydrogen	4½	273	272	10.4	9.6
9	"	4½	418	415	12.0	11.9
10	"	5	370	363	-	-

These results prove beyond doubt that the increase obtained by Man and Gildea is due to oxidation and not to further extraction. The constancy of the lipoid phosphorus even when refluxing is not carried out in hydrogen, is further evidence, for phospholipoids are more closely connected with proteins than any of the other fractions, and are more liable to be retained along with the precipitated material.

Attempts were made to extract a further quantity of fatty acid from the protein precipitate by heating it with alcohol-ether mixture in a Soxhlet after the material had been separated, washed with cold ether once or twice, dried and ground. Results were not uniform but they indicated that about 4% more fatty acid could be obtained. However, washing with ether is an unsatisfactory process, the mass being rather porous, and also there is a considerable amount of pigment extracted (much more so than when the blood and solvent are refluxed together) and this heavily contaminates the precipitate obtained after hydrolysis and acidification. Refluxing of the protein in the original extract is a much more satisfactory process. From these results, it may/

may safely be concluded that Bloor's method of extraction is complete, and that fat-protein complexes, if such exist, are very easily decomposed.

Both sodium ethylate and alcoholic sodium hydroxide have been used for the saponification of the extracted lipoids. Stewart, Gaddie and Dunlop have used the latter while Bloor prefers the former, and the following experiments were designed to test the completeness of the hydrolysis. Normally, with alcoholic sodium hydroxide, the mixture is boiled down to about 1 c.c. on the hot-plate and finally taken to dryness in the steam-oven, care being taken to avoid further heating after the soaps are dry. Hydrolysis may conveniently be prolonged by adding a further 5 c.c. of water plus 5 c.c. of alcohol at the stage when the mixture is ready to go into the oven, and this process may be repeated ad lib. In every case, of course, the soaps are finally dried off. Table III shows the result of varying numbers of such hydrolyses.

Table III /

Table III.

All results are expressed in mgms. per 100 c.c. of whole blood.

Blood sample	No. of hydrolyses		
	1	2	3
A	326	336	-
B	237	230	-
C	279	-	279
D	404	-	398

It is at once obvious that there is no object in increasing the length of time of hydrolysis, and since at the end of each hydrolysis, the alkali becomes very concentrated, it may be assumed that saponification is complete.

Lecithin and kephalin are both readily hydrolysed by alkali; Yasuda (13) has shown that saponification of cholesteryl esters is complete after heating for 20 minutes with sodium ethylate; and it is generally agreed that the common triglycerides are also easily saponified. Man and Gildea (4) have found that with potassium hydroxide, only/

only 82% of the phospholipoid fatty acids can be recovered, while sodium hydroxide will give only 65% of them. Grave doubts may be case upon the accuracy of these results from a theoretical consideration alone. Both sodium and potassium hydroxides in N/10 concentration are almost 100% dissociated and it is the concentration of hydroxyl ions which determines the rate and amount of catalysis - not the concentration or nature of the kation. It is even more peculiar that these same authors in a later publication (16) have used the sodium and not potassium hydroxide for the hydrolysis.

It may be mentioned briefly that sodium ethylate as the saponifying agent gives identical results with those obtained by the use of alcoholic sodium hydroxide. Furthermore, in Stewart, Gaddie and Dunlop's method an excess of N/10 hydrochloric acid in the liberation of fatty acids up to 0.5 c.c. does not interfere with the final titration. A larger excess than this gives definitely higher results.

A study of the figures in Table III reveals a much more important point. So long as the fatty acids remain in an aqueous alkaline medium, there is no/

no tendency towards oxidation of the double bonds to carboxyl groups. It can readily be shown, however, that if the dried soaps be left in the oven for any considerable period, the number of carboxyl groups increases as shown by the volume of N/10 sodium hydroxide required for their neutralisation. On looking into the matter more closely, it will be seen that this process of oxidation will also affect Bloor's method which depends, not only upon the carbon and hydrogen content of the mixture to be oxidised, but also upon its oxygen content. The increase of carboxyl groups, as demonstrated by the titration method, may amount to as much as 50% if the soaps be dried at 90-100°C. for 24 hours. Usually the soaps are dry in 15-30 minutes and if they be removed as soon as all moisture is away, duplicate analyses may be regularly obtained to within 2%. When a series of analyses is carried out on the same extract, the soaps do not all come down to dryness at the same rate and it may be that one will take as much as 10 minutes longer than another. Even then, the same accuracy can be attained showing that the tendency toward oxidation at/

at this stage is not very great. Bloor, however, dries the soaps with a current of air while they are on the steam-bath. This is a procedure to be avoided, and Peters and van Slyke (17) have advocated the use of a current of hydrogen or carbon dioxide for the purpose.

Experiments were attempted in which the flask containing the hydrolysate boiled down to 1 c.c., was filled with hydrogen, evacuated, and the soaps dried by placing in a hot water bath. They were not successful, for, owing to the excessive amount of frothing, the soaps were deposited around the sides of the flasks, thus preventing quantitative liberation of the fatty acid after addition of hydrochloric acid. However desirable such a modification would be, the practical difficulties could not be overcome.

The greatest drawback to Stewart, Gaddie and Dunlop's modification is that it will not estimate soluble fatty acids. It must be remembered that precipitation of the acids is carried out in a solution of sodium chloride and also that they are washed with 5% saline, both of which procedures will tend to reduce their solubility. Since this method gives/

gives lower results than any other, it is possible that here is a considerable source of error.

Man and Gildea (4) have collected the filtrate from several analyses, extracted the soluble fatty acids with ether, and determined their concentration by titration with sodium hydroxide. It is to be noted that they washed the ether extract with water to remove any free hydrochloric acid, so that the bulk of any extracted water-soluble fatty acids would be lost also. Smith and Kik (5) have extracted the fatty acids from the original acidified hydrolysate with benzene, but they also wash the extract with water and undoubtedly lose more material than is lost by filtration and washing with sodium chloride solution. Bloor's method of course will estimate all fatty acids irrespective of their solubility in aqueous media.

It has been found, however, that 1 c.c. of N/10 sulphuric acid may be repeatedly extracted by petroleum ether without any of the mineral acid being removed by the solvent. The ether extract is evaporated to dryness in a 50 c.c. conical flask which is then extracted with boiling alcohol. The alcohol is made up to 10 c.c. of which 2 c.c. are used for titration/

titration with N/10 alkali. It was found that the titration of the 2 c.c. of extract was exactly the same as the titration for a blank consisting of an equal volume of boiled alcohol. Still more important was the observation that neither phosphoric nor lactic acid was extracted by petrol ether. The former will certainly be present as one of the end-points of phospholipoid hydrolysis, while the latter may be derived from any sugar which happens to be in the alcohol-ether blood extract. Similar results were obtained using benzene in place of petroleum ether, but this solvent is more difficult and more objectionable to handle. It follows that it is quite unnecessary to wash either the petroleum ether or the benzene extract containing the fatty acids.

Stewart, Gaddie and Dunlop's method was therefore modified as follows. Immediately the soaps were dry, one drop of an aqueous solution of phenol red was added and the mixture made just acid with N/2 sulphuric acid. After standing overnight, the fatty acids were extracted with petroleum ether (40/60°C. redistilled over solid potassium hydroxide). Four or five extractions were carried/

carried out each with 5 c.c. of the solvent. Only minute traces of the phenol red indicator are extracted by the petroleum ether. The ether was decanted each time into a conical flask, and the whole extract evaporated to about 2 c.c. on the hot plate. The last traces of ether were removed by a current of hydrogen, and the fatty acids extracted with 10 c.c. of alcohol, of which 2 c.c. were titrated in the usual manner. Table IV. shows the comparative results of the modified and original methods, some of the analyses having been carried out in duplicate.

Table IV.

Results are expressed in mgms. per 100 c.c. of whole blood.

Blood sample	(1) By titration method	(2) By extraction method	Percentage increase of (2) over (1)
1	211, 211	211, 216	+ 1.2
2	199	202	+ 1.5
3	221	218	- 1.4
4	495	516	+ 4.2
5	216, 220	225, 222	+ 2.5
6	236, 238	248	+ 4.6
7	244	248	+ 1.6
8	245	250	+ 2.0

The extraction method gives results which are 2-4% higher than those obtained by filtration and in all probability this represents the percentage of soluble fatty acids. The extraction method differs from Bloor's method only in the final stages (it having been shown that there is no difference between sodium ethylate and alcoholic sodium hydroxide as hydrolysing agent). If the fatty acids can be estimated by titration, then they must necessarily be estimated also by the chromate oxidation technique, for Bloor does not purify them further. The titration method is the more specific, for, when Bloor's method is closely examined, it is seen that the blood is first extracted with alcohol-ether, and the hydrolysate is then extracted with petroleum ether. It is assumed that the petroleum ether extract contains no oxidisable substances other than fatty acid or cholesterol. Obviously, any contaminating substance will be put down to the credit of total fatty acid, and it remains to be seen whether one is justified in assuming that the final extract is such a pure mixture of two substances. The hydrolysate contains/

contains glycerol and bases (choline, amino-ethyl alcohol, sphingosine) derived from phospholipoids, pigments or their hydrolytic products and perhaps other unknown substances. All or some of these may be extracted by petroleum ether thus giving high results.

The oxidation stage in Bloor's method is open to another but lesser objection. Results are calculated as oleic acid and theoretically 1 mgm. of oleic acid requires 3.61 c.c. of N/10 dichromate for its complete oxidation but if the process of oxidation be incomplete this figure will be lowered. A considerable amount of confusion has been caused by individual authors finding different factors which suit their particular technique. Thus, for example, Bang (18) and Fleisch (19) use the factor 2.50. Blix (20) uses the factor 2.06; Vasilewska (21) found it to be 2.65; and Sinclair (quoted by Bloor) has even used a factor as low as 1.65. Although Bloor (1) has succeeded in arriving at conditions which give the theoretical value, the absolute values of all the earlier analyses are quite unknown.

In the modified method described above, it was noticed that the residue left after the petroleum ether/

ether had been removed, was not completely soluble in alcohol. The alcoholic extract contained some material in suspension and a white deposit was left in the conical flask. There is good reason for believing that this is organic in nature, on a consideration of the previous treatment, and since it is not soluble in alcohol, it cannot be either cholesterol or fatty acid.

It must be admitted at this stage, that it is not an easy matter to obtain consistent results with the modified titration method where the fatty acids are extracted with petroleum ether. There are sufficient figures in Table IV to show that the two methods are comparable, but occasionally, duplicates by means of the petroleum ether extraction have failed to agree for some unknown reason. Bloor has pointed out that exposure to the air will convert the unsaturated acids to hydroxy acids which are insoluble in petroleum ether. And, moreover, if the petroleum ether extract be allowed to stand, the same oxidation occurs. This may be one of the factors which cause these variable results but it is not the only one since even rapid working and careful avoidance of undue exposure to air may not lead/

lead to an improvement.

The extraction process has never yielded results which are more than 4.2% higher than those obtained by filtration and it may safely be said that 2-3% represents the maximum limit of soluble fatty acids. In most cases, the limit has been found to be of the order of 1-3% and represents an almost insignificant error in Stewart, Gaddie and Dunlop's method. For this reason, and since it can be guaranteed to give excellent duplicates, the method advocated by these authors has been adopted throughout the following work.

Estimation /

Estimation of Phospholipoids in Blood

Three phospholipoids are known to occur in the animal body, viz. lecithin, kephalin and sphingomyelin. Which of them are present in blood and in what proportion, is not yet known. In all analytical methods, it is assumed that the molecules are those of either lecithin or kephalin. Table VI shows that the differences between these three depend upon the known fact that sphingomyelin contains only one fatty acid radical. For the sake of comparison, it has been assumed in the second column of this table that all the fatty acids are oleic acid, although it is known that lecithin and kephalin each contain a saturated acid, and that the acid in sphingomyelin (lignoceric acid) is also saturated. The demonstration of any galactoside in blood will of course considerably alter these calculations.

Table VI /

Table VI.

Phospho-lipoid	Mgms. of phospholipoid which are equivalent to 1 mgm. of lipoid phosphorus	Mgms. of fatty acid which are equivalent to 1 mgm. of phosphorus (expressed as oleic acid)
Stearyl-oleo-lecithin	26.0	18.2
Stearyl-oleo-kephalin	24.0	18.2
Sphingo-myelin	26.8	9.1

From the point of view of the part which they play in fat metabolism, it is essential to know which of the phospholipoids are present in blood. Attempts to answer this question will be described later.

There are two principal methods for the estimation of phospholipoids in blood, (a) isolation and subsequent oxidation by dichromate; and (b) by determining the lipoid phosphorus. The oxidation method was elaborated in 1929 by Bloor (22) and subsequently modified by Boyd (23). Briefly, the method consists of evaporating down an/

an aliquot portion of the alcohol-ether extract of blood and dissolving out the phospholipoids from the dried residue with ether. The bulk of the ether is evaporated off and the lipins precipitated by acetone and the addition of 2-3 drops of a saturated alcoholic solution of magnesium chloride. The precipitate is centrifuged down, washed with acetone, and extracted by ether saturated with water. The ethereal extract is separated, all traces of the solvent are removed and the phospholipoid residue is then estimated by oxidation with silver dichromate in the usual manner. The result is expressed in mgms. of lecithin.

There is a new method for the estimation of "lecithin" published by Lintzel and Monasterio (23a) the basis of which is the estimation of choline by oxidation of that substance by permanganate to trimethylamine which is then distilled off and estimated by titration. Choline, however, is common to both the lecithin and the sphingomyelin molecules and analysis along these lines can have little significance.

As has been mentioned before, there are numerous methods in the literature for the estimation/

estimation of small amounts of phosphorus. One of the earliest micro methods was the nephelometric one of Bloor (24) which has largely been replaced by colorimetric processes, although its use has been supported even up to 1928.

