ON THE PURIFICATION OF PHOSPHATIDES

By HUGH MACLEAN.

*From the Lister Institute, Bio-Chemical Department**(Received June 19th, 1912)*

In the preceding paper on 'The Phosphatides of the Kidney,' it has been shown that the chief part of the lipoid occurring in the alcoholic extract of horse kidney is not a diamino-monophosphatide as described by Erlandsen¹ in heart muscle, but a monamino-monophosphatide which contains nitrogenous impurities. The phosphatide was freed from these impurities by repeatedly emulsifying with water and adding some acetone when the lecithin separated out, leaving the contaminating substance in solution. In order to obtain fairly complete separation of the phosphatide from the water-acetone mixture, it is necessary to have some salt present, otherwise acetone fails to precipitate the lipoid from its aqueous emulsion; a trace of sodium chloride suffices, and it is only necessary to add a few drops of a weak solution of this substance to the water used for the emulsion in order to ensure good separation. It is important to make the emulsion with water which contains traces of sodium chloride, for the addition of salt after the emulsion is formed gives very unsatisfactory results.

The substance can be very easily emulsified by adding at first a very little water and rubbing up thoroughly in a mortar; more water is gradually added and after each addition the whole is well rubbed up. In this way a very fine emulsion approaching to a colloidal solution is formed, and the best results are obtained when great care is taken to have this emulsion as fine as possible. Both the nitrogen impurity and lecithin are precipitated by acetone, but very much less suffices to separate the lecithin than is required for the precipitation of the other substance; only the minimum quantity of acetone necessary to separate the phosphatide is added. The lecithin rises to the top of the liquid and can be easily skimmed off, while any of the impurity that may be precipitated remains suspended in the liquid and gradually falls to the bottom of the vessel. This separation of the phosphatide is by no means quantitative, and the water-acetone solution always contains a certain amount of lecithin. With care, however, the loss in this way is comparatively small.

As the method of purification had been tested only on kidneys which had been already extracted with ether, some experiments were made on other tissues. At the same time an extension of the kidney experiments was carried out. Since the phosphatide present in the ether extract was the same as that contained in the subsequent alcoholic extract, it is obvious that the primary ether extract, the secondary alcoholic extract, and a control alcoholic extract, without primary treatment of the tissues with ether, should on purification all give a substance with an approximate nitrogen to phosphorus ratio as 1 : 1. This proved to be the case.

KIDNEY EXPERIMENTS

Some kidneys were minced and dried in the usual way. Two portions of the dried substance were then extracted as follows:—

- (a) With ether.
- (b) With alcohol.
- (c) With alcohol after ether (a extracted subsequently with alcohol).

(a) *Ether extract*

The ethereal extract was treated as before described in order to separate fat, cuorin and cholesterol. The final lipoid obtained was then subjected to the acetone-water purification, dried and analysed. The results show that the substance was a lecithin.

Analyses

0.4242 gm. used 6 c.c. N/10 H_2SO_4 = 1.9 per cent. Nitrogen.

0.2814 gm. used 21 c.c. N/2 NaOH = 4.1 per cent. Phosphorus.

N : P = 1 : 1.

(b) *Alcohol extract without ether*

The dried tissue was extracted six times, first at room temperature, then at 40° C. and the different extracts mixed together. On standing some time a white precipitate formed which was filtered off. The clear extract was now evaporated to small bulk at 40° C. under reduced pressure. On adding ether to the residue a very muddy liquid was obtained, which was treated with excess of acetone in order to get rid of cholesterol and fat. The precipitate obtained from the alcohol-ether mixture was treated several times with cold acetone and finally dried in vacuo over H_2SO_4 . This crude substance was dissolved in a small amount

of ether and centrifuged, when a white precipitate was obtained. The precipitate was discarded and the clear supernatant fluid treated with acetone. The precipitate was again thoroughly extracted with acetone, dissolved in alcohol and left to stand in the ice-room overnight. A precipitate formed which was filtered off. This process was repeated until a concentrated alcoholic solution produced little or no precipitate when left to stand in the ice-room. The alcohol was then evaporated off and the residue extracted with ether. A fair amount of a slimy substance (κ) remained behind at the bottom of the vessel. The ether solution was now treated with acetone and the precipitate dried. It was then emulsified and precipitated with acetone eight times, extracted with acetone, dried and analysed. It gave the following figures.

Analyses

0.4211 gm. used 6.2 c.c. $N/10 H_2SO_4 = 2.0$ per cent. Nitrogen.

