CCXIV. DETERMINATION OF SMALL AMOUNTS OF PHOSPHATIDES AND CHOLESTEROL IN TISSUES¹.

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(Received October 24th, 1931.)

ALTHOUGH there have been many publications on methods of estimating phosphatides and cholesterol in tissue, the value of many of the methods described remains uncertain. In some cases extraction of lipid substances may be incomplete. Not all of the phosphorus in extracts may be derived from phosphatides. Colorimetric methods of doubtful validity [Nekludov and Chalatov, 1929] are still in use for determining cholesterol. While microgravimetric methods have been worked out for pure cholesterol, they have received little application to tissue-extracts.

Before undertaking analyses of tissues we have thought it advisable to make an investigation of methods of analysis. This is reported here with necessary details, together with such results of the analyses as are of methodical interest.

An account of analytical methods is necessary to make it possible to assess the value of the analytical data given in an accompanying paper [Jowett, 1931]. Some results of interest in themselves have also been obtained.

Extraction of lipid substances from tissues.

In work carried out some years ago in which one of the authors took part, dried tissues were extracted with chloroform². While such a solvent has the advantage that it is likely to extract little phosphorus beyond that contained in phosphatides, an impression prevails that chloroform and ether may not give complete extraction of lipid substances.

We have therefore tested this point. Fresh rabbit liver was minced, rubbed in acetone, and dried as described later. After exhaustive extraction

¹ This investigation was undertaken on behalf of the Liverpool Medical Research Organisation (Director: Prof. W. Blair Bell).

² This method was adopted from Bullock and Cramer [1913-14], who in turn appear to have followed Smith and Mair [1911, 1913]. Dr Cramer kindly sent us a description of the methods used. The results obtained by us have been summarised in several places, *e.g.* Bell [1925, 1930] and Lewis [1927]. They are not further detailed in the accompanying paper [Jowett, 1931], since they are less accurate than the data given there.

in a Soxhlet apparatus with chloroform, the tissue was extracted with absolute alcohol¹. The alcohol extract was taken to dryness and re-extracted with chloroform. If alcohol is assumed to complete the extraction of lipid substances, the results show that chloroform extracts only about 82 % of the lipoids from rabbit liver.

With other tissues the proportion of the lipoids extracted by chloroform may be as low as 50 %. This is suggested by a comparison of two series of estimations on human uterine muscle, which has a fairly constant composition. In the first series, made some years ago with chloroform, the average total lipid content found for five specimens was 3.5 %. In the present work with absolute alcohol as solvent, five specimens gave an average value of 7.2 % for total lipoids.

In the present work we have used absolute alcohol to extract lipid substances from tissues and as the result of some preliminary experiments decided to extract the tissues without drying them thoroughly.

The tissue for analysis, weighing from 5 to 20 g., is cut up, and gross fat, connective tissue and so forth removed as far as possible. It is then finely minced in a Latapie type mincer of small size. The minced tissue is weighed after removing a portion for dry weight determination, and then covered with 20 to 80 cc. of acetone or absolute alcohol, the tissue being well stirred up immediately to avoid caking. When convenient, the material is poured into a Soxhlet thimble of double thickness, and washed with alcohol. The filtrate is retained. The thimble is mounted with wire in a conical flask under a reflux condenser, and its contents are extracted three times, for an hour each time, with boiling absolute alcohol. Fresh alcohol is used for each extraction.

The combined extracts are taken to dryness, alcohol being first distilled off, and the operation then being finished at as low a temperature as possible in a stream of carbon dioxide or nitrogen. If the temperature is too high the lipid material obtained will not dissolve completely in chloroform; if removal of alcohol is incomplete, the chloroform extract may be turbid. The dried alcohol extract is extracted by boiling with three successive portions of chloroform for a few minutes, and the filtered chloroform extract kept for analysis.

The alcohol extraction is fairly complete. In two cases the tissue after alcohol extraction was dried, ground up and extracted with ether for 3 hours. The weight of the ether extract amounted to an additional 0.5 % for some malignant tissue, and 2.4 % for a sample of muscle.

Estimation of dry weight and total lipid content of tissues.