The great majority of the methods, however, depend upon the reduction of phosphomolybdic acid. The blue colour developed in this reduction was observed independently by Bell and Doisy (25) and by Denigés (26), and various reducing reagents have been used, e.g. hydroquinone, stannous chloride and 2:4 aminonaphthol-sulphonic acid. The last of these is undoubtedly the most satisfactory and its use was first described by Fiske and Subbarow (27). Unfortunately, most of the methods for the estimations deal with classes of phosphorus other than lipoid phosphorus and it has been tacitly assumed that they can be readily adapted to the analysis of alcohol-ether extracts. This is not always the case. The underlying principles of the methods for estimation of lipoid phosphorus consist of evaporating down an aliquot portion of blood extract with sulphuric acid. The organic matter is thoroughly charred and the carbon is oxidised by either peroxide or nitric acid/

acid. The residue contains the phosphorus in the form of phosphoric acid; ammonium molybdate is then added, and reduction gives a deep blue colour proportional to the concentration of phosphorus.

There are many factors which come into this analysis and those upon which the colour production depends are(1) the temperature of charring and the degree of heat required to remove all of the oxidising agent; (2) the most important point of all is the concentration of acid present while the colour is developing. Absence of acid results in reduction of the ammonium molybdate itself while excess of acid suppresses the production of all colour; (3) the concentration of molybdate; (4) the concentration of reducing reagent; (5) the temperature at which the colour is developed; and(6) the time required for complete reduction. These are all points which must be taken into account.

While a study of these factors was being made, a method for estimation of total phosphorus in any specimen was evolved and certain doubtful points cleared up. Naturally, the method may be applied to lipoid phosphorus and while each worker would appear to prefer his own method the following can scarcely be equalled for simplicity, speed and/

and accuracy.

The concentration of acid is of primary importance. Sufficient must be used to keep the foot of the tube (6 x $\frac{5}{8}$ pyrex) covered during destruction of organic matter, and to prevent reduction of the molybdate reagent: excess must be avoided otherwise no reduction will take place in the subsequent molybdate treatment. To avoid the tube becoming dry at the foot during incineration at least 1 c.c. of 10 N acid must be used, but this concentration, in a 10 c.c. dilution, is sufficient to inhibit the production of the blue colour. Reduction, however, can still take place if the tubes be placed in a boiling water-bath. Table VII shows the effect of varying concentrations of sulphuric acid, the tubes each being made up to 10 c.c. after addition of all the reagents, but before heating.

Table VII /

Table VII

Each tube contains 0.02 mgms. phosphorus (in the form of potassium hydrogen phosphate).
0.5 c.c. of 2½ % aqueous ammonium molybdate.
0.4 c.c. of aminonaphthol-sulphonic acid made up according to the directions of Fiske and Subbarow (27)

All tubes were heated at 100°C. for 10 min. after addition of the reagents.

Volume of 10 N sulphuric acid in c.c.	Mgms. "P" found i.e. $\frac{St}{Un}$ x 0.02
0.2	.1021
0.3	.0752
0.4	.0348
0.5	.0212
0.75	.0202
1.0 (standard)	.0200
1.2	.0200
1.4	.0098
1.6	.0086
1.8	.0016

When the volume of acid is plotted against the apparent recovery of phosphorus, the graph obtained is that shown in Fig. 1.

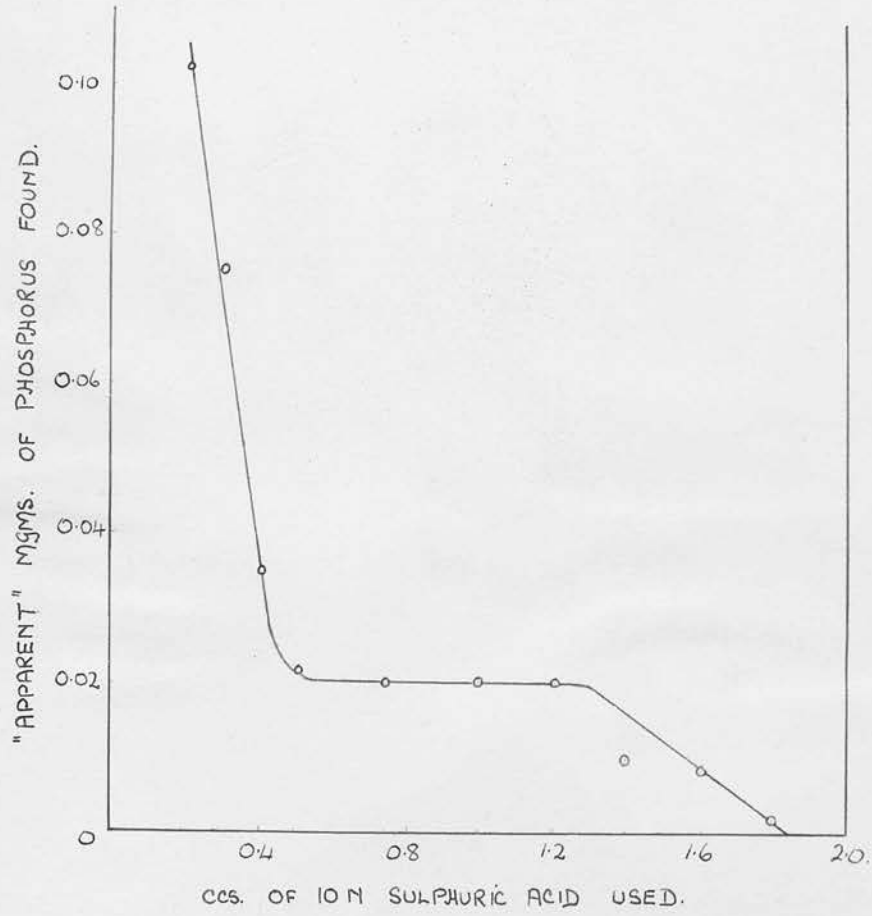

Fig. 1.

If the destruction of organic matter be carried out with 1.0 c.c. of 10 N sulphuric acid, the loss of a little acid in the form of fumes will make no difference unless this loss be greater than 25% . This is of considerable advantage, for the amount of fuming is proportional to the length of time of charring which, in turn, is proportional to the amount of organic matter present. It is with only large quantities of lipoid that sufficient acid is lost to cause an appreciable error.

The next table shows the effect of varying amounts of ammonium molybdate. A neutral aqueous solution/

solution has been used in place of the more usual acid solution, so that all the sulphuric acid necessary will be available for the charring process.

Table VIII.

Each tube contains 1.0 c.c. 10 N sulphuric acid
0.4 c.c. reducing reagent.
No phosphorus present.

All are heated for 10 min. at 100°C.

Volume of 2.5% molybdate in c.c.	Colour produced
0.5	none
0.75	none
1.0	++
1.5	++++

The maximum allowable volume of the molybdate reagent is thus about 0.75 c.c., but in practice 0.5 c.c. is used. This quantity will be quite sufficient for even large quantities of phosphorus, and leaves a safe margin. This point is not of very great importance for the same volume is always added to both standard and unknown, and if there be any excess, then both tubes will be altered to the same extent. If the alteration be small, the error/

error will be negligible.

Three of the remaining factors may be referred to briefly. Excess of aminonaphtholsulphonic acid reagent up to 2.0 c.c. does not have any effect. The colour is not developed in the cold: at 100°C. however, 96% of the colour is produced after five minutes and reduction is complete at the end of ten minutes. If heating be continued longer than this period, no change occurs until after twenty-five minutes when the mixture turns brown and, for colorimetric purposes, is useless.

Details of the method employed.

The material to be analysed (e.g. the alcohol-ether extract of blood, etc.) is evaporated down to dryness in a 6 x $\frac{5}{8}$ pyrex tube graduated at 10 c.c. 1.0 c.c. of 10 N sulphuric acid is added and the mixture heated over a naked micro-burner flame until completely charred. The carbon is removed by successive additions of 1 drop of Merck's Perhydrol until the final mixture is colourless and water-clear. As a rule, a varying amount of acid is lost in the form of fumes, but it has been

shown/

shown that, within limits, this is of no importance. It is essential to remove or decompose all the perhydrol, and to this end, the sulphuric acid is distilled each time up as far as the graduation mark. After cooling, water is added to make the final volume up to about 9.0 c.c.

A standard phosphorus solution is prepared containing 0.0200 mgms. of phosphorus per c.c. Two c.c. of this solution (accurately measured) to which 1.0 c.c. of 10 N sulphuric acid has been added and the mixture diluted to 9 c.c., serves as the standard for comparison. To both standard and unknown are added 0.5 c.c of 2.5% aqueous ammonium molybdate followed by 0.4 c.c. of the reducing reagent. The contents of the tubes are thoroughly mixed, placed in a boiling water-bath for 10 minutes, cooled, made up to exactly 10 c.c., and compared in a Klett colorimeter.

Baumann (28) and others have criticised the above principles on the grounds that phosphoric acid is lost by volatilisation during heating. It has been suggested that an excessive amount of heating/

heating will convert orthophosphoric acid to metaphosphoric acid which is not estimated by the above method. Varying quantities of standard phosphate solution have been heated with and without the addition of organic matter - i.e. glucose and tripalmitin. In some of these cases, an excessive amount of organic matter was added so that several additions of perhydrol were required to remove all the carbon, and yet no appreciable loss in phosphorus occurred. These results are shown in Table IX and in every case the phosphorus was estimated by comparison against a standard which had not been subjected to previous heating with sulphuric acid.

The method can be readily adapted to the estimation of alcohol-ether soluble phosphorus, and duplicate analyses invariably agree to within 2%.

Table IX /

Table IX.

Details	P added mgms.	P recovered mgms.
Phosphate and acid heated alone; no perhydrol required	0.0240	0.0239
do. do.	0.0220	0.0222
do. do.	0.0180	0.0182
do. do.	0.0160	0.0166
5 mgms. glucose added	0.0240	0.0247
do.	0.0220	0.0221
do.	0.0160	0.0162
3 mgms. tripalmitin added	0.024	0.0239
do.	0.020	0.0200
3 mgms. tripalmitin + 5 mgms. NaCl added	0.020	0.0199
do. do.	0.018	0.0187
Average error		1.7%

The use of lipoid phosphorus as a method of determining the phospholipoids is a difficult assumption to justify. Like fatty acids the phospholipoids are completely extracted by alcohol-ether, and other mixed solvents do not increase the yield to any appreciable extent (see Table I). Acetone has/

has the effect of reducing the solubility but even with this solvent quite a large proportion is extracted. It must be remembered, however, that acetone will dissolve a certain amount of phospholipoid if there are other fatty substances in solution. This fact has been noted by Maclean and Maclean (29): and furthermore, if the magnesium chloride be omitted in Bloor's method (22), very low results are obtained. That extraction is complete is confirmed by the figures given in Table II where it is shown that extraction of lipoid phosphorus is not increased by refluxing in an atmosphere of either hydrogen or air.

In spite of the length of the process for the oxidative determination of phospholipoids, it is comparatively easy to handle. Its main drawbacks are (1) that it requires a comparatively large amount of plasma and (2) that while a pair of estimations are quite simple, it is almost impossible to carry out more than four analyses at one time. The completeness of the isolation of the phospholipoids may be gauged by following up the phosphorus contained in the various residues and extracts, and

a/

a study along these lines has yielded interesting results. In all, five phosphorus analyses are necessary in each experiment:-

(1) the "lipoid phosphorus" of the original alcohol-ether extract of blood,

(2) the phosphorus left after the first extraction with ether,

(3) the phosphorus contained in the acetone after precipitation and washing of the phospholipoids,

(4) the phosphorus left in the magnesium chloride residue, and

(5) the true "lipoid phosphorus" contained in the final ether extract.

Details of the method of purification

4 or 5 c.c. of blood are extracted by about 80 c.c. of boiling 3:1 alcohol-ether. After cooling, the mixture is made up to 100 c.c. 75 c.c. of this extract are pipetted into a 150 c.c. conical flask and evaporated to about 10 c.c. on the hot-plate. Care must be taken at this stage to avoid bumping of the liquid. The remainder of the alcohol-ether is evaporated off on the steam-bath and immediately the foot of the flask is dry, a current of purified hydrogen is blown in to remove the last traces of the solvent.

The residue is extracted 4-5 times with 7 c.c. portions of pure diethyl-ether. It is essential,

and/

and it cannot be overemphasised, that this ether must be free from peroxide. Even the slightest traces of ether peroxide are sufficient to give a precipitate of the purified phospholipoids which rapidly darkens in colour owing to oxidation. The ethereal extract which is always more or less turbid is transferred to a 6 x 1 centrifuge tube and the deposit removed by centrifuging. The supernatant liquid is transferred quantitatively to another 6 x 1 centrifuge tube and the deposit washed thoroughly and again centrifuged. The washings are transferred to the bulk of the ether extract. The residue, together with any material in the original conical flask, is dessolved in water and analysed for phosphorus.

