L. ON THE SO-CALLED "ACETONE-SOLUBLE PHOSPHATIDES."

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Though many points relating to the nature and distribution of the phosphatides still await solution, it has recently been shown that certain bodies which were supposed to be definite substances are really nothing more than lecithin contaminated by nitrogenous impurities [MacLean, 1913]. This observation has materially simplified the phosphatide problem, and it seems probable that further research will reduce the number of these bodies to a comparatively few well-defined substances. It is now definitely established that the only alcohol-soluble and acetone-insoluble phosphatide present in the majority of tissues is lecithin. The substance described as a di-amino-monophosphatide, and obtained from ether-extracted tissues by subsequent extraction with alcohol, is not a definite substance but a mixture of lecithin with certain basic bodies. Lecithin can be easily obtained from the mixture by emulsification with water and precipitation with acetone [MacLean, 1912]. The only tissue phosphatides identified with certainty are lecithin, kephalin, cuorin and sphingomyelin, and all these substances differ from ordinary neutral fat and from fatty acids in the fact that they are insoluble in acetone.

Under certain conditions lecithin is precipitated almost quantitatively from its ethereal solution by excess of acetone, and this property is constantly made use of in the separation of the phosphatides from accompanying fats and fatty derivatives.

While it is agreed that phosphatides in general are insoluble in acetone, certain observers have described another distinct class of these bodies—*acetone-soluble phosphatides.*

Thus Fränkel and Pari [1909] extracted ox-pancreas with acetone and from the acetone extract obtained a phosphatide which was isolated as the cadmium chloride combination. This compound, which they called vesalthin, had a nitrogen to phosphorus ratio of 1 : 1 and in many ways did not materially differ from the compound formed by lecithin with cadmium chloride. It had an empirical formula represented by $C_{32}H_{63}O_9NP \,.\, CdCl_2$ and contained

$$1{\cdot}71\ \%\ \text{N and}\ 3{\cdot}78\ \%\ \text{P.}\quad \text{N} : \text{P} = 1 : 1.$$

Erlandsen [1907] obtained a somewhat similar substance from ox-heart; this substance he isolated as the platinum derivative. The dried heart tissue was extracted with ether, the ethereal extract concentrated to a syrup and excess of acetone added; on separating the precipitated lecithin the acetone solution still contained a phosphatide together with other acetone-soluble fatty bodies. On concentration of the acetone solution the phosphatide was not precipitated by the addition of more acetone but easily passed into solution. The residue obtained on evaporation of the acetone was extracted with a little alcohol and the phosphatide precipitated by the addition of an alcoholic solution of platinum chloride. The precipitate was washed with alcohol and dried in vacuo. The platinum chloride combination thus obtained had the empirical formula $(C_{33}H_{62}O_8NP)_2H_2PtCl_6$;

$$\text{N} = 1{\cdot}81\ \%,\quad \text{P} = 3{\cdot}69\ \%;\quad \text{N} : \text{P} = 1{\cdot}09 : 1.$$

Now since lecithin has approximately the formula $C_{43}H_{80}O_9NP$ with 1·8% nitrogen and 4% phosphorus, it is obvious that the phosphatides of the above compounds of Fränkel and of Erlandsen contain a good deal more nitrogen than is present in lecithin. In fact the nitrogen percentage of the above *compounds* is almost exactly the same as that of *free* lecithin, while lecithin-cadmium-chloride contains about 1·4 % nitrogen and 3·90 % phosphorus.

Since it has been shown that lecithin very often contains impurities of a basic nature together with phosphorus compounds, it was possible that the above acetone-soluble phosphatides were nothing but impure lecithin which, owing to some physical or chemical change, had become soluble in acetone.

In order to test this, an ethereal extract of dried ox-hearts was made in the usual way. The greater part of the ether was evaporated, the lecithin precipitated by the addition of a large excess of acetone and the acetone filtrate evaporated to a thick syrup. This syrup contained large amounts of nitrogen and phosphorus indicating the probable presence of a phosphatide, but on the addition of more dry acetone no precipitate whatever was obtained.