Estimation of dry weight. The minced tissue is stirred with a glass rod to a uniform paste, and a portion of about 0.5–1.0 g. removed to a small, light

¹ All solvents used in this work were of good quality; before use they were dried with calcium chloride (quicklime in the case of alcohol), and redistilled using a twelve-pear column.

weighing-bottle. After being weighed, the tissue is covered with acetone, and the acetone is afterwards roughly evaporated off in a stream of nitrogen or carbon dioxide. The bottle is then dried to constant weight in the vacuum oven over sulphuric acid at 40° ; this requires about 5 days. Bottles were weighed on a micro-balance to 0.01 mg., and the change in weight of a bottle to which pure acetone was added was applied as a correction. The weights of empty bottles were reproducible within 0.2 mg., and the weights as corrected are probably more accurate than this. This would suggest that dry weight estimations are accurate within about 0.2 %, but difficulties of sampling and of reproducing the degree of dryness may make the accuracy less than that figure.

Estimation of total lipoids. The chloroform extract from a specimen of tissue is brought to a volume of 100 cc. immediately before an estimation. This is necessary, as chloroform is very expansible and volatile.

A measured volume of the extract is transferred by pipette to a weighing-bottle, which is then immersed in a water-bath at $50-55^{\circ}$, and a stream of carbon dioxide led over the surface of the liquid until at least 10 minutes have elapsed after evaporation of chloroform appears to be complete. After being wiped, the bottle is transferred to a desiccator containing calcium chloride and weighed soon afterwards. Every time an estimation was made, a bottle was put through the same process with pure chloroform, and its change of weight was applied as a correction to the other changes; no regular change of weight took place, and the change was usually less than 0.3 mg. The weight of lipoids taken for an estimation was from 20 to 100 mg. Duplicate estimations, which were always made, checked very closely.

Fresh estimations of the total lipid content of the chloroform solutions were always made when further volumes of the solutions were taken for analysis¹.

The estimation of phosphatides.

It is uncertain that phosphatides are the only phosphorus-containing substances extracted from tissues by absolute alcohol, and we are relying on phosphorus estimations to determine phosphatides. We therefore undertook experiments with the object of separating phosphatides on the small scale from other phosphorus-containing substances.

According to Nerking [1910] acetone does not precipitate lecithin completely. Nor, according to experiments made by us, does cadmium chloride in alcoholic solution completely precipitate small amounts of lecithin.

Nerking states, however, that magnesium chloride can precipitate lecithin completely in an acetone-ether mixture, and we commenced experiments to test this. The work of Bloor [1929] came to our notice soon afterwards, and

¹ Methods of handling small quantities of liquids are described by Szent-Györgyi and Tominaga [1923], and we have found these helpful in designing our methods.

most of our work took the form of testing the modification of Nerking's method described by this author.

Estimation of phosphorus. Our estimations of phosphorus have been made by the colorimetric method of Briggs [1924].

The method was modified by keeping the quinol and the sodium sulphite in the same solution, 0.5 % quinol in 10 % sodium sulphite. 2 cc. of the solution are added for a total volume of 10 cc.

Extensive testing with inorganic phosphate solutions showed us that an accuracy of about 1 % can usually be obtained, when the amount of phosphorus is within some 30 % of that in the standard, which is best taken at 0.06 mg. P (as potassium dihydrogen phosphate). The Klett top-reading colorimeter, provided with a lamp, was used. Preliminary estimations are necessary when the amount of phosphorus to be estimated is quite unknown.

Estimations of organic phosphorus were made after ignition with sulphuric acid and hydrogen peroxide. The chloroform solution is taken to dryness in a 6-inch thick-walled test-tube in a stream of air, 0.3 cc. of concentrated sulphuric acid is added, followed by 0.5 cc. of Merck's perhydrol previously redistilled under low pressure. The ignition is carried out over a small flame, more peroxide being added as necessary, and the tube being rotated to rinse the walls towards the end of the ignition. After addition of water, the acid is neutralised approximately with sodium hydroxide.

Tests on the ignition of known amounts of inorganic phosphate gave complete recovery. With lipid phosphorus the reproducibility was often within 1 %, and usually within 3 %.

Experiments with pure lecithin and magnesium chloride. We shall report experiments only in which the precipitation took place under the conditions used by Bloor, as the variations tried did not greatly affect the results.

From 1 to 3 cc. of an alcoholic solution of lecithin¹ was taken to dryness in a centrifuge-tube, and dissolved in 2 cc. of light petroleum and 7 cc. of acetone. Then was added 0.1 cc. of a saturated solution of hydrated magnesium chloride in absolute alcohol. After thorough stirring of the precipitate—precipitation of the salt takes place independently of the presence of lecithin—it was centrifuged down, the supernatant liquid poured off, and the precipitate washed with 2 cc. of acetone.