The extract is evaporated to about 1 c.c. and 8-10 c.c. of redistilled acetone are then added. This is followed by 0.5 c.c. saturated alcoholic magnesium chloride solution. During the addition of the magnesium chloride, the mixture should be thoroughly rotated as each drop enters. The phospholipoids separate as a white flocculent precipitate which remains pure white for half an hour/

hour provided that the ether was free from peroxide. After this interval of time, the precipitate is centrifuged down and the acetone layer removed. The phospholipoids are washed once with acetone, and then dissolved in ether saturated with water. The acetone washings are combined and analysed for phosphorus. The precipitate is extracted 3-4 times with moist ether and the extract transferred to a 50 c.c. standard flask. The magnesium chloride residue is dissolved out by alcohol and also analysed for phosphorus. In the ethereal solution of the phospholipoids, phosphorus, total fatty acids etc. are then determined. Actually the final ether extract is transferred to a 50 c.c. standard flask and its total volume is usually 35-45 c.c. A silica chip is then added, the flask is heated on an electric plate, so that the volume is reduced to about 15-20 c.c. After cooling it is made up to 50 c.c. with alcohol. This alcohol ether mixture is much easier to pipette than pure ether. The original ethereal solution is cloudy (probably due to magnesium chloride) but addition of alcohol completely dissolves the suspended matter. For the sake of clearness, this solution is referred to as "the final ether extract". It was later observed that if the alcohol/

alcohol were added very slowly to the ether solution when making up to 50 c.c., a precipitate was gradually formed and attained a maximum after the addition of about 20 c.c. of the alcohol; further addition of this solvent rapidly dissolved it and the resulting solution was water-clear and did not precipitate out on standing. According to Maclean and Maclean (29) lecithin is completely soluble in cold alcohol; kephalin and sphingomyelin only slightly so; while galactosides are insoluble. However, as a mixed solvent is being used no conclusion may be drawn for such an observation, except that, if the solution be regarded as a pure solution of phospholipoids (with any galactosides) then the precipitate contains some compound in addition to pure lecithin. It was later observed that the magnesium occurs in this extract at least partly in combination with the phospholipoids; the solubilities of these complexes in pure solvents is not known, and care must be taken in the interpretation of such a solubility phenomenon as is described above. The precipitate might quite easily be a magnesium-lecithin complex.

As is shown in Table XI, the amount of phosphorus/

phosphorus left after the alcohol-ether soluble material of blood had been re-extracted with pure ether, is almost negligible. It is surprising that the alcohol-ether does not extract any inorganic phosphorus although it contains quite a large amount of water coming from the blood or plasma. Were any inorganic phosphate present in alcohol-ether, it would necessarily remain unextracted by ether. This fact is confirmed by experiments in which solid disodium hydrogen phosphate, sodium glycerophosphate, and casein were added to blood, and estimations of the lipoid phosphorus were carried out before and after these additions. The results are shown in the following table:-

Table X.

Nature of the added phos-phate	Mgms. P comp. added to 2.c.c. blood	Conc.of P added (1) mg.%	Original lipoid P. (2) mg.%	Total (1) + (2) mg.%	Lipoid P of the mixture mg.%	%age of added P recovered
Na_2HPO_4	2.85	12.0	11.0	23.0	11.0	nil
do.	7.0	30.3	11.0	41.3	11.0	nil
KH_2PO_4	1.35	15.4	12.14	27.54	12.0	nil
sod. glycero-phosph.	2.0	14.5	12.61	27.11	12.21	nil
	2.0	14.5	11.05	25.6	11.73	4.6
do.	4.7	34.0	11.05	45.1	11.97	5.6
casein	32.5	11.3	11.05	22.4	11.05	nil

In the three cases given in Table X no inorganic phosphorus was recovered but in two of the cases where sod. glycerophosphate was used, 5% of the added phosphorus was recovered. It should be noted, however, that in both of these cases, especially the second, the added phosphorus is far in excess of the concentration that would ever be found in blood. These figures, plus the fact that the alcohol-ether-soluble phosphorus is also completely soluble in pure ether are sufficient to warrant the conclusion that no appreciable amount of inorganic phosphorus is extracted.

In Table XI is given a selection of typical results of the losses in phosphorus occurring in Bloor's method of isolating the phospholipoids by precipitation with acetone in presence of magnesium chloride. The analyses recorded were carried out on whole blood.

Table XI /

Table XI.

All results are expressed in mgms. per 100 c.c. of whole blood.

P in original alcohol-ether extract	P remaining after ether ext.	P in acetone washings	P in $MgCl_2$ residue	P in final ether ext.	%age of original P recovered in all fractions
9.66	0.05	0.00	0.27	9.06	97.1
12.71	0.07	0.00	0.32	12.06	98.0
11.56	0.05	0.00	0.42	10.55	95.5
12.48	0.06	0.00	0.22	11.91	97.6
10.45	0.08	0.00	0.28	9.92	98.3
12.77	0.05	trace	0.23	12.22	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. It is observed that no loss occurs except at the stage where the purified phospholipoids are extracted with moist ether. One would scarcely expect almost the whole error to be concentrated at this point, for 99.5% of the total phosphorus was initially extractable with ether.

The/

The most obvious, and probably the correct, explanation lies in the assumption that decomposition has occurred to a slight extent. 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 phospholipoid-magnesium chloride complex since acidification will not increase the quantity of phosphorus extracted. Having regard to the notorious instability of the phospholipoids lecithin and kephalin, especially when they are in the pure state, it is almost certain that after precipitation, part of the molecules will decompose to inorganic phosphorus, or some other ether-insoluble fraction, and the corresponding amount of fatty acid will be lost in the acetone washings. Even without such an assumption, 95% of the original "lipoid phosphorus" has turned out to be true lipoid phosphorus and there are indications, as noted above, that the percentage is even greater. It is also seen in the above table that a small loss (2-3%) arises from the difficulties of manipulation, and this is probably also "true" lipoid phosphorus.

One may now proceed to draw a comparison between/

between the two methods of estimating phospholipoids. When the estimation is based on the phosphorus content of the alcohol-ether extract, the analyses are very simple and 15-20 estimations may be carried out together in a very short time. Furthermore, a minimum of 95% of the phosphorus so estimated occurs in the form of phospholipoid, hence the error is comparatively small. An additional advantage is obtained inasmuch as the result is expressed in terms of phosphorus without committing oneself to the nature of the phospholipoid. On the other hand, Bloor's oxidative method requires a far greater amount of manipulation during which there is not only the possibility of slight losses of material, but also of oxidation. It is much less convenient to carry out a large series of analyses by it; and it finishes with a chromate oxidation which, although it can be controlled perfectly, is open to the same objection as the corresponding method for estimation of fatty acid plus cholesterol namely, there is no guarantee of the purity of the material analysed. In considering the oxidation method for the estimation of phospholipoids, the following table is interesting. It will be seen that the volumes of N/10 dichromate required for the/

the various phospholipoids are remarkably close together.

Table XII.

Lipins	Vol. N/10 dichromate per mgm.
Lecithin (oleic, stearic)	3.08
do. (oleic, palmitic)	3.035
Kephalin (oleic, stearic)	3.08
do. (oleic, palmitic)	3.04
Sphingomyelin	3.24
Phrenosin	3.19
Average	3.11

These values are so extraordinarily close together that they would form an ideal method of estimation, but it remains to be seen if the precipitate is sufficiently pure to warrant their use.

There are two lines along which the study of phospholipoids may be advanced. After purification by means of Bloor's precipitation method, the phospholipoids are contained in an alcohol-ether extract to the extent of over 95% of the original phosphorus, the remainder having been lost by decomposition/

decomposition(which will result in a loss of an equivalent amount of fatty acid etc. in the acetone) and during manipulation. The nature of the phospholipoids may be partly elucidated by carrying out phosphorus and fatty acid estimations together on the ether extract. From this, may be calculated the quantity of fatty acid which is equivalent to 1 mgm. of lipoid phosphorus and it is an essential step in the solution of problems dealing with the part played by phospholipoids in fat metabolism.

By itself, however, the equivalent of 1 mgm. of lipoid phosphorus in terms of fatty acid will convey little even if the factor comes out to 18.2 (see Table VI). The lipins obtained by Bloor's method presumably include any galactosides which may be present, and the whole group may be classified as follows for the present purpose:-

A. Lecithin
Kephalin containing 1 atom phosphorus and 2 fatty acid residues

B. Sphingomyelin containing 1 atom phosphorus and 1 fatty acid residue

C. Galactosides containing no phosphorus and 1 fatty acid residue.

Any/

Any factor may be expressed as representing any mixture of these three classes.

Equally important information may be obtained by carrying out simultaneous estimations of phosphorus and nitrogen on the purified phospholipoid solution. Estimation of nitrogen has been carried out by the microkjeldahl method which is sufficiently accurate to give trustworthy results. The lipins may be again classified according to the nitrogen-phosphorus ratio as follows:-

A. Lecithin) N : P ratio = 1 : 1
Kephalin)

B. Sphingomyelin N : P ratio = 2 : 1

C. Galactosides N : P ratio = 1 : 0.

Estimations of the phospholipoid fatty acids were carried out by the filtration method described above. It was observed that the final alcoholic solution of the free fatty acids (i.e. immediately before titration) always contained a precipitate which, however, does not interfere with the titration. On examination, this precipitate was found to be partly organic in nature, but also gave an inorganic residue upon ignition. This inorganic residue contained magnesium. The precipitate is

quite/

quite insoluble even in 10 N sulphuric acid which eliminates the possibility of it being a magnesium phosphate. The most obvious conclusion, and the one which is almost certainly correct, is that the moist ether removes some of the magnesium which reacts with the soaps liberated during hydrolysis to form magnesium soaps which are totally insoluble either in alcohol or in dilute acids (hydrochloric or sulphuric). The acid added after hydrolysis does not decompose them and there is certainly some error although not very large if one may judge from the bulk of the precipitate.

Numerous devices were tried before this source of error was eliminated and while all but one proved to be failures, quite a large amount of information was obtained in the experiments.

In the first place, it was thought that the water contained in the ether dissolved out some magnesium chloride and that the use of pure dry ether would be a considerable advantage. It was found that a considerable amount of material containing phosphorus would not dissolve in dry ether. This is curious since, in one of the previous stages, it was shown that the whole of the phosphorus could be extracted from the dried alcohol-ether extract

of/

of blood by means of pure dry ether. At this stage, however, there were other lipoids present, e.g. cholesterol, which may considerably modify the solubility of the phospholipoids. The insolubility in dry ether points to the presence of kephalin which exhibits the curious property of being insoluble in dry ether, but very easily soluble in moist ether. Precipitation by acetone alone without addition of magnesium chloride results in a considerable loss of phosphorus (9-16%) in the acetone washings.

In some cases, it was noticed that only a very small precipitate was obtained by the addition of the acetone alone. As soon as the magnesium chloride was added, however, precipitation was complete and the bulk of the precipitate increased several hundred per cent. Omission of the magnesium chloride and mere fractionation with acetone, which has been used by several of the earlier workers, will lead to considerable error. In place of the magnesium chloride various other salts were used:- two drops of a saturated aqueous solution of sodium chloride were added, but this modification was equally useless since even the small/

small amount of water present in these two drops slowly dissolves the precipitated phospholipoids. The magnesium chloride was then replaced by an equal volume (1) of a saturated alcoholic solution of lithium chloride and (2) of a saturated alcoholic solution of cadmium chloride. In both cases, there was again a considerable loss of phosphorus in the acetone washings. It is to be noted that cadmium chloride has been extensively used by Levene and his co-workers in the preparation of pure specimens of various phospholipoids. It is very doubtful if it has any effect in reducing the solubility and certainly it is a very poor precipitating agent compared with magnesium chloride.

Since all of these modifications proved to be failures, the only alternative was to remove the magnesium before estimation of fatty acids. It was found that it could not be removed quantitatively by the addition of either phosphates or 8-hydroxyquinoline, although the former reagent (ammonium phosphate plus one drop of dilute ammonia) certainly removed part of the magnesium when the mixture was allowed to stand overnight. The next attempt, and indeed, the only possible method left, was a result of/

of the observation that during the early stages of hydrolysis, a white gelatinous precipitate slowly formed. This precipitate is best obtained by heating the ether extract of phospholipoids along with 5 c.c. of alcohol and 5 c.c. of N/10 sodium hydroxide in a centrifuge tube placed in a water-bath at 70°C. The precipitate is centrifuged off after half an hour at this temperature, washed with alcohol and the supernatant liquid plus washings analysed for fatty acid. With the introduction of this modification, the free fatty acids are completely soluble in alcohol. The precipitate is white, typically gelatinous and is completely soluble in one drop of 30% hydrochloric acid. It does not therefore contain any magnesium soaps, and actually consists of magnesium hydroxide.

As has been mentioned above, this precipitate of magnesium hydroxide appears only slowly on heating the phospholipoid extract with the alkali. Furthermore, the magnesium is not precipitated quantitatively, either by phosphate in presence of ammonia or by 8-hydroxy-quinoline. All these facts point to the magnesium being present at least partly in/

in some combination with the phospholipoid, so that the magnesium chloride is not merely present as a source of inorganic ions, but is required to form some insoluble magnesium-phospholipoid complex.

When analyses of the same extract for fatty acids were carried out (a) with the magnesium present and (b) after removal of magnesium, it was found that there was very little difference between the two. As a rule, the latter was about 1-3% higher which is of the order expected from the size of the precipitate of magnesium soaps.

The following table shows the equivalent of 1 mgm. of lipoid phosphorus in terms of fatty acid. They may be compared with the theoretical values listed in Table VI.

Table XIII/

Table XIII.

All values are expressed in mgms. per 100 c.c. whole blood.

Lipoid P in the final ether extract	Phospholipoid fatty acids (as oleic acid)	Equivalent (in mgms. of fatty acid) of 1 mgm. lipoid P.
10.00	137	13.70
14.58	196	13.45
11.19	164	14.70
9.75	123	12.60
12.20	174	14.25
9.75	137	14.05
9.06	114	12.58
12.06	148.5	12.31
10.55	141	13.36
11.91	136	11.41
	Average	13.24

In these analyses the magnesium was not removed before fatty acid determination. In the first two experiments, about 1.0 mgm. pure cholesterol was added during saponification. It has been mentioned by Stoddard and Drury (2) that cholesterol/

cholesterol has the effect of hardening the fatty acid precipitate, and thus holding back any globules of liquid fatty acids. Its addition will improve the filtration, but it is hardly necessary, for, in its absence, the precipitate of fatty acid appears to be very solid. This is unexpected for the phospholipoids have always been associated with unsaturated fatty acids.

