CL. THE ETHER-SOLUBLE SUBSTANCES OF CABBAGE LEAF CYTOPLASM.

IV. FURTHER OBSERVATIONS ON DI- GLYCERIDEPHOSPHORIC ACID.

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In a previous paper [Chibnall and Channon, 1927] it was shown that the ether extract of cabbage leaf cytoplasm contains very little, if any, phospholipin, in the commonly accepted use of this term. The major part of the ether-soluble phosphorus was present as the calcium salt of a diglyceridephosphoric acid of the type

Sufficient evidence was adduced to substantiate this structural formula, but the small amount of material then at our disposal did not enable us to obtain all the information which we considered was necessary for establishing the relationship of this acid to the two commoner phospholipins, lecithin and kephalin.

During the present summer we have prepared nearly 60 g. of the crude calcium salt from about 220 kg. of cabbage leaves, and have been able to investigate further two of the questions that had perforce been left open.

(1) In all our earlier work on this diglyceridephosphoric acid we had been troubled by the persistence with which small amounts of iron and nitrogen clung to what were considered purified products. We have now prepared this acid free from iron by dissolving it in a small volume of cold absolute alcohol. There remains undissolved a substance containing 8.9 % of iron and no nitrogen, which is extremely soluble in ether and is completely precipitated from ethereal solution by the addition of only one volume of alcohol. This explains why our previous efforts to purify the diglyceridephosphoric acid

by precipitation of its lead salt from ethereal solution by alcohol were unsuccessful. The yield of this unknown iron-containing substance is extremely small, 1.1 g. from 220 kg. of leaves. In view of the known connection in chlorotic leaves between chlorophyll synthesis and iron, we are undertaking a further investigation of this substance.

Although we have thus prepared the diglyceridephosphoric acid free from iron, we are still unable to remove the small amount of nitrogen (0.11 %). As to the nature of the substance giving rise to this nitrogen, we have no information. The natural suggestion would be that lecithin or kephalin was responsible for it. From the method of preparation, it seems improbable that the nitrogen was due to the presence of lecithin. In the preparation of the diglyceridephosphoric acid, the material precipitated from ether by acetone was repeatedly extracted with boiling acetone to remove certain other substances, the nature of which will be subsequently discussed. This treatment would tend to remove lecithin in the small amounts in which it could be present, for lecithin has a definite solubility in that solvent. Further, in this and previous work, we have purified the diglyceridephosphoric acid by repeated precipitation of its lead salt from ether solution by alcohol. This treatment would certainly have removed lecithin, and it seems probable that the small amount of kephalin which would be necessary to give rise to this percentage of nitrogen would have been removed also. The diglyceridephosphoric acid is also very soluble in cold acetone. In addition, as was previously reported, we have been unable to obtain at any time a precipitate with alcoholic cadmium chloride, and the nitrogen after the baryta hydrolysis in the preparation of the barium glycerophosphate remains insoluble in water. Hence we have no evidence whatever in favour of this 0.1 % of nitrogen being due to the presence of lecithin or kephalin, and it seems probable that there is some other ether-soluble nitrogen-containing substance present. This question must remain open until material is available in greater bulk.

(2) The nature of the fatty acids resulting from hydrolysis of the diglyceridephosphoric acid required more detailed investigation. We have now been able to show that the saturated acids are probably stearic and palmitic and that the unsaturated acids are linolic and linolenic. Oleic acid has not been identified, though that acid may be present in small amounts. A distinguishing feature is the low proportion of saturated acids. Levene, in his papers on lecithins and kephalins of animal origin, has shown that the acids consist of equal molecular proportions of saturated and unsaturated acids. But his more recent work on soya bean phosphatides has shown that this relationship may not hold in the plant, for his soya bean lecithin contained a low proportion of saturated acids. This is likewise the case with the diglyceridephosphoric acid. In the first place, the high degree of unsaturation of the mixed acids (iodine value, 143) is greater than that found in the acids from liver lecithins, which are characterised by the presence of arachidonic acid, which has four double bonds. We have found no trace of an acid more un-

saturated than linolenic in the mixed acids under discussion. In the second place, the fractionation of 13.2 g. of the mixed acids by means of the lead soaps yielded 2.85 g. of solid acids of iodine value 44, and 9.35 g. of liquid acids of iodine value 173. The major part of the latter fraction must have been linolic acid, for only 2.1 g. of hexabromostearic acid, equivalent to 0.8 g. of linolenic acid, were subsequently isolated from it. Therefore, the amount of saturated acids remaining in the liquid acids must have been small, and there is no doubt that the proportion of saturated acids in the original mixed acids was considerably less than one half.