0.2204 gm. used 16.5 c.c. $N/2 NaOH = 4.1$ per cent. Phosphorus.

$N : P = 1.08 : 1.$

This substance was therefore a monamino-monophosphatide and so far agreed with that obtained from the ether extract.

(c) Extract with alcohol after ether

The different processes utilised for the separation of the phosphatide are the same as described for the alcohol extract above. The final product obtained was treated with water and acetone eight times.

Analyses

0.6241 gm. used 9.5 c.c. $N/10 H_2SO_4 = 2.1$ per cent. Nitrogen.

0.3216 gm. used 24 c.c. $N/2 NaOH = 3.9$ per cent. Phosphorus.

$N : P = 1.2 : 1.$

A comparison of these three substances shows that the analytical figures are practically the same for all three preparations. The slight differences are not greater than can be accounted for by experimental error.

COMPARISON OF THREE PREPARATIONS

	Ether extract	Alcohol extract	Alcohol extract after ether	
			I	II*
Nitrogen ...	1.9	2.0	2.1	1.97
Phosphorus...	4.1	4.1	3.9	4.07
$N : P$ ratio ...	1 : 1	1.08 : 1	1.2 : 1	1.07 : 1

* Sample described in paper on 'The phosphatides of the kidney.'

The results prove that the chief phosphatide of the kidney is lecithin or a lecithin-like substance, and that the method of purification adopted is capable of separating off the impurities associated with this phosphatide.

MUSCLE

Experiments similar to the above were carried out on horse muscle, and three substances corresponding to the fractions described above were obtained.

- (a) Ether extract.
- (b) Alcohol extract without ether.
- (c) Alcohol after ether.

The treatment of the muscle tissue prior to the separation of the different fractions was almost entirely the same as that described for kidney. It is interesting to note that while much 'white substance' was present in the kidney alcoholic extract, little or none of the diamino-monophosphatide was found in muscle.

(a) *Ether extract.*

The phosphatide present, after being separated in the usual way and purified three times with water and acetone, gave the following figures.

Analyses

0.6212 gm. used 8.5 c.c. N/10 H_2SO_4 = 1.9 per cent. Nitrogen.

0.2164 gm. used 15.5 c.c. N/2 NaOH = 4.0 per cent. Phosphorus.

N : P = 1.05 : 1.

(b) *Alcohol extract without ether.*

The crude alcohol extract here contained a great amount of a slimy substance insoluble in ether, similar to a corresponding substance mentioned under kidney. The final impure phosphatide obtained was purified with acetone and water in the usual way, and samples examined at different stages in order to determine how many times it was necessary to emulsify the substance. The crude product, which contained originally nearly 4.0 per cent. of nitrogen, gave the following results:—

Substance emulsified and precipitated once.

Analyses

0.3562 gm. used 5.7 c.c. N/10 H_2SO_4 = 2.24 per cent. Nitrogen.

0.4245 gm. used 30 c.c. N/2 NaOH = 3.9 per cent. Phosphorus.

N : P = 1.3 : 1.

Thus a single treatment reduced the nitrogen percentage from

4 per cent. to 2·24 per cent., and gave a substance having a N : P ratio nearly the same as that of lecithin.

Although this sample of crude phosphatide had been prepared in the usual way, previous to the emulsification treatment it contained over 50 per cent. of its weight of a substance soluble in water. This was obtained from the water-acetone solution by evaporation = substance (κ). The following results were obtained after the phosphatide had been purified four and eight times respectively.

Substance emulsified and precipitated four times.

Analyses

0·3802 gm. used 5·3 c.c. N/10 H_2SO_4 = 1·95 per cent. Nitrogen.

0·2609 gm. used 18·7 c.c. N/2 NaOH = 4·14 per cent. Phosphorus.

N : P = 1·04 : 1.

Substance emulsified and precipitated eight times.

Analyses

0·3750 gm. used 5·1 c.c. N/10 H_2SO_4 = 1·9 per cent. Nitrogen.

0·1790 gm. used 12·8 c.c. N/2 NaOH = 3·97 per cent. Phosphorus.

N : P = 1·06 : 1.

From these results it appears that the first purification by this method is sufficient to remove all but a small amount of impurities. Naturally it becomes much more difficult to get rid of these traces, but the substance obtained after four precipitations was just as pure, judging from the N : P ratio as that obtained after eight precipitations. If care is taken to make a good emulsion, it is probable that only traces of impurities remain after four successive treatments. A fine emulsion is essential in order to provide access of the water to impurities enclosed in the fatty globules.

(c) *Alcohol extract after ether.*

This was purified six times in the usual way and gave the same figures as the other samples.

