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

II. CALCIUM SALTS OF GLYCERIDEPHOSPHORIC ACIDS.

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INTRODUCTION.

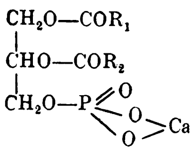
IN the previous paper the methods for preparing the ether-soluble substances of the cytoplasm from leaf cells on a large scale were described, and an account given of the general nature of the substances obtained from the green leaves of unheaded cabbage.

Determinations of the phosphorus content of the fatty material showed that this element is present in varying amounts. Thus batches *E*, *F*, *G* and *H* contained respectively 1·0, 1·6, 1·89, 1·1 % of phosphorus. It was mentioned in that communication that if four volumes of acetone be added to the solution of the ether-soluble substances in anhydrous ether in order to remove the phospholipin fraction, a large proportion of the material was precipitated. Thus from different preparations, 41, 42, 41 and 44 % of the ether-soluble material was precipitated in this way. Although the amount of phosphorus in the batches varied considerably, it will be seen that the amount of precipitate obtained by the addition of excess of acetone was constant. Now since the phosphorus was found to be almost quantitatively removed from the ether solution by precipitation with acetone, it follows that the phosphorus content of the acetone precipitate must vary in proportion to the phosphorus content of the ether solution from which it is derived. If we assume, therefore, that only one compound containing phosphorus is present in the ether solution, it follows that the acetone precipitate must necessarily contain at least one substance which is phosphorus-free. Actually it will be shown that there are present in it at least one solid hydrocarbon and one higher alcohol, both of which can be removed by treatment with boiling acetone or alcohol and subsequent filtration. It is thus apparent that the constant percentage of the ether-soluble substances precipitated from dry ether solution by the acetone is fortuitous, because this precipitate is made up of at least three substances which happen to be present in varying amounts, but the sum total of which is constant.

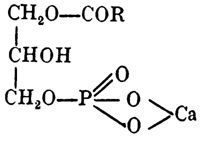
This communication deals only with the material which remains undissolved when the precipitate obtained by adding excess of acetone to the ether solution has been exhaustively extracted with boiling acetone. This fraction which is insoluble in hot acetone contains virtually all the phosphorus of the original ether extract; further every preparation made from different batches of leaves had a similar elementary composition. Thus the phosphorus content of the material from batches *D*, *F* and *H* was 4·9, 4·86 and 4·59 %; they contained 0·22, 0·23 and 0·22 % of nitrogen.

The appearance of this material, its rapid oxidation in air and the fact that it yielded about 70 % of its weight of fatty acids on hydrolysis, made it appear probable that it was of phospholipin character. Against this view however was the fact that its nitrogen content was very low, and it was certain that if this nitrogen were represented by lecithin, the bulk of the material must be of a different nature, as 0·2 % of nitrogen will only account for 10 % of the phosphorus present if calculated on the basis of lecithin. The possibility of the material being a uniform substance with an N:P ratio of 1 : 10 was considered highly improbable. It was therefore argued that this mixture probably contained two substances, one containing nitrogen and possibly phosphorus, but the bulk made up of a substance containing over 4 % of phosphorus and being nitrogen-free. If the molecule of the latter substance were built up in any way similarly to what we accept as the structure of lecithin, there must be some grouping to replace the choline radicle and the grouping must be nitrogen-free. The probability of it being a sugar was negatived, because hydrolysis with methyl alcoholic HCl gave no products which would reduce an alkaline copper solution; nor were any carbohydrate reactions obtained. It seemed probable, therefore, that a metal was replacing choline. The likely metals are K, Mg, Na, and Ca; and the last was the one actually found to be present in the molecule. Further, determinations of the calcium and phosphorus present, showed that in every preparation made, these substances bore an atomic ratio in all cases very close to unity.