The figures in Table XIII show that the phospholipoids cannot be regarded as pure lecithin and this is supported by various qualitative observations mentioned above. The average variation from the mean is 6% in these ten analyses, but they were carried out on specimens of which many came from pathological cases.

If the magnesium chloride be omitted, it is true that part of the phospholipoid is lost, but, on the remainder, the ratio of phosphorus to fatty acid is about the same as is shown above. Removal of the magnesium either partly as phosphate or wholly as hydroxide does not affect the value to any appreciable extent. Table XIV shows the result of such experiments.

Table XIV/

Table XIV.

Method	Equivalent (in mgms. of fatty acid) of 1 mg. lipoid P.
No $MgCl_2$ used during precipitation	12.23
do.	13.80
Mg partly removed as phosphate	13.09
do.	11.93
do.	13.83
do.	13.42
Mg wholly removed as hydroxide	12.00
do.	12.80
do.	13.85
Average	13.00

The analyses given in Tables XIII and XIV make it obvious that the phospholipoids of whole blood are a composite mixture, and that the quantity of fatty acid in such combination cannot be calculated from the blood lipoid phosphorus by the use of any previously known factor. Nor can it be/

be calculated from the phospholipoid which is estimated by Bloor's oxidation method. The lowering of the theoretical equivalent of 18.2 mgms. of fatty acid for lecithin (or kephalin) may be caused by sphingomyelin, galactosides, or a mixture of both.

Galactosides, if present, will presumably be precipitated by acetone and magnesium chloride along with the phospholipoids. The part which they play in fat metabolism is quite unknown.

It was observed that the extract of purified phospholipoids gave, in some cases, a reduction with Fehling's solution and a positive Molisch's reaction. The sugar which is responsible for these reactions may be derived from galactosides or it may be some of the normal blood glucose which has survived all the stages of purification. This second suggestion is not so likely in view of the purification which the material has undergone, but it is by no means impossible. Molisch's test was always used since it is more delicate, and more specific than any of the other common tests for sugar. The reaction obtained was fairly strong, weak, or in a few cases, entirely absent. Further purification/

purification of the phospholipoids may be carried out by evaporating the moist ether extract to 2 c.c. and reprecipitating with acetone and magnesium chloride. In four analyses in which this was done, three of the specimens gave a positive Molisch reaction after the first purification, but all were negative after a second precipitation. This argues in favour of the view that the sugar is blood glucose, but it is not proof, for although Bloor's purification gives a very good yield of phospholipoids, it may fail to recover galactosides quantitatively.

Estimation of the amount of reducing substance by the method of Hagedorn and Jensen for blood sugars, gave very disappointing results. The values obtained on different blood extracts were so variable that they were quite useless as an indication of the quantity of sugar present. In galactosides - if we may accept the formulae generally given - the aldehyde group of the sugar residue is combined with the sphingosine radical, and may be set free by hydrolysis with dilute acid or alkali. Estimation of the sugar before hydrolysis gave such widely varying results that little reliance could be placed upon the difference between them and

analyses/

analyses after hydrolysis. In all cases, only a small amount of reducing substance was set free by heating with N/10 hydrochloric acid, but it should be noted that in Hagedorn and Jensen's method (as in all methods of estimating sugar) the mixture is heated in alkaline solution and this itself might quite well cause a certain amount of hydrolysis, thus giving a high value for the preformed reducing material.

Unsatisfactory as these experiments undoubtedly are, the conclusion is arrived at that galactosides are either absent or present in only very small amounts. This is based wholly upon the qualitative observations for little reliance can be placed upon the sugar estimations.

The final extract of purified phospholipoids gives a completely negative Liebermann-Burchard reaction. This test is very delicate, and what is more important, is given by cholesteryl esters as well as free cholesterol. The whole of the cholesterol, free and esterified, has been removed in the acetone washings, so that the figures given in Tables XIII - XIV are not complicated by fatty acids combined with sterol.

In/

If one accepts the view that the blood phospholipoids are a mixture of lecithin, kephalin and sphingomyelin only, then the ratio of nitrogen to phosphorus must have a minimum value of 1:1 for pure lecithin or kephalin, and a maximum value of 2:1 for pure sphingomyelin. Since 1 mgm. of lipoid phosphorus is equivalent to 13.12 mgms. fatty acid (the average of all figures quoted above) one would expect the ratio to lie between these two values. Channon and Collison (15) have found that in partly purified phospholipoids from ox blood, there is considerably more nitrogen than is required for the lecithin bases on the estimation of lipoid phosphorus. In a series of twelve analyses there was an average of 332% more nitrogen than would be required for the phosphorus if all the phospholipoid were lecithin (or kephalin) with a N:P ratio of 1:1. In other words, the N:P ratio actually found is 3.33:1. Similar results have been obtained by Maclean (33) who found a N:P ratio varying from 1.5:1 to 4.0:1 in the phospholipoid extract from horse kidney. He has suggested that unknown nitrogenous substances are carried along with the phospholipoids and act as though they possessed all the properties of/

of phospholipoids. It is claimed that they can be removed by precipitation of the phospholipoids from an aqueous solution by means of acetone but a considerable amount of material is lost.

This excess of nitrogen has also been found in the present experiments working with what is probably a purer extract than was employed by these authors. The ratios found are given in Table XV.

Table XV.

Ratio N:P (in atoms) in the phospholipoid extract of whole blood.

	3.48 : 1
	3.02 : 1
	2.57 : 1
	2.90 : 1
	2.35 : 1
	3.67 : 1
	1.99 : 1
	2.56 : 1
	2.33 : 1
Average	2.76 : 1

In the last three of these determinations, the analyses were carried out on a phospholipoid extract which had been reprecipitated by acetone and magnesium chloride. They are slightly lower than the values obtained after only a single precipitation, but the final average value of 2.76:1 makes it quite clear that there are substances present other than the three phospholipoids. It is very doubtful, and indeed may be regarded as impossible, that the increase is due wholly to galactosides. If the latter are present at all, they are certainly not the cause of such a high ratio. The obvious inference is that the final phospholipoid extract contains some material, evidently rich in nitrogen, as an impurity, and such a conclusion would argue strongly against the use of Bloor's oxidation process for the estimation of this class of lipoids.

It is impossible to calculate the amount of impurity from such data, but Bloor (22) has shown that there is a fair agreement between the phospholipoid (calculated as lecithin) which is estimated by the oxidation procedure and that which may be calculated from the alcohol-ether lipoid phosphorus. Of the two, the latter appears to be preferable

and/

and theoretically sounder since phosphorus is an essential constituent of this class of substance and it has been shown that, within experimental limits, the material (i.e. the alcohol-ether extract of blood) in which phosphorus is determined contains all the phospholipoid phosphorus and no other phosphorus.

What is far more important, however, is the discovery that the phospholipoids of blood consist of almost equal quantities of sphingomyelin and lecithin (or kephalin). No distinction has been made between lecithin and kephalin, for they differ only in the choline and amino-ethyl alcohol residues which, for the purposes of the part played by them in fat metabolism is immaterial.

Cholesterol and Cholesteryl Esters

There are two methods in use for the estimation of cholesterol. The method of Myers and Wardell (30) consists of drying the blood either by spreading on a fat-free filter paper or by means of calcium sulphate, followed by extraction with dry chloroform. The cholesterol in the extract gives a greenish-blue coloration in presence of acetic anhydride and concentrated sulphuric acid. The depth of colour is proportional to the amount of cholesterol present and may be estimated by comparison against a standard solution which has been treated with the same reagents. In this way both cholesterol and its esters are determined and the development of colour probably depends upon the unsaturated linkage.

The second micro-method depends upon the oxidation of cholesterol digitonide and was first put forward by Okey in 1930 (12). The blood is extracted with alcohol-ether in the usual fashion an aliquot portion of the extract is evaporated to dryness with 3 c.c. of a 0.5% alcoholic solution of digitonin. Excess of digitonin is removed by boiling/

boiling with water and the mixture filtered through a good crucible containing purified asbestos. The precipitate is thoroughly washed with ether and after complete removal of the latter, the crucible plus contents are oxidised by dichromate in the usual manner. From the amount of dichromate required, the concentration of cholesterol may be calculated. This method estimates only free cholesterol since combination between cholesterol and the digitonin occurs at the hydroxyl group. Before total cholesterol can be estimated, the esters must be hydrolysed. The hydrolysate is acidified, extracted with petroleum ether and the procedure is then the same as for free cholesterol.

In place of filtering, Turner (31) separates the precipitate by centrifuging, but on account of the lowering of the surface tension due to the excess of digitonin, the procedure is very slow and the precipitate may not separate quantitatively. To overcome the difficulty of reduced surface tension, Yasuda (13) has added an equal volume of acetone to the aqueous suspension of the digitonide. This addition serves two purposes in an admirable fashion: firstly, it prevents frothing, and secondly it helps to dissolve out lipoids other than the digitonide/

digitonide. The precipitate is separated using a sintered glass filter, washed with acetone, finally with ether, and is then dissolved in absolute alcohol prior to oxidation.

All of these modifications have been attempted in the course of the work described here. Turner's modification is very tedious and centrifuging of the precipitate requires to be carried on for about an hour before separation is complete. Yasuda's method of dissolving out the digitonide in boiling absolute alcohol was not successful, for the solubility in this liquid appears to be very slight. In her work, Okey has adopted the addition of acetone, but has also found difficulty in dissolving the precipitate and prefers the original method of filtering through purified asbestos. An attempt to separate the digitonide by centrifuging the aqueous suspension in presence of acetone was made. This was quite successful at the first stage and the precipitate settles down rapidly in a mixture of acetone and water. It was found, however, that the suspension remained perfectly stable during the washing with acetone, and no devices attempted were able to persuade it to come down. Obermer and Milton(32) have/

have come up against the same difficulty and avoided it successfully by adding a little aluminium hydroxide which, they claim, carries down the precipitate completely. Repetition of this work failed to yield the desired results and estimations have therefore been carried out using Okey's original method of filtering after the addition of acetone.

The presence of asbestos undoubtedly accelerates the decomposition of the dichromate, but the error is very small (about 1-2%) and is adequately compensated by running a blank which also contains asbestos.

Only one really useful improvement may be suggested here. Bloor has advocated the carrying out of the oxidation at 124° ± 2°C. since the dichromate mixture is very active at this temperature and spontaneous loss of oxygen is negligible. Many devices have been used to obtain a constant temperature and a considerable amount of work has been expended with this aim, for the maintenance of the correct temperature is highly important.

A rectangular copper vessel measuring 12 x 5 x 5 inches, is covered with a lid in which a dozen holes are bored of a size sufficient to admit a 6 x 1 pyrex tube. The bath is then filled to a depth/

depth of about 3 inches with a saturated solution of calcium chloride. The strength of the solution is adjusted so that the boiling point is exactly 124°C. The bath is heated by a ring burner and kept boiling fairly vigorously. The temperature is recorded by two thermometers, one dipping into the liquid, and the other held in a 6 x 1 tube containing about 15 c.c. of glycerine. Within 5 minutes of the boiling point being reached, both thermometers will register the same. Tubes containing the cholesterol analyses are then inserted, the liquid is kept boiling, and the temperature occasionally adjusted to 123°C. by the addition of a little cold water. In this way, the temperature in the tube containing the glycerol may be kept constantly between 123° and 124°C. with very little attention. This calcium chloride bath has been found to be most effective.

It has been shown in Table I that extraction of cholesterol from blood may be carried out with either 3:1 alcohol-ether, 1:1 alcohol-chloroform or 1:1 alcohol-petroleum ether, which argues in favour of complete extraction in all cases.

Haematocrit Determinations.

In a study of the distribution of lipoids between plasma and corpuscles, a knowledge of the relative volumes occupied by these two fractions is necessary. From time to time, these haematocrit determinations have been subject to criticism on several grounds. The best way in which to settle the question is to make separate determinations of some constituent which occurs both in cells and in plasma. From a knowledge of those two factors and a determination of the cell volume, the concentration may be calculated for whole blood and should agree with the value found experimentally. These principles have been successfully used by Greenberg and his co-workers (33a) to determine the distribution of magnesium.

Such experiments are not only useful in checking the haematocrit readings, but also form an excellent guarantee of the ability of any method to give reproducable results. In the present series of experiments they have been successfully used in finding the distribution of lipoids between plasma and corpuscles.

Technique/

Technique employed.

In all cases, neutral potassium oxalate in the minimum amount was used as anticoagulant, and precautions were taken to avoid the presence of ether or water in syringes and tubes. Under these conditions, haemolysis can be completely avoided. The haematocrit tube consisted of about 15 cm. of capillary tubing sealed off at one end. The blood is introduced by means of a still finer glass capillary which reaches down to the bottom of the tube. Blood may be transferred to the haematocrit tube in a matter of a few seconds and no error is introduced even with bloods of a high sedimentation rate. The inside of the closed end is conical in shape, but this does not affect the reading, for, after centrifuging, it is filled with a deposit of calcium oxalate which presents a more or less flat surface.

The maximum amount of plasma is obtained after 25-30 minutes centrifuging at 2000 R.P.M. and in normal cases varies from 56-58% of the total. In lipoid estimations, part of the blood was removed for analysis and cell volume determination

and/

and the rest swung at 2000 R.P.M. for about 20 min. 4 c.c. of the plasma were removed for analysis and the rest of it drawn off by suction with a filter pump. The cells were then mixed with an equal volume of normal saline, avoiding undue shaking. The mixture was centrifuged and the cells again washed. Finally, they were made up with an approximately equal volume of normal saline and 4-5 c.c. of the mixture were extracted with alcohol-ether. At the same time a second haematocrit was carried out on the cell-saline mixture, and from the percentage of cells which it contains, the actual volume of cells taken for analysis can be calculated.