This acetone was evaporated under reduced pressure and the syrup obtained allowed to flow slowly drop by drop into a large bulk of acetone in a tall cylinder. For each cc. of syrup about 200 cc. of acetone were taken. Immediately on the addition of the first few drops the liquid became hazy and then opalescent. After adding the calculated amount of syrup, the cylinder was shaken thoroughly, when a well-marked bulky flocculent precipitate separated out, leaving the yellowish tinged acetone quite clear. After standing overnight the supernatant fluid was separated, the acetone again evaporated and the syrup tested for nitrogen and phosphorus. As both were still present in considerable amount, the syrup was again added drop by drop to excess of acetone. Again a slight haziness appeared, followed by the formation of a small amount of flocculent precipitate. A third repetition of this process gave a very slight precipitate, but on the fourth attempt, the acetone to which the syrup was added remained perfectly clear, not a trace of precipitate being produced. On evaporation of the acetone the residue still gave distinct reactions for nitrogen and phosphorus. For purposes of description later on this residue will be called *syrup M*.

The different precipitates obtained above were collected and dissolved in a small amount of ether. On addition of acetone a well-marked precipitation at once took place and the substance behaved in all respects like lecithin. On analysis, however, the nitrogen content was found to be much too high, so the syrup was purified by emulsification with water and precipitation with acetone [MacLean, 1912]. A considerable amount of some basic impurity together with some phosphorus was separated off. The purified phosphatide was then dissolved in ether, precipitated with acetone and dried in vacuo over H_2SO_4. Analysis gave

$$N = 1{\cdot}81\ \%\ ;\quad P = 3{\cdot}96\ \%\ ;\quad N : P = 1{\cdot}01 : 1.$$

It was soluble in ether, alcohol, chloroform and benzene but quite insoluble in acetone. Its alcoholic solution was precipitated by cadmium chloride and by platinum chloride. On hydrolysis, choline was obtained. Its behaviour, in all respects like ordinary lecithin, left no doubt that this "acetone-soluble" substance was really lecithin.

An attempt was now made to investigate the substance present in the acetone syrup M mentioned above. When added drop by drop to pure dry acetone not the faintest haze appeared. Since it is well known that the presence of electrolytes often plays an important part in the precipitation of lecithin, it was thought that the addition of a trace of electrolyte to the

acetone might give a precipitate. Since the acetone used in these experiments was carefully dried over calcium chloride and distilled immediately before use, no electrolyte could be present. A trace of calcium chloride was now added to the acetone and the syrup run in drop by drop as before. Immediately a marked opalescence appeared, followed by a very distinct precipitate. This process was repeated as described above until no more precipitate could be obtained. The final acetone solution, on evaporation to a syrup, was found to be *free from nitrogen and contained only a very faint trace of phosphorus.* Thus the whole of the phosphatide was precipitated by acetone in the presence of a minute quantity of an electrolyte.

The different precipitates were mixed together, dissolved in ether, filtered and precipitated by acetone. The precipitate obtained was purified in the usual way with water and acetone, dissolved in ether, again precipitated with acetone and dried in vacuo. Analysis gave:

$$N = 1{\cdot}86\ \%\ ;\quad P = 3{\cdot}98\ \%\ ;\quad N : P = 1{\cdot}03 : 1.$$

In solubilities and other physical and chemical properties, it behaved exactly as ordinary lecithin. The whole of the phosphatide present in the original solution was therefore lecithin.

In another experiment, two ox-hearts were extracted with alcohol, the alcohol evaporated, the residue taken up with ether and precipitated with acetone as described. The acetone solution was allowed to stand in the ice-chest over night when a slight precipitate formed. This was filtered off, the acetone evaporated and the residue added drop by drop to excess of acetone containing *a trace of calcium chloride.* The proportions taken were 4 cc. syrup to 100 cc. acetone. A very marked flocculent precipitate was obtained. On separating the precipitate, evaporating the acetone and repeating the above process, another well-marked precipitate separated. On the other hand when a small amount of the syrup was added to pure acetone free from electrolytes the fluid remained quite clear.

The process was repeated six times, when it was found that practically no substance separated on the addition of the syrup to the acetone containing calcium chloride.

The syrup now obtained on the evaporation of the acetone contained 0·025 % phosphorus and 0·362 % nitrogen, *i.e.*, N : P = 11 : 1. The original syrup was divided now into parts:

A	*B*
Residual syrup which gave no precipitate when added to excess of acetone containing an electrolyte.	Precipitate obtained by adding syrup to acetone containing an electrolyte.