Since the second substance has a phosphorus content of about 4 %, and a calcium content corresponding to the same number of atoms of that element as of phosphorus, and since the percentage of fatty acids obtained on hydrolysis was very similar to that which such a body as lecithin would yield, we based our work on the hypothesis that it was the calcium salt of a diglyceride-phosphoric acid of the type



The analyses quoted later show that the crude calcium salt is a mixture of at least two substances, one the calcium salt of the diglyceridephosphoric acid just outlined, the presence and structure of which has been definitely established, and the other probably the calcium salt of a monoglyceridephosphoric acid of the type



At least three-quarters of the material insoluble in boiling acetone consists of the calcium salt of the diglyceridephosphoric acid: it is for this reason that we have made the preparation and analysis of the free diglyceridephosphoric acid the main thesis of this paper. The possible presence of the monoglyceridephosphoric acid and the absence of lecithin are discussed in two appendices.

In discussing the analytical data which follow, it will be simpler if the figures required by the calcium salt of distearylglycerophosphoric acid are taken as the theoretical basis. This is not strictly correct, for as appears later the fatty acids present in the molecule are mainly unsaturated; and hence the values required by theory will not be in strict accord with those obtained experimentally. Since a number of fatty acids seem to be present, and the crude calcium salt is thus really a mixture of substances containing different fatty acids, there is no alternative but to adopt some basis for theoretical calculation, and figures for the calcium salt of distearylglycerophosphoric acid have therefore been used.

Isolation of the crude calcium salt.

Batch H. Total weight of ether-soluble substances 62·3 g. : P 1·10 %; Ca 1·43 %. Ratio Ca to P is approximately 1:1.

Fraction precipitable by acetone. The fat was dissolved in 400 cc. of anhydrous ether and 1600 cc. of pure acetone added; the solution was left for 1 hour on ice and filtered. The residue was ground with acetone and refiltered. The mother-liquor was reduced *in vacuo* to a volume of 200 cc. and four volumes of acetone were again added and, after standing on ice overnight, the solution was filtered. The combined precipitates, which weighed 28·1 g., were redissolved in ether and again precipitated with acetone. Experiments with earlier samples had shown that repetition of this procedure, although reducing the weight of material finally obtained, makes no appreciable difference to its phosphorus content. In other words, both the phosphorus-containing substance and the other substances present have similar solubilities in the ether-acetone mixture. Another method of purification was thus necessary, and finally exhaustive extraction with boiling acetone was adopted. The substances extracted by this hot solvent crystallise as a pale green powder as soon as the temperature of the filtrate falls to 40°. The acetone extraction of this 28·1 g. was continued therefore until no crystalline material appeared on cooling the filtrate. (The filtrate will always go cloudy on cooling even though all the crystalline substances have been removed, for the phosphorus compound has a definite, though small, solubility in the hot solvent.)

By this procedure two fractions were obtained.

(1) The fraction insoluble in hot acetone. This fraction contains the calcium and the phosphorus. Weight 11·46 g. (18·6 % of total ether-soluble substance).

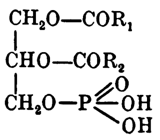
(2) The fraction soluble in boiling acetone. This consists chiefly of one or more hydrocarbons and alcohols, contaminated with traces of (1). The weight of this crystalline material was 13·0 g. (21 % of total ether-soluble substance); its nature will not be discussed further here, but will form the subject of a later communication.

Crude calcium salt H. The fraction insoluble in hot acetone, which we shall call the crude calcium salt, gave the following analysis:

0·0727 g. substance : 0·1524 g. CO_2, 0·0572 g. H_2O, 0·0142 g. ash.
0·4316 g. „ 0·0712 g. $Mg_2P_2O_7$.
0·4316 g. „ 0·0862 g. $CaSO_4$ and 0·0065 g. Fe_2O_3.
0·0888 g. „ required (micro-Kjeldahl) 1·60 cc. 0·01 *N* acid.

	C	H	Ca	P	Ash	Fe	N
Found	57·16	8·74	5·88	4·59	19·54	1·08	0·25
Theory	63·10	10·10	5·39	4·18	17·20	0·0	0·0

It will be seen that although the calcium and phosphorus are present in the ratio 1:1 the values obtained for these elements are too high for the calcium salt of a saturated diglyceridephosphoric acid (containing two C_{18} acids), and, accepting the existence of the latter, it is to be surmised that a second substance containing calcium and phosphorus is also present. A substance of the type postulated above will readily lose its calcium when shaken in ether solution with aqueous mineral acid; there will then be produced an acidic substance of the type



which is a diglyceridephosphoric acid and which we may term the free acid.