The further evidence submitted in the present paper confirms our original contention that the free acid has the structural formula already given. It is therefore necessary to consider in some detail the class of substances to which this free acid and its salts are to be assigned. The free acid is an oil which is freely soluble in organic solvents, but has a very low solubility in water. The sodium salt is insoluble in ether, has a low solubility in cold alcohol and is soluble in water. The barium, calcium and lead salts are plastic solids which are insoluble in water, very readily soluble in ether, and are precipitated from ethereal solution by both acetone and alcohol. These properties, together with the fact that the acid and its salts readily darken on exposure to air, are essentially those of kephalin.

It is to be remembered that Thudichum divided the substances which give rise on hydrolysis to fatty acids, nitrogen and phosphorus, into two classes, the phosphatides and the cerebrosides. Leathes [1910] put forward reasons for altering these terms to phospholipin and galactolipin respectively. Thudichum's classification [1884] was based primarily on whether or not the substance contained phosphorus. His cerebrosides were nitrogen-containing but phosphorus-free fatty substances, while his phosphatides, as their generic name indicates, contained phosphorus but did not necessarily contain nitrogen. Thus, although his kephalophosphoric acid, the existence of which has yet to be proved, was nitrogen-free, it was classed as a phosphatide.

In his discussion of Leathes' nomenclature, MacLean [1918] defines the term lipins as "substances of a fat-like nature yielding on hydrolysis fatty acids or derivatives of fatty acids, and containing in their molecule either nitrogen or nitrogen and phosphorus." The essential point of the classification of Leathes is thus the nitrogen content of the substance, and from this point of view it seems to us the less satisfactory. Since Thudichum's classification is based on the phosphorus content of the fat, *i.e.* phosphatides are "phosphorised" fats, whether they contain nitrogen or not, the substance discussed in this paper readily finds a place in it, while it remains outside that of Leathes. There is the further point that the terms phosphatide and cerebroside have been in international use since 1880 and are in use to-day. They are wider terms and can be used to embrace a greater number of similar substances than those suggested by Leathes, and until research has further elucidated the difficult question as to what further compounds of these types exist, it

seems to us preferable to keep to Thudichum's original definitions. If this be done the diglyceridephosphoric acid and its salts are phosphatides. Since the acid is chemically the parent acid of the two more commonly occurring phosphatides lecithin and kephalin, we propose to call it *phosphatidic acid* and the calcium salt found by us in the plant becomes calcium phosphatide.

As the latter may thus be regarded as the simplest of the phosphatides so far reported, the question arises as to whether it may not be the precursor of lecithin and kephalin in the animal body. It is to be remembered that kephalin preparations have been reported to be contaminated by calcium. Thus Diaconow [1867] reported that the kephalin fraction (ether-soluble, alcohol-insoluble) from eggs contained calcium which can be removed by shaking its ethereal solution with HCl, and that the acid solution contained no phosphoric acid, showing that the calcium was not present in inorganic combination. Thudichum reported that after treatment of an ethereal solution of kephalin with HCl, the latter on evaporation yielded an ash containing calcium and iron. Parnas [1909] made similar observations and stated that an ethereal solution of crude kephalin loses its fluorescence when it is shaken with HCl. This fluorescence returns if the calcium-free ether solution is shaken with lime water. There would thus seem to be sufficient evidence that calcium in chemical combination with organic material in a form which is soluble in ether does exist in those tissues from which phosphatide preparations are usually made. This ether-soluble calcium appears in the kephalin fraction and can be removed by simple shaking with dilute mineral acid. In other words, it has similar solubility properties and is acted on by mineral acid in the same way as calcium phosphatide. Further, Levene and Komatsu [1919], in their studies on cuorin, found that by careful fractionation of the phosphatide preparation from heart muscle they were able to obtain fractions which were beginning to approximate to various simpler kephalin derivatives, such as a monoglyceridephosphoric acid, etc. These may be decomposition products and this view is the one usually adopted; but there seems to us no sufficient reason why we should expect to find only pure kephalin and lecithin in such a tissue as, say, heart muscle. Because these phosphatides are extremely difficult to prepare in an analytically pure state, and because of the great ease with which they are oxidised, the conception has arisen that every so-called impurity in such preparations is the result of decomposition of the material through chemical manipulation or enzyme action. May not the alternative view be the more probable, namely, that these simpler products are normally present to some extent in such tissues, and that their presence is the cause of the variation in the N : P ratios on which the existence of so many phosphatides has been postulated, only to be overthrown on more careful examination?