Phosphorus was estimated in the supernatant liquid and wash liquid; it was usually necessary to unite the liquids from several precipitations and to add some inorganic phosphate after ignition, in order to obtain enough phosphorus to estimate.

A "blank" correction was necessary; when no lecithin was used in the precipitation and washing, phosphorus was apparently present in the supernatant and wash liquids.

¹ The lecithin was made according to the method of Levene and Rolf [1927] by Dr H. I. Price, to whom we are indebted for it.

The figures obtained are given in Table I.

Table I. *Precipitation of lecithin by magnesium chloride.*

Mg. P taken	Mg. P not precipitated	Mg. P (blank)	Corrected mg. P not precipitated	% of P not precipitated
0.111	0.0086	0.0022	0.0064	6
0.333	0.0231	"	0.0209	6
0.114	0.0099	"	0.0077	7
0.114	0.0096	"	0.0074	7
0.342	0.0435	"	0.0413	12
0.113	0.0080	"	0.0058	5
0.113	0.0108	"	0.0086	7
0.067*	0.0045	0.0026	0.0019	3
0.067*	0.0047	"	0.0021	3
0.134*	0.0085	"	0.0059	4
0.134*	0.0058	"	0.0032	2
0.067*	0.0037	0.0024	0.0013	2
0.067*	0.0039	"	0.0015	2

* For these estimations 200 mg. of lecithin were precipitated with MgCl_2 and washed as in the micro-estimations. The precipitate was extracted with ether and chloroform, and the extract finally dissolved in chloroform together with a little alcohol.

Evidently about 93 % of the lecithin is precipitated, or about 97 % when the lecithin has been purified.

Experiments were then made to test whether the reason for incomplete precipitation was solubility of the lecithin in the precipitant mixture. Magnesium chloride precipitates, after the usual washing, were washed again with a mixture of 7 cc. of acetone and 2 cc. of light petroleum, and phosphorus estimated in this liquid. When 0.113 mg. of lecithin-P was taken initially, and two washings made, the uncorrected P from two washings was about 0.0061 mg. as a mean. Subsequent pairs of washings gave about the same value. The value suggests that most of the lecithin that remains unprecipitated does so on account of its solubility.

According to Bloor the lecithin may be extracted from the magnesium chloride precipitate with moist ether, solution being, however, a slow process. The water in the ether dissolves the magnesium chloride to form a separate phase.

We have found it very difficult to extract all the lecithin with ether. It does not dissolve completely, but can be decanted suspended in ether. From three to five washings with ether are necessary. The recoveries of phosphorus were then 88, 96, 91 and 89 % of that taken. These values are moderately satisfactory, but the procedure is too laborious to be a practical method.

Extraction with chloroform was then tried: the solvent was brought to boiling-point and extraction made three or four times. This is a simpler method, but the recovery is variable. The percentage recoveries found were 88, 86, 85, 96, 94, 89, 88, 83, 75, 92 and 78.

We have also tried estimating the phosphorus in the magnesium chloride precipitate by igniting this directly. The recovery is usually from 95 to 102 %, but the ignition in presence of magnesium chloride is lengthy and laborious.

Our conclusion is that the most satisfactory way of using the magnesium chloride method is to estimate total phosphorus and subtract from it phos-

phorus not precipitated by magnesium chloride. This variant has been used in the work of the following section.

Experiments with lipid mixtures and magnesium chloride.

The magnesium chloride precipitation has been made on tissue extracts, containing phosphatides, cholesterol and fats.

The data obtained are given in Table II. "Blank" corrections were not applied, and if the behaviour were the same as it is for pure lecithin about 5 % of the phosphorus would have remained unprecipitated.

Table II shows that in some cases the percentage not precipitated is much higher. It is highest when the fat content of the extract is high (shown by a high value of the ratio of total lipoids to total phosphorus).

Table II. *Precipitation in lipid mixtures.*

Tissue extract	Total lipoids	% P not precipitated
	Total P	
2 A	125	26.0
2 B	317	20.0
2 C	343	18.5
6 B	175	15.2
5 B	43	11.3
5 A	50	9.3
7 B	41	8.6
3 A	48	5.9
5 C	33	5.6

Now there is no special reason to believe that, in the presence of fat, alcohol will extract from tissues non-phosphatide-phosphorus that would otherwise remain unextracted.

The most probable conclusion is that fat interferes with the precipitation of phosphatides by magnesium chloride.

If this is so, Bloor's method for estimating phosphatides will tend to give low results, quite apart from the fact that the ether extraction in our hands has not given complete recovery.