With a view to obtaining some information regarding the possible second substance in the crude calcium salt a portion of about 5 g. of the latter was dissolved in ether, shaken with dilute HCl several times and the ether solution was well washed with water. By this procedure there were obtained 3·62 g. of free acid.

Crude free acid H:

0·3625 g. substance : 0·0505 g. $Mg_2P_2O_7$.
0·0727 g. „ required (micro-Kjeldahl) 1·50 cc. 0·01 N acid.

	P	N
Found	3·87	0·29
Theory	4·40	0·0

These figures show that a large proportion of the phosphorus has become water-soluble and been removed. The significance of this, together with the reasons for the phosphorus value being so low, will be discussed later. 0·727 g. of this free acid in ether solution was shaken out several times with lime water and the ether solution washed, reduced to a volume of 10 cc., and four volumes of acetone added to precipitate the regenerated calcium salt.

Regenerated crude calcium salt H:

0·1340 g. substance : 0·0184 g. $Mg_2P_2O_7$.
0·1340 g. „ 0·0265 g. $CaSO_4$.

	P	Ca
Found	3·83	5·82
Theory	4·18	5·39

It will be seen from these figures that the calcium : phosphorus ratio is now 1 : 1·18, in other words excess of calcium is present. This high value for calcium suggested that some other calcium-phosphorus-fatty acid compound in the original crude salt had broken down on being shaken out with acid. As a result the phosphorus has passed into the aqueous layer and been lost, whereas the fatty acid has remained in the ether layer along with the diglyceridephosphoric acid. On conversion of the latter into the calcium salt this contaminating fatty acid has been converted into the calcium soap. Hence the loss of phosphorus and increase in calcium could be explained.

Preparation of the lead salt of a diglyceridephosphoric acid.

As a means of preventing the possible contamination of the regenerated product, it was decided to make the lead salt after the crude free acid had been first obtained. 2·6 g. of the crude calcium salt were converted into the corresponding free acid, and the latter in ether solution was shaken several times with an aqueous solution of lead acetate containing a little acetic acid. If some substance had been previously hydrolysed to yield fatty acids these would not be capable of decomposing lead acetate. Hence they would remain as free acids along with the lead salt of the diglyceridephosphoric acid in the ether, and when the salt was precipitated by the addition of four volumes of alcohol, they would remain in the ether-alcohol mother-liquor (see Appendix 2). (It was found that the ether-alcohol mother-liquor retained the deep red colour which had been characteristic of the solution of the crude and regenerated calcium salts mentioned above.) The precipitated lead salt was greyish and dissolved in ether to give a pale yellow-brown solution. On precipitating a second time the ether-alcohol mother-liquor was water-clear.

The yield of the lead salt after two precipitations from the ether-alcohol was 1·707 g.

Lead salt of diglyceridephosphoric acid H:

0·0755 g. substance : 0·1414 g. CO_2, 0·0493 g. H_2O, 0·0240 g. ash.
0·1707 g. „ 0·0550 g. $PbSO_4$.
0·1707 g. „ 0·0207 g. $Mg_2P_2O_7$.

	C	H	Pb	P	Ash
Found	51·07	7·38	22·02	3·43	31·60
Theory	51·47	8·14	22·80	3·41	32·33 (calc. as $Pb_2P_2O_7$)

This lead salt was contaminated with 0·65 % of Fe determined colorimetrically by comparison with standard $Fe_2(SO_4)_3$ treated with $K_4Fe(CN)_6$: it also contained 0·17 % of N determined by the micro-Kjeldahl method. The significance of the presence of these elements will be discussed later (see Appendix 1).

These analytical results were considered satisfactory for a compound of such a type as this. This preliminary work established a method of purifying the crude calcium salt and the next batch of material was accordingly treated in the same way.

ANALYSIS AND CONSTITUTION OF THE FREE DIGLYCERIDEPHOSPHORIC ACID.