Work is in progress with a view to obtaining further information as to the possible occurrence of nitrogen-free phosphatidic substances in animal tissues. Certain of the difficulties which will arise are obvious, not the least

of them being the necessity of ensuring that any such substance isolated has not arisen through the decomposition of the primary substances present. Recently a paper has appeared [Wadsworth, Maltaner and Maltaner, 1927], in which the authors claim to have obtained a nitrogen-free phosphatidic substance from heart muscle, after previous treatment of an extract with ammonia and calcium chloride. To this substance they attribute the structure of calcium phosphatide. The chemical evidence which these authors have so far produced is wholly unsatisfactory. We shall defer detailed criticism of this paper until the results of our investigations on heart muscle, which are among the tissues which we have been studying for some time from this point of view, are more complete.

In discussing the analytical data which follow, we have continued to use the figures for distearyl-glycerophosphoric acid as a basis for theoretical calculation.

EXPERIMENTAL.

Preparation of the crude calcium salt.

220 kg. leaves from winter-sown cabbage picked in May were treated as described in the earlier paper. 220 g. ether-soluble substances were extracted, from which 58 g. crude calcium salt were obtained after precipitation with acetone and extraction of the insoluble material with hot acetone.

Crude calcium salt R.

0.1008 g. substance: 0.2077 g. CO_2 , 0.0763 g. H_2O , 0.0222 g. ash.

0.2903 g. substance: 0.0043 g. Fe_2O_3 and 0.0506 g. $\text{Mg}_2\text{P}_2\text{O}_7$.

0.2902 g. substance: 0.0628 g. CaSO_4 .

0.2903 g. substance: 0.0632 g. AgI .

0.1160 g. substance required (micro-Kjeldahl) 2.0 cc. 0.01 *N* acid.

	C	H	Ca	P	Fe	Ash	Glycerol	N
Found	56.19	8.40	6.37	4.86	1.00	22.03	8.54	0.24 %

2.90 g. on saponification yielded 1.77 g. of fatty acids or 61.1 % and 0.0512 g. of unsaponifiable matter or 1.8 %.

Isolation of phosphatidic acid.

50 g. crude calcium salt in 800 cc. ether were shaken once with 400 cc. $\text{N}/2 \text{H}_2\text{SO}_4$ and four times successively with 200 cc. $\text{N}/4 \text{H}_2\text{SO}_4$ to remove the calcium. The ether solution of the free acids was shaken four times with an aqueous solution of lead acetate, the ether solution of lead salts washed twice with water, and the solution concentrated to 300 cc. *in vacuo*. Two volumes of absolute alcohol were then added and the precipitated lead salts filtered off. These were dissolved in 200 cc. ether and reprecipitated with alcohol: yield, 36.0 g.

Lead salt of phosphatidic acid R.

0.0907 g. substance: 0.1675 g. CO_2 , 0.0405 g. H_2O , 0.0288 g. ash.