Analyses

0·6428 gm. used 8 c.c. N/10 H_2SO_4 = 1·8 per cent. Nitrogen.

0·5069 gm. used 3 c.c. N/2 NaOH = 3·9 per cent. Phosphorus.

N : P = 1·02 : 1.

The chief phosphatide of muscle is therefore a lecithin, and the lipid

of the alcoholic extract is the same as the alcohol soluble one present in the ether extract—a monamino-diphosphatide. In muscle a great amount of water soluble substance is present in the alcohol extract, but this does not appear to be a lipid, though it is precipitated by excess of acetone from both its alcohol and aqueous solutions. A comparison of the analytical figures with those obtained from the purified lecithin of kidneys suggests that the phosphatide present in both cases is the same.

On the nature of the nitrogenous impurity

As already described, on emulsifying the phosphatide obtained from the alcohol extract of muscle and precipitating with acetone, a considerable amount of material remained in solution. This substance was dark in colour, sticky to the touch, and exceedingly tenacious; on drying it formed a hard substance, but could not be powdered. It was exceedingly soluble in water, and in alcohol containing traces of water, but much less soluble in absolute alcohol. It appeared to be absolutely insoluble in ether, by which it was precipitated from solution as a dark, sticky mass. On adding some alcohol to its clear alcoholic solution, a faint white precipitate formed, causing an opalescence which soon cleared up again.

Analyses

Nitrogen—

0.4202 gm. used 18 c.c. N/10 H_2SO_4 = 6.0 per cent. Nitrogen.

0.2231 gm. used 9.8 c.c. N/10 H_2SO_4 = 6.15 per cent. Nitrogen.

Phosphorus—

0.3513 gm. used 9.1 c.c. N/2 NaOH = 1.44 per cent. Phosphorus.

Another sample gave 6 per cent. of nitrogen and only 0.5 per cent. phosphorus, so that it is probable that the latter is present as an impurity. On standing, its aqueous solution deposited small minute white round balls which were but slightly soluble in cold water, but dissolved easily on heating. The substance was practically insoluble in cold, but somewhat soluble in hot alcohol. In ether, chloroform and benzene, it was insoluble. With mercuric chloride it gave a voluminous precipitate. On recrystallising twice from water and alcohol, a pure white substance was obtained. This contained no phosphorus. Analysis gave the following figures:—

0.0934 gm. used 16.6 c.c. N/10 H_2SO_4 = 24.9 per cent. Nitrogen.

Its melting-point was indistinct, but the high nitrogen content and other reactions suggest that it is of the nature of a purin base. Further

experiments on this substance are in progress. After the separation of the white substance, the residue obtained by evaporation of the mother liquor was treated as follows:—An alcohol solution was made and ether added when a precipitate was obtained; this was repeated twice. The substance was now dissolved in water and again precipitated by acetone; the residue was then extracted thoroughly with acetone and ether and dried. On analysis it was found to contain 6 per cent. nitrogen and only about 0·3 per cent. phosphorus. As it has been found that tissues contain a substance which cures beri-beri, some of this material was given to pigeons suffering from polyneuritis in order to test its effect. The result was very marked, as three pigeons exhibiting very severe symptoms were completely cured in a short time. Partially purified lecithin had a certain effect on these pigeons, but a dose of 1 gm. of such lecithin resulted only in an amelioration of the symptoms and no cure was obtained. As many observers have placed it on record that lecithin is more or less efficient in curing beri-beri, it is probable that here we have an explanation of their results. The curative substance is not lecithin, but is present in ordinary lecithin as an impurity. It can be separated, however, by the method above described, and a lecithin so purified has but little effect in curing beri-beri. The whole subject is being investigated and the results will be published at a later date.

CONCLUSIONS

The alcohol soluble phosphatide of kidney and muscle is lecithin with a N : P ratio of 1 : 1.

(1) Though substances containing different amounts of nitrogen are obtained in the different extracts, the application of the method described—emulsification and precipitation with acetone—gives a single product of the nature of an ordinary lecithin. No alcohol soluble substance having a higher percentage of nitrogen than that of lecithin has been found.

(2) From an aqueous extract of the nitrogenous impurity of lecithin a substance of basic nature crystallises out; after the separation of this substance the mother liquor is very effective in curing polyneuritis (beri-beri).

(3) This explains the anomalous results of many observers who have endeavoured to cure pigeons suffering from beri-beri by lecithin.

REFERENCE

1. Erlandsen, *Ztsch. f. physiol. Chem.*, LI, p. 71, 1907.