Syrup A. From the relation of the nitrogen to phosphorus it was obvious that only a small portion of the nitrogen, if any, was present in phosphatide form. On addition of an alcoholic solution of cadmium chloride no precipitate was obtained unless excess was added, when a slight opalescence occurred. It cleared up immediately on the addition of excess of alcohol. The same phenomenon occurred when platinum chloride in alcoholic solution was added.

When three drops of a 1 % alcoholic solution of lecithin were mixed with 5 cc. of the syrup, the addition of a few drops of cadmium chloride gave a distinct precipitate which did not disappear in excess of alcohol. Thus when a trace of lecithin was added the behaviour of the syrup was entirely different from that of the control, showing that the substance originally present was not lecithin. The reactions of the syrup with cadmium chloride and platinum chloride were exactly the same as those obtained with a basic water-soluble impurity which I had previously shown to be constantly associated with tissue lecithin [MacLean, 1913].

On extracting the syrup three times with water, the aqueous solution contained all but a trace of the nitrogen of the syrup, while no phosphorus could be detected. The part insoluble in water gave no precipitate whatever on the addition of cadmium chloride or platinum chloride in excess and was practically free from nitrogen. It is, therefore, clear that the nitrogen of the syrup was present in some water-soluble combination which contained no phosphorus or at most only a trace of phosphorus. This entirely excludes phosphatides.

Precipitate B obtained by adding syrup to acetone containing an electrolyte.

This substance was dissolved in ether and precipitated with acetone. It was then purified three times with acetone and water, and a water-insoluble residue obtained. The acetone-water solutions were mixed together and evaporated to a syrup. Substance *B* was thus divided into two parts:

(1) A portion insoluble in water.

(2) A water-soluble portion.

(1) *The water-insoluble portion.*

This constituted the greater part of the substance *B*. It was soluble in alcohol, ether, chloroform and benzene. From its ethereal solution it was precipitated almost quantitatively by acetone even in small amount,

It contained choline and its physical characteristics appeared identical with those of lecithin. On analysis it gave:

$$N = 1{\cdot}85\ \%;\quad P = 4{\cdot}05\ \%;\quad N : P = 1{\cdot}01 : 1.$$

From its analyses and reactions there can be no doubt that the substance was ordinary lecithin.

(2) *The water-soluble portion.*

This had all the properties of the water-soluble substance first described. It was insoluble in ether, soluble in alcohol containing a trace of moisture, but practically insoluble in water-free alcohol. On hydrolysis, no fatty acids were produced. Analysis gave:

$$N = 2{\cdot}05\ \%;\quad P = 0{\cdot}43\ \%;\quad N : P = 10 : 1.$$

A third experiment carried out with an old extract of heart on the lines indicated gave similar results. A substance was obtained from the acetone extract which had all the reactions of lecithin. Analysis gave:

$$N = 1{\cdot}89\ \%;\quad P = 3{\cdot}94\ \%;\quad N : P = 1{\cdot}05 : 1.$$

From these results it is clear that the ' acetone-soluble ' phosphatide described as obtained from ox-heart is really ordinary lecithin associated with a nitrogenous impurity. This lecithin is easily precipitated by acetone when certain electrolytes are present, but in the absence of these electrolytes the presence of fatty acids and fats renders it soluble in acetone. When separated, it behaves in all respects like ordinary lecithin both towards acetone and other reagents.

It is very probable that all phosphatides are insoluble in acetone, and that an application of the above methods to other tissues would result in eliminating from the literature certain substances which are supposed to be individual phosphatides, but which are really nothing more than impure lecithin.

Summary.

(1) The so-called acetone-soluble phosphatide of heart is impure lecithin. This substance can be easily separated from accompanying fats and fatty acids by means of acetone *containing a small amount of some electrolyte, such as calcium chloride.* In this condition it is associated with a basic impurity. When purified it has all the reactions of lecithin and is quite insoluble in acetone.

(2) The basic impurity is not a phosphatide; it is soluble in water and contains no fatty acids, while only a small and variable amount of phosphorus is present.

(3) It is probable that all acetone-soluble phosphatides described in the literature are merely lecithin contaminated with the nitrogenous impurity mentioned.

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