	C	H	Ash
Found	50.36	7.41	31.75 %
Theory	51.47	8.14	32.30 %

When concentrated *in vacuo* the alcohol-ether mother-liquors gave a further precipitate of lead salt weighing 1.4 g., making a gross yield of 37.4 g. 34.9 g. of this lead salt in 900 cc. ether were shaken four times with $N/4$ HCl to remove the lead, washed four times with water and concentrated *in vacuo* to a syrup. 300 cc. cold absolute alcohol were then added to dissolve the phosphatidic acid. The insoluble material was removed by centrifuging, dissolved in ether and again shaken with $N/4$ HCl as before. A small amount of lead as chloride was removed, and, after washing, the ethereal solution was concentrated to 20 cc. *in vacuo*. Two volumes of alcohol were added with stirring, when the precipitated material collected as a brown gummy mass around the stirring rod. The yield of this insoluble material, which is the iron-containing material referred to in the introduction, was 1.1 g. The alcoholic solution on evaporation yielded 24.7 g. of phosphatidic acid. It is a pale olive-brown oil which flows slowly at room temperature, and clings tenaciously to traces of organic solvents. For analysis it was dried at 100° *in vacuo* for one hour, a treatment which does not affect its solubility in ether.

Phosphatidic acid R.

0.0771 g. substance: 0.1795 g. CO_2 , 0.0663 g. H_2O .

0.2346 g. substance: 0.0374 g. $\text{Mg}_2\text{P}_2\text{O}_7$.

0.2306 g. substance (Zeisel-Fanto): 0.0728 g. AgI .

0.0938 g. substance required (micro-Kjeldahl) 0.80 cc. 0.01 N acid.

0.4692 g. substance required for neutralisation 13.4 cc. 0.1 N NaOH .

0.0938 g. substance absorbed 0.1079 g. iodine (Wijs); iodine value 115.

	C	H	P	M.W. (dibasic acid)	Glycerol	N
Found	65.71	10.09	4.42	700	12.5	0.11 %
Theory	66.57	10.94	4.40	704	13.1	0.0 %

3.51 g. on saponification with alcoholic potash gave 2.76 g. fatty acids of iodine value 143 and neutralisation value 199. Yield of fatty acids 78.6 %, theory for C_{18} saturated acid 80.6 %.

The total yield of phosphatidic acid represents 50 % and of the lead salt 55 % of the total phosphorus present in the original crude calcium salt. In view of the inevitable losses it is probable that about 60 % of the original phosphorus was present as calcium phosphatidate. The nature of the other 40 % of the phosphorus needs further investigation.

Products of hydrolysis of phosphatidic acid.

1. *Glycerophosphoric acid.* 15 g. phosphatidic acid dissolved in a small volume of alcohol were emulsified with water and added slowly to 750 cc.

boiling water in which had been dissolved 15 g. barium hydroxide. After standing on the water-bath for half an hour the precipitated barium soaps were filtered off, ground up with a further 300 cc. of baryta and boiled for one hour. This operation was repeated. Excess of barium was removed from the combined filtrates with carbon dioxide, and the filtrate from the barium carbonate concentrated to 50 cc. Two volumes of alcohol were added and the precipitated barium glycerophosphate filtered off. It was redissolved in water and boiled with charcoal until the filtrate was water-clear. The optical rotation was

$$[\alpha]_{\text{Hg green}}^{20} = +1.3^{\circ} (l = 2, c = 3).$$

This rotation confirms our earlier finding, but, as we mentioned in the previous paper, we consider that Levene's lengthy lead treatment should be applied before the question of the true rotation of this substance can be answered. We have not yet accumulated sufficient material for this purpose.

2. *Saturated fatty acids.* The barium soaps from the above hydrolysis were converted into the free acids and saponified with alcoholic potash to make certain that all the phosphatidic acid had been decomposed. 10.7 g. fatty acids obtained in this way, together with 2.5 g. from a previous saponification, 13.2 g. in all, were converted into the lead soaps and extracted with ether. The ether-insoluble lead soaps yielded 2.85 g. solid acids, iodine value 44. From 2.47 g. of these acids 1.66 g. of crystalline fatty acids were prepared by fractionation from 95 % methyl alcohol. This material was further separated by means of absolute methyl alcohol into two fractions—*A*, weighing 0.80 g. and *B*, weighing 0.53 g. These analysed as follows:

A. 0.0702 g. substance: 0.1937 g. CO₂ and 0.0810 g. H₂O.

0.2855 g. substance required for neutralisation 10.55 cc. 0.1 *N* alcoholic NaOH.