Duplicate readings of the cell volume may be regularly obtained with an accuracy of not less than 0.5%. Such a procedure has been found to be very satisfactory for the determination of blood lipoids, and examples of analyses based on this technique are to be found in the following pages. It will be observed that there is no pipetting of pure cells - always a difficult and unsatisfactory process.

In the Annual Review of Biochemistry (1934), H.D.Kay/

H.D. Kay states that "with regard to the organic phosphorus compounds, the reviewer must once more protest against the far too frequent occurrence in the literature of statements as to changes in these phosphorus compounds "in blood" after various experimental procedures, and in various diseases. It is, or ought to be, well known by all working in this field that, apart from phospholipins, the organic phosphorus compounds..... are practically entirely confined to the red blood cells, so that any changes reported "in blood" may merely be evidence of anaemia or polycythaemia, unless the analyses are accompanied by determinations of changes in haemoglobin or the percentage volume occupied by the red cells, or by other information as to possible blood dilution or concentration".

Such remarks apply to the distribution of lipoids in general but particularly to the phospholipoids the concentration of which in corpuscles is usually double the concentration in the corresponding plasma. The analyses given in Table XVI are obtained by the use of the methods described above. They are determinations of the lipoid fractions in two/

two cases of pernicious anaemia in which it is stated that the "blood" lipoids are generally low.

Table XVI.

	Whole blood mgms. %.	Lipoids in plasma from 100 c.c. blood. mgms.	Lipoids in cells from 100 c.c. blood. mgms.	Total: Cells + plasma	Plasma mgms. %.	Cells mgms.%.
Case W.A. Cell volume = 22.7%. Iron = 31.0 mgms./100 c.c. blood.						
Total fatty acids	289	255	55	310	330	244
Lipoid P	9.93	6.21	3.27	9.48	7.93	14.41
Total chol-esterol	169	131	31	162	169	136
Case M.C. Cell volume = 28.1%. Iron = 37.0 mgms./100 c.c. blood						
Total fatty acids	289	198	73	281	275	259
Lipoid P	10.5	5.90	3.50	9.40	8.21	12.30
Total chol/ esterol	171	126	40	166	174	141

The concentrations of total fatty acids and cholesterol are more or less normal; the lipoid phosphorus of blood is low due to the relatively small proportion of corpuscles which contain the greater concentration of the lipoid phosphorus.

The Lipoids of Blood.

The generally accepted values for blood lipoids and their distribution between cells and plasma is that quoted by Peters and van Slyke (17) who give the following figures:-

Table XVII.

Lipoids of normal blood in mgm. per 100 c.c.

	Whole blood	Plasma	Corpuscles
Total lipoids	-	570-820	-
Fatty acids	290-410	190-420	280-450
Lipoid P	10-18	7-14	15-23
Total cholesterol	100-230	100-230	100-230
Free cholesterol	-	30-60%	Almost 100%
Ester cholesterol	-	40-70%	Almost none

The variations in these fractions after ingestion of fat, and in pathological cases has been the/

the subject of much discussion. The next table gives a summary of the findings of various workers during the last twenty years, on the changes in these blood lipoids after feeding of fat.

The current theory on the subject is that of Bloor (1915) who found that in eleven experiments on dogs, all of them showed definite, and in some cases very large, increases in the blood fatty acids during absorption of olive oil. The variations in cholesterol were negligible, but the lipoid phosphorus increased almost parallel with the total fatty acids. In later experiments, it was found that the increase in lipoid phosphorus did not occur in the plasma, and it was concluded that the bulk of the fat, if not all of it, was converted into phospholipoids which were carried through the blood stream by the corpuscles. When the absorption processes are finished, the lipoid phosphorus returns to normal about eight hours after the fat has been taken. Part of the theory was later abandoned by Bloor (36) who stated that unreported work in his laboratories had not borne out his original findings, and that in some cases, the feeding of olive oil causes a rise in the plasma phospholipoids while in other cases the cell phospholipoids are increased. Bloor's theory has been rejected by several authors/

authors, notably Vahlquist (37) who finds that the increase in lipoid phosphorus after feeding of olive oil to dogs, invariably occurs in the plasma and that the cell lipoid phosphorus remains quite constant. Leaving the question of where the increase occurs, out of account, it is instructive to analyse the data of other workers on the changes of blood lipoids after ingestion of fat. Their results may be tabulated as follows:

Table XVIII /

Table XVIII.

Observer and reference	Year	Experimental animal	Nature of fat fed	Changes in: (a) Fatty acid	Changes in: (b) Lipoid P.
Bloor (10,11)	1915	dog	olive oil	increase	increase
Zucker (34)	1919	"	"	"	"
Bodansky (35)	1930	"	"	"	"
Vahlquist (36)	1931	"	"	—	"
Knudson (37)	1917	"	"	increase	"
Bang (38)	1918	" men	" "	" "	" no change
Man and Gildea (16)	1932	"	fat meal	"	increase
McClure and Huntsinger (39)	1928	"	fat oleic acid olive oil	" " —	— increase no change
Hiller et al(40)	1924	"	butter	increase	"
Greenwald (41)	1915	"	—	—	"
Page et al(42)	1930	"	olive oil	increase	"

These findings show that ingestion of fat will result in an increase in the blood fatty acids, but as far as phospholipoids are concerned, it will produce

an/

an increase in this fraction in the blood of dogs, but apparently not necessarily in the blood of human beings. The increase found by Man and Gildea (16) is almost certainly due to experimental error, for their technique is open to many objections to which reference has already been made. McClure and Huntsinger found that of several diets, only oleic acid which is not a fat, produced an increase in the blood lipoid phosphorus. Much the same conclusion has been arrived at by Eckstein (43) who found that feeding of olive oil or palmitic acid did not increase the lecithin content of the lymph of dogs, but that this fraction was increased by the feeding of oleic acid. He suggested that the increase was due to the toxic action of that acid and not to the synthesis of phospholipoids, but his reasons for coming to such a conclusion are not very clear.

Apart from Bloor's theory, it has been found by Knudson (37) that when dogs are fed olive oil, there is an increase in the percentage of esterified cholesterol both in the plasma and in the cells, mainly in the latter although the total cholesterol remains unaltered. All authors are agreed that there is, at the most, only very small variations in/

in the total cholesterol of blood after feeding of fat, and therefore the quantity of fatty acid which can combine with sterol is strictly limited. Combination as cholesteryl esters can therefore account for only a small fraction of the total fatty acid increase.

Since there are no methods for the direct estimation of triglycerides, the part which these substances play in fat metabolism is very obscure and they generally represent the fatty acid which cannot be accounted for by the other fractions. There is no record in the literature of any study of the soaps and free fatty acids of blood during fat absorption although they are known to be present in normal fasting blood (Stewart and White, 14).

The studies in fat metabolism presented here can be regarded only as preliminary work based on the methods and findings given above.

The average for total fatty acids and the corresponding lipoid phosphorus have been found in the following 28 cases of normal fasting human blood.

Table XIX(a) /

Table XIX(a).

	Maximum Mg. %.	Average Mg. %.	Minimum Mg. %.	Average deviation from the mean.
Total fatty acids	322	276	208	9.5%
Lipoid P	13.7	11.8	10.6	5.8%
Ratio:				
$\frac{\text{Fatty acids}}{\text{Lipoid P}}$	30.4	23.4	16.8	9.8%

In a series of 75 pathological cases (the greater part of which were diabetics) the corresponding values were as follows:-

Table XIX(b).

	Maximum Mg. %.	Average Mg. %.	Minimum Mg. %.	Average deviation from the mean
Total fatty acids	1027	336	205	-
Lipoid P	21.0	12.1	7.2	-
Ratio:				
$\frac{\text{Fatty acids}}{\text{Lipoid P}}$	49.0	27.8	21.4	11.1%

For the reasons mentioned above, the values for the lipoid phosphorus are misleading inasmuch as no account has been taken of changes in cell volume. This objection does not apply to the normal cases given in Table XIX(a) where the cell volume was always strictly within the normal limits. The analyses in Table XIX(b) included those from eight cases of anaemia which will reduce the average value for the lipoid phosphorus. Since the fatty acids in these cases are also reduced (although not by an exactly proportional amount) the ratio fatty acids: lipoid phosphorus will be almost independent of the cell volume. The difference between the ratios for the normal and pathological series (15%) indicates that a change in fatty acid is closely followed by a corresponding change in lipoid phosphorus.

The relationship between these two fractions is more clearly established by following up a single case of any disease where the blood lipoids are disturbed. The points brought out by H.D. Kay are very clearly demonstrated by the graphs in Fig. 2 from a case of diabetes complicated by anaemia.

Two/

Two factors are influencing the changes in the lipoid phosphorus, (1) the decreasing fatty acid concentration following the use of insulin, and (2) the increasing cell volume due to the treatment of anaemia, with the consequent increase in cell phospholipoids and cell fatty acids.

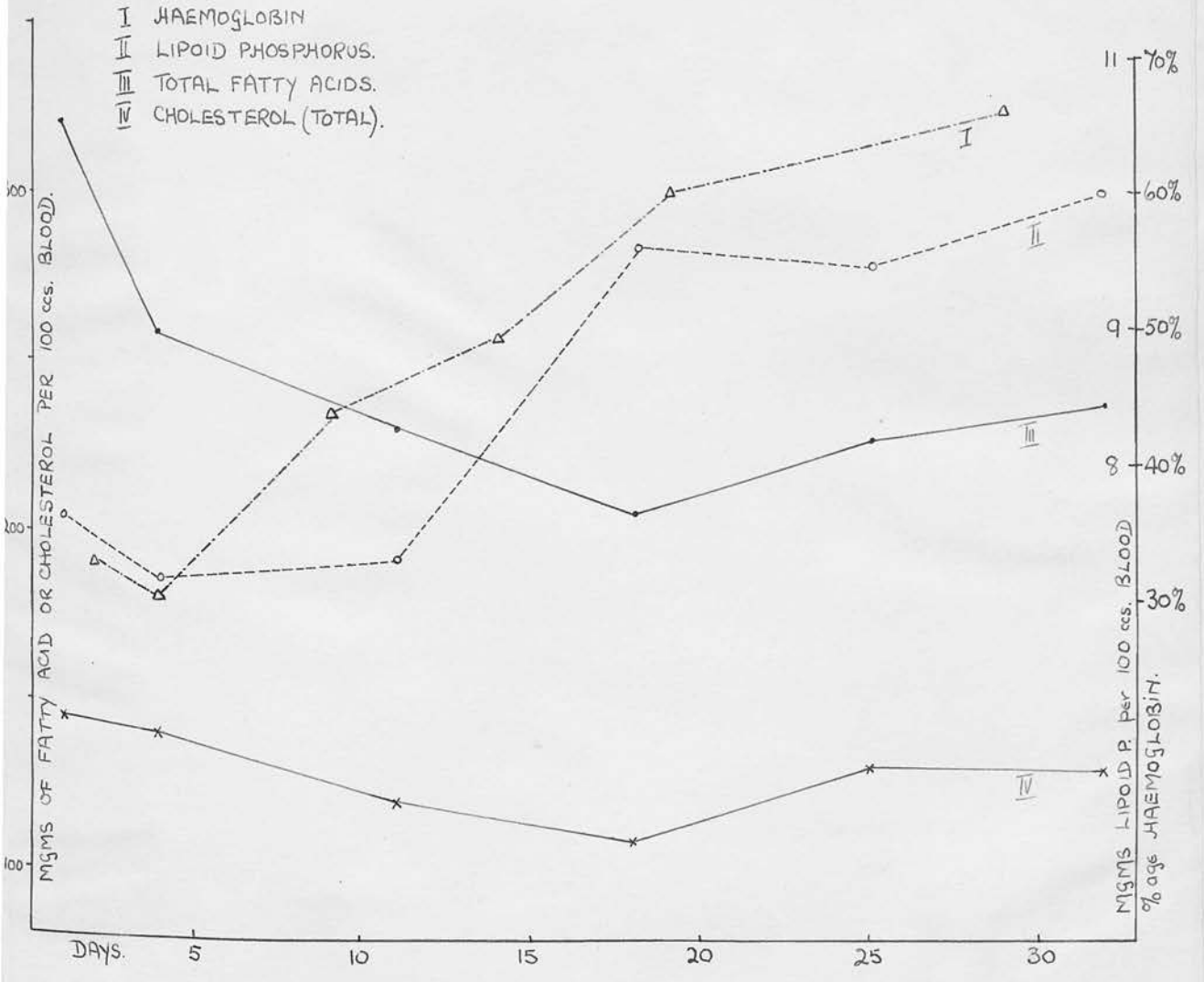

FIG. 2.

So long as there is a marked tendency towards anaemia, the lipoid phosphorus is very low, returning almost to normal when the haemoglobin reaches a value of about 70%. Initially, the blood fatty acids are greatly elevated(when it is considered that only a small fraction of them can be present in the form of phospholipoid) and they return to a figure definitely below normal (205 mgm.%) as insulin treatment proceeds. The lipaemia has been removed by this time, and the subsequent increase in fatty acids is to be ascribed mainly to the increasing cell volume and consequent rise in the blood phospholipoid concentration. It is not until the haemoglobin has reached a value of about 60% that the fatty acids and lipoid phosphorus show any degree of parallelism. Unfortunately this is the only case of such a type which has been available.

The more common type of graph which is encountered in diabetes mellitus is represented by the typical examples in Figs. 3 and 4.