Conclusion. We have been forced to assume that the whole of the phosphorus in our extracts represents phosphatides. The assumption is probably not much in error, if we may judge from a comparison between the results of the magnesium chloride method on pure lecithin and on tissue extracts containing little fat. This comparison confirms the general impression that anhydrous solvents extract little phosphorus-containing material other than phosphatides.

We have calculated the phosphatide content of our extracts on the basis that the phosphorus content of phosphatides is 4.00 %. This figure may be a little too high—Levene and Rolf [1927] find about 3.9 % for pure lecithin, and Levene and West [1916] about 3.7 % for kephalin—but the error introduced should tend to offset any error in assuming that all the phosphorus found represents phosphatide.

The phosphorus percentage will vary somewhat in phosphatides from

different tissues, but the available data suggest that the variation is not great. The objections that have been raised to assuming a constant composition seem to have been over-emphasised.

The estimation of cholesterol.

The gravimetric method, in which cholesterol is combined with digitonin, has been used.

The compound has been precipitated in a manner similar to that employed by Szent-Györgyi [1923]. A quantity of material containing from 0.5 to 3 mg. of cholesterol is taken to dryness in a 10 cc. centrifuge-tube, dissolved in 2 cc. of acetone, and 1 cc. of digitonin solution added (1 g. of commercial digitonin dissolved in 50 cc. of 80 % alcohol). After being stirred and warmed, the liquid is evaporated to about one-half in a stream of air. After about 15 minutes the precipitate is washed in the centrifuge-tube, in a manner not very different from that of Tominaga [1925], twice with 7 cc. of ether, and the supernatant liquid poured off into the filter-tube. Three washings are then given in the heat with 7 cc. (each time) of a mixture containing 80 % of acetone and 20 % of water. Of the wash-liquids it is only the 80 % acetone that removes much of the excess digitonin.

The precipitate is then washed on to the filter with about 10 cc. of pure acetone, followed by 6 cc. of ether. The filter-tube is then left in an electrically heated oven at 105–110° for an hour. On removal it is capped and left in a calcium chloride desiccator for 20 minutes, after which it is weighed.

Experiments were first made with Jena sintered glass filter-tubes. Unfortunately the filters with the finest pores (G 4) often became clogged with the precipitate, while those with larger pores (G 3) allowed the precipitate to pass through to some extent. The use of sintered glass filters was therefore abandoned.

The type of filter-tube described by Szent-Györgyi [1923] was employed, but lighter caps were used which were flat at the top and had somewhat flattened sides. The filter-tubes weighed, complete, about 5 to 6 g. The asbestos used was Powminco Grade xx; a little solid asbestos was followed by an aqueous suspension, and stronger suction succeeded gentle suction. After washing with water, acetone and ether the tubes were heated and then weighed; this procedure was repeated until the weight became approximately constant. The weight usually becomes constant within 0.04 mg., but further washing would result in further loss of weight.

Similarly when cholesterol digitonide is on the filter, continued washing leads to a larger and continued loss of weight. Any method of using the filter-tubes has to be established empirically.

To test the method described above a series of estimations was made on pure cholesterol¹. A solution in absolute alcohol containing 1.021 mg. per cc.

¹ We are indebted for the cholesterol to Mr W. Doran, Department of Organic Chemistry, University of Liverpool.

was used to obtain the data in Table III. The average deviation of the estimations from the "theoretical" value (taking the precipitate to contain 25 % of cholesterol) is about 3 %. Tominaga claimed an accuracy within 1 %. Probably we could have obtained greater accuracy if every filter had been prepared with extreme care, many being rejected, and if those accepted had been used for only a few estimations. We have used a filter about eight times before renewing the packing.

Table III. *Weight of digitonide (mg.) from cholesterol.*

	1 cc.	2 cc.	3 cc.
	4.04	8.50	11.95
	3.91	8.66	12.58
	3.96	8.12	—
	4.11	7.94	—
	3.87	—	—
Mean	3.98	8.30	12.26
Theoretical	4.08	8.17	12.25
Average percentage deviation from theoretical	2.8	3.4	2.6

Estimations on tissue extracts. Although several papers have been published on estimation of small amounts of cholesterol by the gravimetric method, and the methods were apparently designed for estimating cholesterol in tissue extracts, the method seems to have been little applied to that purpose.

Our experience of the application of the method has not been very satisfactory.