B. 0.1039 g. substance: 0.2871 g. CO₂ and 0.1150 g. H₂O.

0.2351 g. substance required for neutralisation 3.47 cc. 0.25 *N* NaOH.

Calculated C₁₆H₃₂O₂: C, 74.92; H, 12.58; M.W. 256; M.P. 63–4°.

Calculated C₁₈H₃₆O₂: C, 75.93; H, 12.78; M.W. 284; M.P. 70–71°.

Found (*A*) C, 75.25; H, 12.82; M.W. 280; M.P. 57°.

(*B*) C, 75.37; H, 12.33; M.W. 271; M.P. 56°.

These results suggest that the saturated acids consist of about equal parts of stearic and palmitic acids.

3. *Unsaturated fatty acids.* The ether-soluble lead soaps yielded 9.35 g. of liquid acids, iodine value 173. An attempt was made to fractionate these acids as barium soaps by benzene containing 5 % of 95 % alcohol, but no insoluble fraction could be obtained. Prolonged centrifuging deposited a little slime which was not worth separating, showing that the original liquid acids contained a small residue of saturated acids and only a small amount, if any, of oleic acid. The free acids were regenerated from the barium soaps, and brominated in anhydrous ether at 0°. The ether-insoluble precipitate,

after well washing with ether to remove excess bromine, weighed 2.1 g. and analysed as follows:

0.1487 g. substance: 0.1582 g. CO_2 and 0.0560 g. H_2O .

0.0992 g. substance required (Stepanoff) 7.85 cc. *N*/10 thiocyanate.

Calculated $\text{C}_{18}\text{H}_{30}\text{O}_2\text{Br}_6$: C, 28.49; H, 3.99; Br, 63.26; m.p. = 180–1°.

Found C, 29.01; H, 4.18; Br, 63.23; m.p. = 180°.

The substance is hexabromostearic acid, showing the presence of linolenic acid in the original mixed fatty acids.

The brominated acids soluble in ether (12.7 g.) were concentrated *in vacuo* to a syrup and extracted with hot light petroleum. From the ether-light petroleum solution there were obtained by alternate concentration *in vacuo* and cooling crops of white crystalline material which together weighed 0.84 g. After recrystallisation from light petroleum, the material melted at 110–111°, and analysed as shown below. A sample recrystallised twice from ether melted at 113° and analysed as follows:

0.1058 g. substance: 0.1358 g. CO_2 , 0.0560 g. H_2O .

0.1023 g. substance required (Stepanoff) 6.95 cc. *N*/10 thiocyanate.

Calculated $\text{C}_{18}\text{H}_{30}\text{O}_2\text{Br}_4$: C, 36.01; H, 5.38; Br, 53.28; m.p. 113–4°.

Found C, 35.80; H, 5.89; Br, 54.41; m.p. 113°.

The substance is tetrabromostearic acid, showing the presence of linolic acid in the original mixed fatty acids.

The ether-light petroleum mother-liquor contained 5.38 g. of acids containing 45.0 % Br, showing that a small amount of oleic acid may have been present in the original mixed fatty acids.

SUMMARY.

Further observations on the calcium salt of a diglyceridephosphoric acid prepared from cabbage leaf cytoplasm have been made.

Reasons are given for considering the free acids and its salts as phosphatides.

The properties of the acid and its salts are discussed: since the acid is chemically the parent acid of lecithin and kephalin, the name phosphatidic acid has been assigned to it.

Phosphatidic acid differs from lecithin and kephalin, in which saturated and unsaturated fatty acids are present in equimolecular proportions, in that the greater part of the fatty acids present in the molecule is unsaturated.

The saturated acids are palmitic and stearic; the unsaturated acids are linolic and linolenic; oleic acid has not been identified; arachidonic acid is absent.

The possible occurrence of calcium phosphatide in animal tissues is briefly discussed.

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