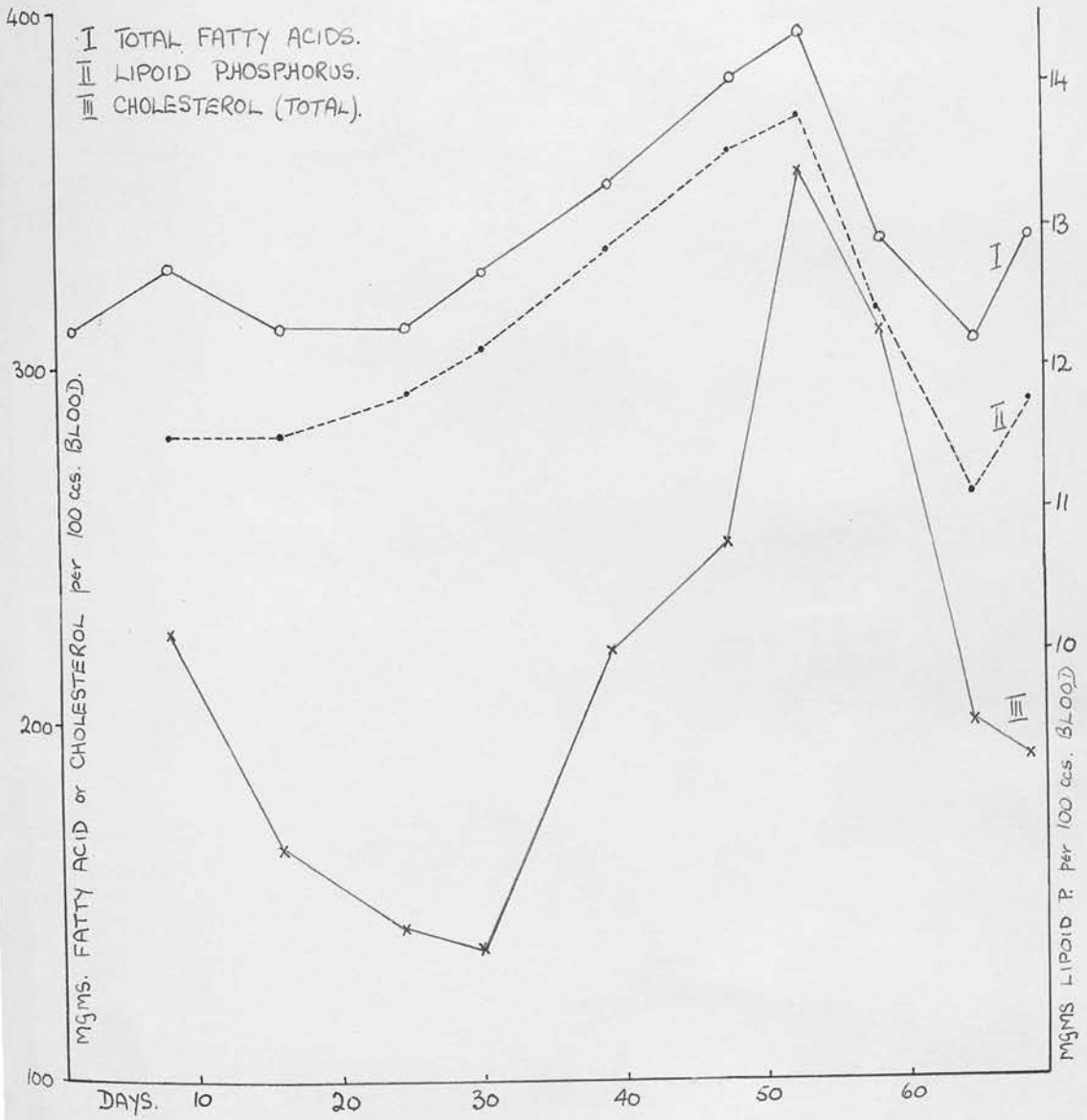
I TOTAL FATTY ACIDS.
II LIPOID PHOSPHORUS.
III CHOLESTEROL (TOTAL).
400
300
200
100
MGMS. FATTY ACID or CHOLESTEROL per 100 ccs. BLOOD.
MGMS LIPOID P. per 100 ccs. BLOOD
14
13
12
11
10
I
II
III
DAYS.
10
20
30
40
50
60

FIG. 3.

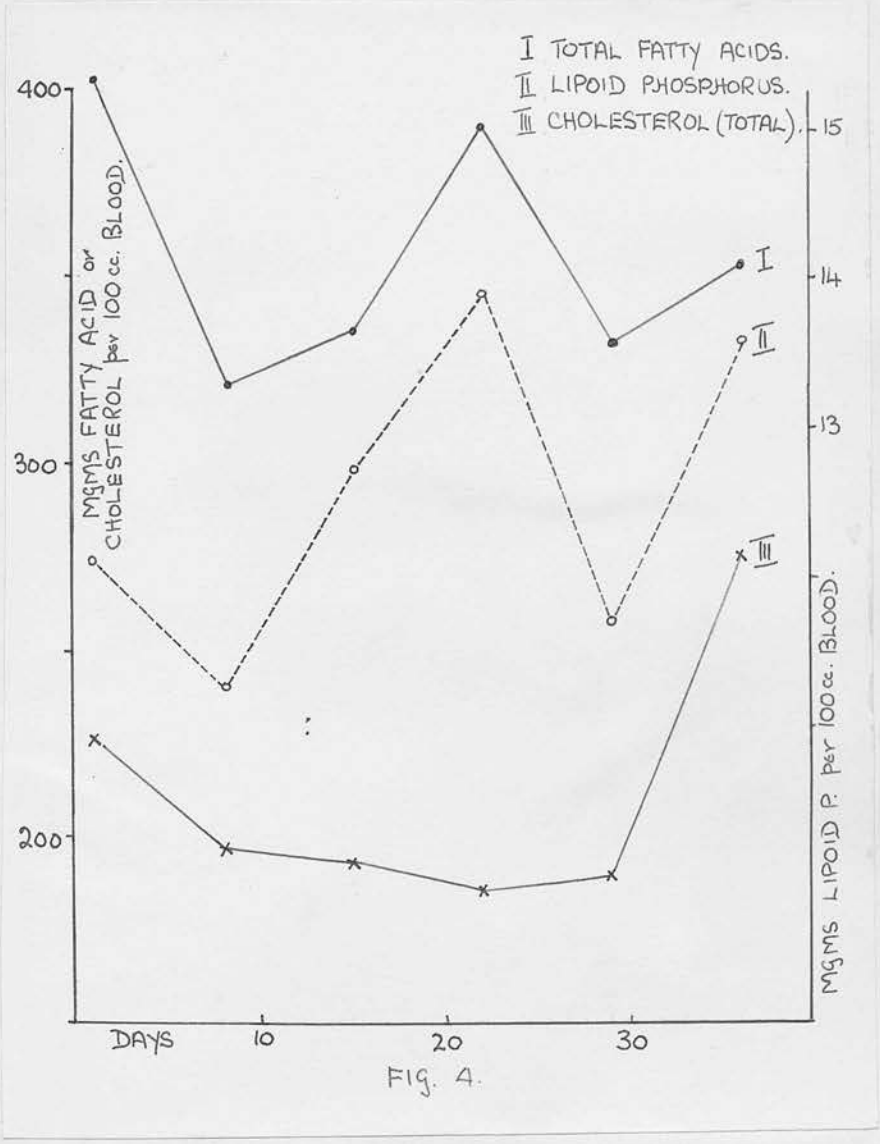
I TOTAL FATTY ACIDS.
II LIPOID PHOSPHORUS.
III CHOLESTEROL (TOTAL).
400
300
200
MGMS FATTY ACID or CHOLESTEROL per 100 cc. BLOOD.
15
14
13
MGMS LIPOID P. per 100 cc. BLOOD.
I
II
III
DAYS
10
20
30

FIG. 4.

These graphs are typical examples of the variations which have been found in the blood lipoids during the treatment of diabetes. In all cases the lipoid phosphorus has run closely parallel to the total fatty acids, and the variation in cholesterol usually follows them, although not so closely.

The results are concordant with the theories that the phospholipoids play an active part in the transport of fat through the blood stream. Considering the average figures given in Table XIX(a) for normal bloods, a lipoid phosphorus of 11.8 mgm.% will correspond to 157 mgm. of fatty acid per 100 c.c. in the form of phospholipoid (cf. pages 62, 64) which is 57% of the total fatty acids. The remainder (119 mgm.%, or 43% of the total) is to be accounted for by cholesteryl esters, soaps, free fatty acids and triglycerides.

In comparing the fatty acids and cholesterol, it has been necessary to separate the figures from those in Table XIX since the cholesterol analyses do not always correspond to the fatty acid values recorded there. The following are the averages found in a series of 25 normal fasting cases:-

Table XX(a) /

Table XX(a).

	Maximum. mgm.%	Average. mgm.%	Minimum. mgm.%	Average deviation from the mean.
Total fatty acids	332	274	229	6.5%
Total cholesterol	210	166	130	10.3%
Ratio: Fatty acids / Cholesterol	2.10	1.65	1.50	7.7%

In a series of 100 cases of diabetes mellitus, the figures were as follows:-

Table XX(b).

	Maximum. mgm.%.	Average. mgm.%	Minimum. mgm.%	Average deviation from the mean.
Total fatty acids	2501	315	160	-
Total cholesterol	1429	217	130	-
Ratio: Fatty acids / Cholesterol	3.17	1.45	0.78	18.7%

The average ratio of fatty acids: cholesterol is/

is very near the ratio found in normal cases, but the deviation from the mean is sufficiently large to cast doubts upon the significance of any relationship between the concentrations of these two fractions. In general, however, a high fatty acid concentration is accompanied by a high concentration of cholesterol and vice versa.

The variations of total fatty acid and phospholipoids are particularly interesting in view of the role which the latter are supposed to play in the transport of fat. From a consideration of figures and graphs recorded here there is no doubt that they have a most important function in blood fat metabolism, but whether this bears any relationship to the increase in blood phospholipoids after feeding of fat to dogs, is not certain. It is most likely that the two phenomena are quite distinct, since all the evidence (as summarised in Table XVIII) goes to show that feeding of fat to human subjects does not result in an increased concentration of blood phospholipoids during absorption.

SUMMARY /

SUMMARY and CONCLUSIONS.

It has been shown that the mixed solvents, 1:1 alcohol-chloroform and 1:1 alcohol-petroleum ether, extract an amount of lipoid from blood which is strictly comparable with that extracted by the more usual 3:1 alcohol-diethyl ether. This, in itself, is an indication that Bloor's method of extraction is complete. Refluxing the blood in 3:1 alcohol-ether produces an increase in the carboxyl groups of the fatty acid fraction, which may be due either to increased extraction of lipoids yielding fatty acids upon hydrolysis, or to oxidation of double bonds present in these molecules giving rise to a more or less constant increase in the number of carboxyl groups. Strong evidence that extraction is complete, is afforded by experiments in which the blood was refluxed in an atmosphere of hydrogen, when no increase of carboxyl groups was observed. In all experiments in which the blood was refluxed, either in an atmosphere of air or of hydrogen, the so-called lipoid phosphorus remained fairly constant. The three facts together amount to conclusive proof that/

that Bloor's method of extracting blood fatty acids is entirely satisfactory.

Attention has been concentrated upon the method of estimating fatty acid by hydrolysis of lipoids, acidification of the soaps, filtration of the free acids and final estimation by titrating with standard sodium hydroxide. All the important factors have been studied and the results show that hydrolysis, as ordinarily performed with alcoholic sodium hydroxide or sodium ethylate, is complete. The various factors influencing the oxidation of fatty acids during the procedure have been discussed.

The possibility of the occurrence of water-soluble fatty acids has been investigated, and particular attention has been paid to this point since they will not be estimated by the filtration method. Water-soluble fatty acids undoubtedly occur, and the ordinary method has been modified in order to estimate them. Such a modification shows that they occur to the extent of less than 3% of the total fatty acids. This figure is rather less than has been given by other authors. The modified method is very similar to Bloor's oxidation technique, but/

but has the advantage of being more specific, for the latter will estimate any impurity which will finally be expressed as fatty acid. The hydrolysate, after addition of acid, may be either filtered or the fatty acids may be extracted with petroleum ether which will also extract water-soluble acids. Of these two alternatives, the former is preferable since it gives more reliable results and is easier and quicker to carry out.

As a preliminary to the study of blood phospholipoids, a new set of conditions has been put forward for the colorimetric determination of phosphorus by reduction of phosphomolybdic acid with 1:2:4-amino-naphthol-sulphonic acid. The most important factor is the concentration of acid present and it is found that the quantity necessary to destroy organic matter is sufficient to inhibit colour production at room temperature. The colour may be developed, however, by heating in a water-bath at 100°C. but it still depends upon the concentration of acid. Various other factors are discussed and the average error of the method is found to be less than 2% when working with quantities of phosphorus of the order of 20 γ.

The/

The validity of the use of lipoid phosphorus as a means of estimating the phospholipoids in blood, is discussed at length. It is shown that inorganic phosphate, glycerophosphate and casein are not extracted by 3:1 alcohol-ether. Furthermore, the phospholipoids have been purified by extraction of the dried alcohol-ether extract with pure ether, followed by precipitation with acetone and magnesium chloride, washing of the precipitate by pure acetone, and finally, extraction with ether saturated with water. In such a process, loss of material is unavoidable when working with such small quantities, but the loss amounts in this case to only 2-3%. Of the total phosphorus in the blood alcohol-ether extract (the so-called "lipoid phosphorus") about 94-96% appears in the final extract. The other 4-6% is lost mainly in the magnesium chloride residue and it is suggested that a small amount of decomposition may occur at this stage.

Information concerning the nature of the phospholipoids may be derived from simultaneous estimations of the phosphorus and fatty acid in the extract of purified phospholipoids. It is shown that 1 mgm. of phosphorus is equivalent to 13.12 mgm. fatty acid. If the phospholipoid were pure/

pure lecithin (or kephalin) the equivalent would be 18.2 mgm., or, if sphingomyelin were present alone, it would be 9.1 mgm. Magnesium has been shown not to interfere with the estimation of the fatty acids. The various attempts, of which all but one were unsuccessful, to remove this element revealed the fact that several other alcohol-soluble salts are incapable of completing the precipitation from acetone solution. The magnesium cannot be removed merely by precipitation with phosphate or 8-hydroxy quinoline and it is most probably in some combination with the phospholipoids. Omission of the magnesium chloride results in a considerable loss of material.