Free cholesterol. When much fat in relation to cholesterol is present, the precipitate is contaminated with fat. We have not judged it wise to remove, prior to the estimation, the fat which often precipitates on cooling a hot acetone solution, as this may contain cholesterol. The fat is removed by washing the precipitate as described, removal being most rapid in the heat.

An examination of the results of a series in which two estimations were made on each of a number of tissue extracts showed that the presence of much fat is associated with less accurate agreement between the results. When the ratio of total lipoids to cholesterol was over 20 (averaging about 50), the average difference between the two values was 8.7 %. With lower values for the ratio (about 8–12), the average difference between the two values was only 4.3 %. Evidently the presence of much fat leads to irregular values.

When several estimations on the same tissue extract have been made with different quantities, there is a certain tendency for lower relative values to be found, the more the material taken for an estimation. This appears to indicate that fat tends to inhibit formation of a digitonide precipitate that is retained by the filter.

Total cholesterol. A portion of the tissue extract is taken to dryness, and then there is added to it 1 cc. of ether followed by 1 cc. of 15 % sodium ethoxide in absolute alcohol. After being warmed to dissolve the lipid material, the solution is allowed to stand at least 24 hours. According to

Gardner and Fox [1924], hydrolysis of cholesterol esters should then be complete. The solution is taken down to dryness, and 2 cc. of 2*N* sulphuric acid are added. After shaking, the material is extracted four times with ether in the test-tube used. The ether extract is evaporated to dryness.

Estimation of cholesterol is then carried out in the same way as for free cholesterol, except that the extract has been dissolved in 2 cc. of ether instead of acetone. Ether was used instead of acetone with a view to lessening the interference of fatty material with the precipitation, as most of the fat then stays in solution. The precipitate of digitonide is more flocculent with ether.

Preliminary estimations, made to test whether the use of ether gives the same results as acetone on free cholesterol, are given in Table IV. The data also serve to show the kind of reproducibility the methods give. The acetone and ether methods give very similar results, as far as the data go. Further tests would be worth while.

Table IV. *Comparison of acetone and ether methods on free cholesterol.*

	mg. precipitate acetone method	mg. precipitate ether method
Pure cholesterol	12.79	{12.56 12.67
Tissue extract containing much fat	{6.87 6.64	{7.03 7.09
Tissue extract containing little fat	{8.16 8.01	{7.50 7.82
" "	{8.52 8.84	8.78
" "	{12.94 13.47	{13.42 12.90

An examination of results, similar to that made in the case of free cholesterol determinations, showed that the extracts containing much fat did not tend to give more irregular results than the others.

The series shows an average difference of 5.4 % between the results of the two estimations on each extract; for the free cholesterol series the corresponding figure was 5.8 %. When the estimations on pure cholesterol in Table III are taken in pairs the average difference between the results for the two members of each pair is 4.0 %. The mean result of each pair of estimations diverges on the average from the theoretical value by 1.8 %, or by half the previous figure.

The values obtained from the pairs of estimations on free and bound cholesterol in tissues should therefore be on the average less than 3 % from the true value, apart from the existence of a systematic error, such as a tendency of fat to produce low values. It must be pointed out, however, that further repetitions of some of the estimations sometimes show much larger discrepancies.

Comparison with other work. Osato and Heki [1930] have made microgravimetric estimations of cholesterol in tissues, but do not yet appear to have published details of their method. The only other work on this subject

we are aware of is that of Onizawa [1928], who extracted cholesterol from tissues by a modification of Fox's method, thereby removing fats. His results on pure cholesterol and on tissues are apparently more reproducible than ours. The only apparent difference in the estimation of cholesterol is that this author washed his precipitates finally with water and dried them in a vacuum oven, as did Szent-Györgyi. Our results suggest that removal of fat prior to the estimation should lead to greater reproducibility.

SUMMARY.

Methods are described for the extraction and estimation of phosphatides and cholesterol in 5–20 g. of tissue.

Chloroform does not extract lipid substances completely from dried tissues. Magnesium chloride precipitates pure lecithin almost completely, but the presence of much fat appears to interfere with precipitation of phosphatides. The analytical method described by Bloor [1929] probably gives low results. The phosphorus in our extracts is probably almost entirely derived from phosphatides.

The micro-gravimetric method has been tested on pure cholesterol and applied to tissue extracts. Much fat lessens the accuracy of free cholesterol determinations, but in the presence of ether does not appear to interfere with estimations of total cholesterol.

In conclusion the authors wish to acknowledge their indebtedness to Prof. W. C. M. Lewis for his interest in this work, and to Prof. H. J. Channon for some helpful advice.

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