Substances which give positive sugar reactions may also occur in small amount in the purified extract - a considerable amount of this sugar is probably derived from the blood glucose for the amount of reducing material set free by hydrolysis is insignificant. It is therefore concluded that if galactosides be present at all, they occur only in very small quantity. Their estimation by means of Hagedorn-Jensen's method, is not satisfactory.

In/

In common with the other authors, it has been found that the N:P ratio is much larger than could be accounted for even on the assumption that the phospholipoid present was pure spingomyelin. The ratio, however, is lower than those previously published, since estimations were carried out on a much purer extract. There is little doubt that the purified extract contains nitrogenous substances other than phospholipoid; since it also contains sugar; the values obtained by Bloor's method of micro-oxidation must be regarded as too high, and all the evidence is in favour of their estimation via the phosphorus content.

It is concluded that the blood phospholipoids represent an almost equal amount of sphingomyelin and lecithin (or kephalin).

In a series of normal and pathological cases, it has been shown that the variations in blood fatty acid are paralleled by similar variations in blood phospholipoid. The agreement is such that any change in fatty acid is largely a function of this change in phospholipoid which is responsible for an average of 57% of the total fatty acids in normal blood. Assuming that 30% of the cholesterol is esterified, 13% of the total fatty acid will be present/

present as cholesteryl esters, and according to Stewart and White (14) about 14% of the total fatty acids are in the form of soaps. The balance (16%) must be present as glycerides or other unknown lipoid complexes.

The cholesterol in pathological cases appears to run fairly closely to the fatty acids but the evidence of any correlation between these two is uncertain.

These relationships are shown both by a consideration of the average values in a large series of cases, and also, by a study of the variations in individual cases.

REFERENCES.

1. W.R.Bloor. J. Biol. Chem. 1928, 77, 53.

2. J.L. Stoddard and P.E. Drury. Ibid. 1929, 84, 740.

3. C.P. Stewart, R. Gaddie and D.M.Dunlop. Biochem. J. 1931, 25, 733.

4. E.B.Man and E.G. Gildea. J. Biol. Chem. 1932-33, 99, 43.

5. M.E.Smith and M.C.Kik[illegible]. Ibid. 1933, 103, 391.

6. W.R.Bloor. Ibid. 1914, 17, 377.

7. C.P. Stewart and A.C.White. Biochem. J. 1925, 19, 840.

8. D.H. Collins. Quart. J. Med. 1933, 2, 267.

9. E. Backlin. Biochem. Z. 1930, 217, 483.

10. W.R.Bloor. J. Biol. Chem. 1915, 23, 317.

11. Idem. Ibid. 1916, 24, 447.

11a. I. Bang and J. Forsmann. Beitr. chem. Physiol. 1906, 8,238.

12. R. Okey. J. Biol. Chem. 1930, 88, 367.

13. /

13. M. Yasuda. J. Biol. Chem. 1931, 92, 303.

14. C.P.Stewart and A.C.White. Biochem. J. 1929, 23, 1263.

14a. W.H.Howell. Amer. J. Physiol. 1912, 31, 1.

15. H.J.Channon and G.A.Collinson. Biochem. J. 1929, 23, 663.

16. E.B.Man and E.G.Gildea. J. Biol. Chem. 1932-33, 99, 61.

17. J.P. Peters and D.D. van Slyke. "Quantitative Clinical Chemistry", 1932, vol. 2.

18. I. Bang. Biochem. Z. 1918, 91, 86, 235.

19. A. Fleisch, Ibid. 1926, 187, 453.

20. G. Blix. Skand. Arch. Physiol. 1926, 48, 279.

21. J. Vasilewska. Chem. Zbl. 1925, 1, 2715.

22. W.R.Bloor. J. Biol. Chem. 1929, 82, 273.

23. E.M. Boyd. Ibid. 1931, 91, 1.

23a. W. Lintzel and G. Monasterio. Biochem. Z. 1931, 241, 273.

24. W.R. Bloor. J. Biol. Chem. 1915, 22, 133.

25 /

25. R.D. Bell and E.A.Doisy. J. Biol. Chem. 1920, 44, 55.

26. G. Déniges. C.R. Acad. Sci. Paris, 1921, 17, 1974.

27. C.H.Fiske and Y. Subbarow. J. Biol. Chem. 1925, 66, 375.

28. E.J. Baumann. Ibid. 1924, 59, 667.

29. H. Maclean and I.S. Maclean "Lecithin and the Allied Substances". Longmans, Green and Co. 2nd edition, 1927.

30. V.C.Myers and E.L. Wardell. J. Biol. Chem. 1918, 36, 147.

31. M.E.Turner. Ibid. 1931, 92, 495.

32. E. Obermer and R. Milton. Biochem. J. 1933, 27, 345.

33. H. Maclean. Ibid. 1912, 6, 333.

33a. D.M. Greenberg et al. Ibid. 1933, 100, 139.

34. T.F. Zucker. Proc. Soc. Exp. Biol. Med. 1919-20, 17, 89.

35. M. Bodansky. Ibid. 1930-31. 28, 628.

36. B. Vahlquist. Biochem. J. 1931, 25, 1628.

37 /

37. A. Knudson. J. Biol. Chem. 1917, 32, 337.

38. I. Bang. Biochem. Z. 1918, 91, 104, 111.

39. C.W.McClure and M.E.Huntsinger. J. Biol. Chem. 1928, 76, 1.

40. A. Hiller, G.C. Linder, C. Lundsgaard, and D.D. van Slyke. J. exp. Med. 1924, 39, 931.

41. I. Greenwald. J. Biol. Chem. 1915, 21, 29.

42. I.H.Page, L. Pasternack and M.L. Burt. Biochem. Z. 1930, 223, 445.

43. H.C.Eckstein. J. Biol. Chem. 1925, 62, 743.

EXPERIMENTAL: PART II.

Arsenic in Keratin Tissues.

Arsenic in Keratin Tissues.

The keratin tissues, hair, nails and skin, are of considerable importance in connection with arsenic poisoning from a physiological as well as a medico-legal point of view. In many of the published cases, the presence and distribution of arsenic has been of major importance in the investigation, and it seemed to be worth while obtaining experimental information on certain points in connection with the effects of arsenic on these tissues. It is obvious that a knowledge of the mechanism of arsenic fixation may give important insight into the body's defence against the poison, and that examination of hair in certain cases of arsenical poisoning may be expected to yield information of great value.

The experimental work presented here was undertaken as a direct result of the problems presented by the Hearne case in 1930, where the finding of arsenic in the hair of the deceased led to much controversy. From the medico-legal aspect it would seem that the finding of arsenic in the

hair, and especially if it were found there in greater quantities than in the other tissues, would be strong evidence of the administration of that poison; but before the analyses may be so interpreted, it is necessary to enquire into a number of other possibilities. For example, one must decide whether arsenic found in the hair may have been derived from liquids bathing the shaft or whether it must necessarily come, via the bulb, from the body fluids bathing the hair root. The importance of such a point may be illustrated by reference to two cases.

In the Seddon case the victim died about fourteen days after the first dose of arsenic and the poison was found not only in the proximal (3 mgms. per 100 gm. hair) but also in the distal (0.2 mgms. per 100 gm. hair) part of the hair which was about 12 inches long. The presence of arsenic in the distal three inches of hair raised the presumption that arsenic had been administered about a year before death. Willcox, however, suggested that the arsenic in the distal ends might have been absorbed by the blood-stained body fluids which had come into contact with the hair, and showed

by/

by *in vitro* experiments that such an explanation was possible. On the other hand, in the Hearne case the presence of arsenic in the hair was interpreted as indicating the administration of arsenic over a considerable period, whereas it may well have been due to contamination from water which had soaked into the coffin after percolation through the arsenic-containing soil.

It is well known that arsenic may be found in the hair after all traces of it have disappeared from other organs of the body, but it is desirable to ascertain whether the quantity found indicates a preferential absorption or merely a trapping of arsenic in non-vascular tissue. A consideration of the results dealt with in the Report of the Royal Commission on Arsenical Poisoning (1) shows that the keratin tissues do take up more arsenic, weight for weight, than any of the other tissues of the body. A number of individuals were given 5 mgm. of arsenic per day for a period of two months. On examination, the hair of these men was found to contain from 2-5 mgm. of arsenic per 100 gm. The total quantity of arsenic taken by each individual

was/

was approximately 300 mgms. and if this amount (without allowing for excretion which would probably account for the greater part of it) be supposed to be distributed equally over the body, the concentration would be about 0.5 mgms. per 100 gms. of body weight, i.e. about one-fifth of the quantity actually found in the hair. Similar figures can be shown for nails and to a lesser extent, for skin. It is this preferential absorption by keratin tissues which has been the subject for study.

Experimental

All analyses were carried out by means of the electrolytic Marsh apparatus, which is shown in the following diagram:

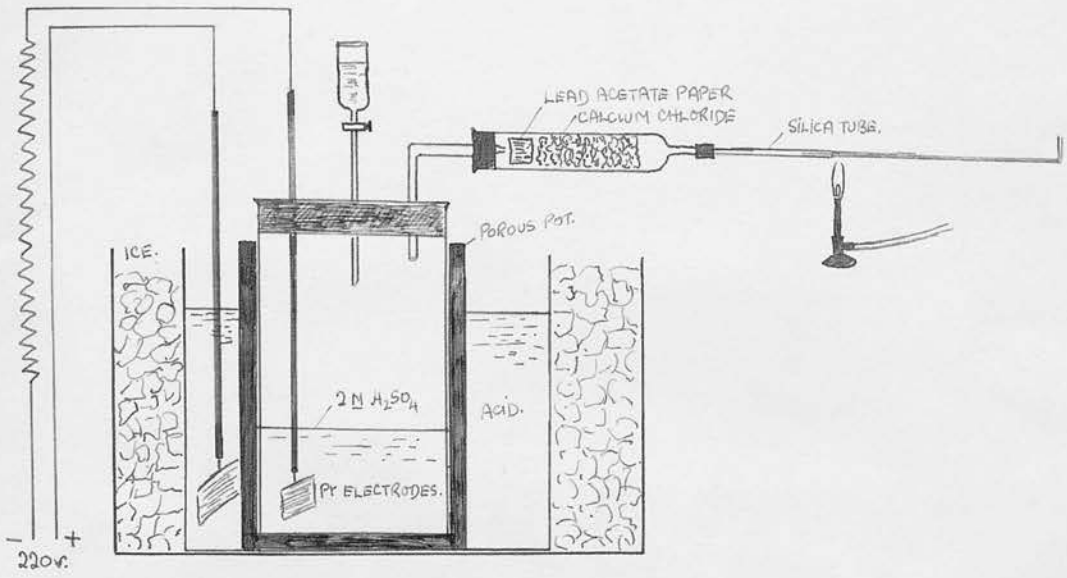

FIG 5. THE ELECTROLYTIC MARSH APPARATUS.

There are several points of importance about the manipulation of this piece of apparatus. In the first place, all reagents must be absolutely free from arsenic. It was for this reason that the ordinary method of generating hydrogen by the action of dilute hydrochloric acid on zinc had to be abandoned, for no specimen of zinc out of several different samples could be relied upon to give a complete blank during the half hour necessary for the experiment. The electrolytic method is much cheaper/

cheaper and more convenient to handle. At the start of the experiment, the porous pot is filled with sufficient sulphuric acid (2N) to cover the negative electrode completely, since one of the many causes of explosions is a small spark developed by an imperfect join in the platinum foil connection. The drying tube contains a roll of lead acetate paper followed by about 1.5 inches of granulated calcium chloride. This calcium chloride must be renewed after every experiment since it soon becomes capable of absorbing small quantities of arsenuretted hydrogen (2). The combustion tube is made of transparent silica and the tubes must all be of approximately the same diameter.

After the current has been started, at least 10 minutes must be allowed to elapse before the jet of hydrogen may be safely lighted and it is advisable to wait another five minutes before addition of the material to be analysed, so that all the air may be removed and the formation of a film of arsenic oxide avoided. The material is admitted by means of the tap funnel, care being taken to allow no air to enter, and washed in with two portions of distilled water. The experiment is allowed to run for a further/

further half hour when the maximum yield of arsenic is obtained.

Destruction of Organic Matter.

The best method of destroying organic matter is to char with sulphuric acid followed by oxidation of the carbon by means of concentrated nitric acid. In this way it is possible to obtain a water-clear solution from liver, skin, hair, nails, gut etc., which contains all of the arsenic. Anything up to 5 grams of the material is placed in a 3½ inch porcelain basin and heated with about 5 c.c. of pure, concentrated sulphuric acid. When the material is thoroughly charred, concentrated nitric acid is carefully added, 0.5 c.c. at a time. Before addition of the nitric acid, the temperature should be lowered so that the acid is not immediately driven off without having had time to attack the carbon. Several additions of acid are necessary, and as much as 15 c.c. may be required for one analysis. Finally, a dark brown, water-clear solution is obtained. Excess of the oxidising agent must be removed and this is carried out by boiling the mixture with about 5 c.c. of water.

Nitric/

Nitric acid is never completely removed by heating with pure sulphuric acid alone.

Before addition of the mixture to the Marsh apparatus, the arsenic must be reduced to the trivalent state. Several reducing agents have been employed, but the one most frequently mentioned in the literature is stannous chloride. Two serious objections have been found to this reagent. In the first place, it has been exceedingly difficult to obtain a sample which is free from traces of arsenic. In the second place, towards the end of an estimation in which stannous chloride is used, there appears a black deposit very close to the flame in the combustion tube. Unlike the arsenic film, this deposit cannot be removed by moving the flame along the tube. If this film be heated in an atmosphere of oxygen, or air, it instantly turns white and the tube is ruined, for this white deposit apparently combines with the silica and cannot be removed. The black film from stannous chloride is not soluble in bleaching powder solution, and in fact, is a deposit of metallic tin and gives all the commoner reactions of/

of that metal. The current of hydrogen carries over tin in the form of the hydride - SnH_4.

For the purposes of reducing the arsenic and removing the last traces of nitric acid, the introduction of about a gram of solid potassium metabisulphite has been found to be very satisfactory. It is necessary, of course, that all the bisulphite be decomposed by boiling the acid solution before Marshing, otherwise hydrogen sulphide is generated and gives rise to a dense yellow film of sulphur.

Preparation of Standards.

Standard films are prepared from a solution of sodium arsenite obtained by dissolving the calculated amount of arsenious trioxide in dilute sodium hydroxide. The solution used contains 2 gamma of arsenic per c.c. and from it, standard films are prepared containing 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 γ of arsenic. The smallest quantity of arsenic which can be detected by this method is of the order 0.5 γ and films containing more than about 12 γ are too dense to be of much use for accurate comparison. The films, if sealed off with wax at both ends, will keep for about 3 months provided they are stored in

a/

a cool place. With sufficient practice it is possible to read comparisons to within about 0.5 γ so that the limit of accuracy is about 5%. However, this accuracy can be reproduced regularly and it is seldom that duplicates vary by a greater amount. Before starting the analyses proper, it is, of course, essential to show that all reagents used are completely free from arsenic. In general, the A.R. reagents which have been used in the course of this work give no trace of a film after a blank analysis has been running for three-quarters of an hour.

Having shown as a preliminary experiment that hair soaked in a solution of sodium arsenite will absorb arsenic, it is desirable to ascertain whether, under such conditions, the hair can reach or surpass the concentration of arsenic in the surrounding medium. For this purpose, a series of tubes were set up each containing 0.5 gm. of hair and 10 c.c. of a solution of sodium arsenite containing 10 γ of arsenic per c.c. The liquid was decanted from each at definite intervals and an aliquot was analysed. The hair was at the same time removed

and/

and quickly rinsed with six portions of distilled water, being allowed to drain as completely as possible after each washing. It was then destroyed by the method outlined above, and an aliquot of the material analysed. The results are shown in Table I where the density of human hair is taken to be 1.00. Since part of the absorbed arsenic might conceivably be due to simple imbibition of the arsenite solution, a known weight of hair (0.3036 gms.) ~~of hair~~ was allowed to soak in water for 72 hours; at the end of this period, it was removed, dried as thoroughly as possible with filter paper, but after this treatment it still felt damp. The increase in weight was only 0.0221 gms.(7.3%). When it was allowed to stand in the balance case for one half-hour, by which time it was apparently quite dry, the increase was reduced to 0.0040 gms. (1.3%). This increase is neglected in the calculations.

Table I /

Table I.

Length of time of soaking in hrs.	As remaining in 10 c.c.of solution γ	As found in the 0.5 gm. of hair γ	Conc.of As in the solution	Conc. of As in the hair	%age As recovered
0	100	nil	-	-	-
20	90	12	9 γ/c.c.	24 γ/c.c.	102
44	80	15	8 "	30 "	95
118	70	25	7 "	50 "	95
145	80	25	8 "	50 "	105
234	70	25	7 "	50 "	95

The hair absorbs arsenic in increasing amounts gradually attaining a concentration of arsenic about seven times that of the surrounding medium. It may be remarked that the general figure for the concentration of arsenic in hair appears to be a time-maximum. It is greater than the figure hitherto reported: it is unaltered by increasing the time of soaking beyond 72 hours, and is not increased by soaking the hair in a stronger solution of arsenite.

In/

In the cases quoted in the Royal Commission Report, the level of the arsenic in the hair runs fairly uniformly at about 2-3 mgm. per 100 gm. not only in those cases where small doses have been given over a long period, but also in cases where the hair has been examined at a long interval after a single dose. In all the published cases of arsenic poisoning in which the hair has been analysed, the level of arsenic concentration runs about the same figure.

In the case of human nails, a correction has to be applied for the uptake of water. An original weight of 0.330 gm. of nails increased by 38.2% after 144 hours soaking and after standing in the balance-case for 3 hours, this increase was reduced to 17.6%. The results obtained by soaking nails in an arsenical solution are shown in Table II where the density is taken to be 1.20 and a correction has been applied on the assumption that 0.5 gm. of nails take up 0.19 c.c. of the solution or 1.9 γ of arsenic by simple imbibition.

Table II /

Table II

Length of time of soaking in hrs.	Conc.of As left in solution γ per c.c.	Conc. of As in the nails γ per c.c.	Conc.of As in the nails (corrected) γ per c.c.	Percentage of As recovered
0	10	nil	-	-
67	7	84	79	105
93	6	84	79	95
116	7	107	103	115
188	1.8	192	185	98

It is a remarkable fact that the nails take up arsenic to a concentration of about 100 times that of the surrounding medium and in these experiments, had not even then reached a maximum.

Evidently both nails and hair, removed from the body and soaked in a solution containing arsenic in the form of sodium arsenite, can absorb that element until its concentration is far in excess of the surrounding medium. In other words, they exhibit/

exhibit preferential absorption. The fact that this absorption is not simply a case of surface adsorption is shown by the following experiments. 0.5 Gm. of hair was soaked for several days in sodium arsenite solution; a series of tubes being put up in this manner. Thorough washing, instead of mere rinsing makes no difference to the arsenic content, nor does rapid washing with a dilute solution of sodium hydroxide. Washing in several changes of alkali solution removes a small part of the arsenic but this is only to be expected. Prolonged soaking in water will also remove part of the arsenic but not all of it, as is shown in Table III. In these experiments the hair which had taken up its maximum amount of arsenic, was allowed to soak in distilled water which was changed every 24 hours. It was finally rinsed several times before being destroyed for analysis.

Table III /

Table III.

Length of time of soaking	Percentage of the original As removed
71 hours	35
91	60
91	58
144	72
212	72
333	59
358	69

The importance of these observations is apparent, for it has been customary to consider a higher concentration of arsenic in the keratin tissues as proof that the arsenic was absorbed by these tissues during life; that assumption is shown to be false.

Under the conditions of the experiments recorded above, the arsenic is evenly distributed along the length of the hair, but if the poison is being absorbed into growing hair or nails from living body fluids, a different distribution is to be/

be expected. At first, arsenic should be found only in the proximal end. As the hair grows either (1) an arsenic-containing band grows with it, in the case of the poison being administered over a short period, being followed by an arsenic-free proximal zone, or (2) in the case of long continued administration or continued presence of arsenic in the blood owing to slow excretion, more and more of the hair will contain arsenic until eventually, through cutting or falling, the distribution becomes uniform. In the majority of cases of poisoning one would expect to find more arsenic in the proximal end of the hair than in the distal, but for a variety of reasons, this distribution is not absolute. For one thing, the relative lengths of the arsenic-containing and the arsenic-free parts will vary from case to case with the time of administration of the poison, the rate and amount of excretion in the vomitus, urine and faeces, the rate of growth of the hair, and the time and frequency of hair cutting. Moreover, as Willcox (3) has pointed out,"sweat and sebaceous secretion contain arsenic, and as the whole of the length of the hair comes in contact with these secretions, the limitation of arsenic to the/

the proximal portions of the hair is relative, not absolute". However, in cases of absorption in vivo some such distribution may generally be expected, and the finding of more arsenic in the proximal parts than in the distal is strong evidence that the arsenic has been absorbed from body fluids - provided it can be shown that no external contamination can account for it. The expectation of a uniform distribution of arsenic in hair is dependent on the supposition that the arsenic remains fixed and can neither travel along the shaft nor be re-absorbed by the body. Experiments to justify this supposition have hitherto been lacking.

The following experiments were carried out in vitro to determine whether or not arsenic could travel along a hair fibre. A lock of human hair about 10 cm. long and held in a vertical position was placed so that the cut ends were allowed to dip to the extent of about 4 cm. into a solution of sodium arsenite containing 0.1 mgm. of arsenic per c.c. The solution was contained in a test tube and the hair arranged round a tightly fitting cork so that 5 cm. of it were completely out of contact with/

with the fluid. Within 24 hours it was observed that the hair above the stopper was damp for a length of about 1.5 cm. It was allowed to stand thus for three weeks and at the end of that period was divided into five approximately equal sections. These were weighed and analysed without previous washing. An inspection of the hair showed that it was moist for a length of about 2 cm. but owing to evaporation, the solution had not travelled further. The results of analyses are shown in Table IV.

Table IV.

Weight of section in gm.	Total As found γ	Notes
0.144	500	Section nearest the solution - very wet.
0.122	150	Damp
0.104	trace	Slightly moist in one part.
0.100	0	Section quite dry.
0.067	0	Section furthest from the solution. Quite dry.

Two important points emerge from these results. The hair not immersed in the liquid, but wetted/

wetted with it by capillary action actually contains more arsenic than the hair in contact with the solution (which has been shown to be, at the most, about 5 mgm. per 100 gm. of hair). This is due to the continuous evaporation of the arsenical fluid and consequent deposition of arsenic on the outside of the hair fibre. Secondly, local contamination at the proximal end has resulted in that gradual distribution which is to be expected if absorption takes place from the body fluids; but it is evident that it may equally occur after death, if, by any chance, the hair is soaked in an arsenical solution at the proximal end only.

In order to prevent the solution travelling up the outside of the fibre, the above experiment was repeated with a thin, unbroken layer of paraffin wax laid round the top of the cork. The hair was again allowed to stand for three weeks with the cut ends dipping into a solution containing 0.1 mgm. arsenic per c.c. No moisture was visible outside the tube and the 3 cm. of hair, each weighing 0.1 to 0.2 gm. nearest the liquid contained no arsenic. This/

This experiment was repeated several times, always with the same result.

The experiments indicate beyond doubt that local wetting of the hair shaft with arsenite solution causes arsenic to be absorbed over a length of hair by creeping of the solution and absorption from outside. They show equally definitely that travel of the arsenic along the inside of the fibres does not take place, and that therefore the graduated distribution of arsenic is legitimately to be attributed to *in vivo* absorption, only if contamination from some external liquid source can be definitely excluded. They suggest, moreover, that arsenic once deposited in hair and nails is finally lost to the body - a suggestion which is *a priori* indicated by the fact that hair shafts, the keratin layers of epidermis, etc. are really dead tissues, and are, indeed, excretions. However, there appears to be a division of opinion on the subject for whereas Willcox (3) states that "it is possible that during the prolonged administration of arsenic a certain amount becomes deposited in the skin, hair, and/

and nails, that this is gradually re-absorbed, being excreted by the urine and faeses", Althausen and Gunther (4), on a basis of a study of excretion of arsenic before and after treatment with sodium thiosulphate, have shown that arsenic is not reabsorbed from the hair.

In the early experiments, the fact that it was possible to rinse arsenic-soaked hair several times with water and even with dilute caustic soda solution without appreciable loss of arsenic, suggests that reabsorption of arsenic from hair to the living body is at any rate a slow and difficult process. Quantitative experiments on this point, however, showed that arsenic-soaked hair containing the maximum of 5 mgm. per 100 gm. could lose part of the arsenic after prolonged soaking in distilled water. After six days soaking in this manner, about 70% of the arsenic had been removed. Further soaking would not remove the other 30%. (Table III).

It was found that arsenic in the hair of a person receiving arsenic injections was not removed to any appreciable extent by prolonged soaking in distilled water. Table V shows analyses in a typical/

typical case. The patient had received a total of 4.3 gm. of arsenic in the form of tryparsamide over a period of 18 weeks.

Table V.

Length of time of soaking in hours.	Mgm. As per 100 gm. hair.	
0	0.36 mg. %	Av. of 3 analyses
96	0.30 "	Av. of 2 analyses
116	0.36 "	Av. of 3 analyses

This suggests the basis of a method of differentiating between arsenic absorbed in vivo and arsenic derived from some external source of contamination, a point of great medico-legal interest.

It is not easy to account for this difference in behaviour between arsenic derived from the body and that coming from an external source. It probably lies in the greater opportunity of the former for becoming chemically fixed in the keratin tissues during their formation, whereas the arsenic absorbed from outside can, at the most, interact with only the surface layers and may therefore be more easily removed.

SUMMARY.

It has been shown that the keratin tissues, hair and nails, when soaked in arsenical solution exhibit the phenomenon of preferential absorption of arsenic. The arsenic so absorbed can be partly removed by very prolonged soaking in distilled water, but rapid rinsing with either distilled water or dilute caustic soda solution does not affect the arsenic content. By this method the maximum arsenic content of hair is found to be about 5 mgm. per 100 gm. and much higher values can be obtained in the case of nails.

In vitro experiments have shown that arsenic can travel along a hair fibre only if "creeping" of the solution along the outside of the fibre is possible.

It has been demonstrated that arsenic absorbed into hair *in vivo* cannot be removed by prolonged soaking, and this fact is tentatively put forward as the basis of a method of distinguishing between arsenic absorbed *in vivo* and arsenic resulting from external contamination.

Details of the method of destroying organic matter previous to analysis, and the accuracy of the Marsh test using the electrolytic process, are also discussed.

REFERENCES

1. Royal Commission Report on Arsenic Poisoning, 1904.

2. Dr Dyer, The Analyst, 45, 1920, 17.

3. W.H. Willcox. Brit.med. J. 22nd July,1922.

4. T.L. Althausen and L. Gunther. J. Amer. med. Ass. 1929, 2002.