Preparation of the free diglyceridephosphoric acid.

Batch M. From 64 g. of original ether-soluble material, 7·80 g. of crude calcium salt were obtained by the method already described. This sample had Ca 6·13 %, P 4·78 %; Ca : P = 1 : 1. The whole of this salt in ether solution was shaken out with dilute sulphuric acid to convert it into the corresponding acid, which in turn was shaken with lead acetate containing a little free acetic acid. The washed ether solution was concentrated to 250 cc. and two volumes of absolute alcohol added. The lead salt was filtered off, redissolved in 70 cc. of anhydrous ether and reprecipitated by the addition of three volumes of absolute alcohol. The yield of purified lead salt was 5·00 g.

Lead salt of diglyceridephosphoric acid M:

0·0769 g. substance : 0·1432 g. CO_2, 0·0526 g. H_2O, 0·0234 g. ash.

	C	H	Ash
Found	50·06	7·49	30·00
Theory	51·47	8·14	32·30

4·80 g. of this salt yielded 3·51 g. of free acid.

Diglyceridephosphoric acid M:

0·0832 g. substance : 0·1997 g. CO_2, 0·0742 g. H_2O.
0·2140 g. „ 0·0334 g. $Mg_2P_2O_7$.
0·0507 g. „ required (micro-Kjeldahl) 0·85 cc. 0·01 *N* acid.
(Fe 0·59 %)

	C	H	P
Found	65·48	9·91	4·34
Theory	66·57	10·94	4·40

Analysis of the diglyceridephosphoric acid. 3·06 g. of the acid dissolved in 40 cc. of mixed alcohol and ether were emulsified with 20 cc. of water and slowly run drop by drop into 200 cc. of boiling water containing 6 g. of barium hydroxide. The boiling was continued for 2 hours and on cooling the barium soaps were removed by filtration. The barium soaps were ground twice with water and filtered. The combined filtrate and washings were treated with CO_2 to remove excess barium, the barium carbonate filtered off and washed with water. The combined filtrate was boiled to precipitate any bicarbonate, cooled and filtered. The final filtrate was reduced to a volume of 25 cc. *in vacuo*, and 2½ volumes of absolute alcohol added. The precipitate, which appeared as a curd, was washed with alcohol and ether and dried *in vacuo*. The yield of crude barium glycerophosphate was 0·73 g. (55 % of theory). A control experiment in which 1 g. of barium glycerophosphate, prepared from commercial glycerophosphoric acid, was used, gave a similar percentage yield when submitted to exactly similar treatment.

Analysis of the barium glycerophosphate. 0·73 g. of the salt dissolved readily in about 10 cc. of water, giving a slightly yellow and faintly opalescent solution. It was filtered and two volumes of absolute alcohol were added to the filtrate. The precipitate was curdy as before and after washing was dried at 107°. Yield 0·63 g.

0·0860 g. substance : 0·0368 g. CO_2, 0·0201 g. H_2O, 0·0624 g. ash.

0·1582 g. substance on ignition gave 0·1122 g. ash, equivalent to 0·0155 g. P or 9·81 %: converted to the sulphate it gave 0·1154 g. $BaSO_4$, equivalent to 0·0679 g. Ba or 42·86 %. This amount of Ba is equivalent to 0·1110 g. $Ba_2P_2O_7$.

	C	H	Ba	P
Found	11·66	2·59	42·86	9·81
$C_3H_7O_6PBa\frac{1}{2}H_2O$	11·40	2·53	43·42	9·81

It was necessary to boil the aqueous solution twice with charcoal before it became clear enough for the optical rotation to be determined.

$$[\alpha]_D^{18} = +\ 1{\cdot}0^\circ\ (l = 2,\ c = 3).$$

Fränkel and Dimitz [1909] isolated a dextrorotatory barium glycerophosphate from brain cephalin and Trier [1913] a similar product from seed lecithin. In each case the salt was purified by repeated solution in water and precipitation with alcohol. Levene and Rolf [1919, 1926] have since shown that in both these cases the dextrorotation was due to an impurity which cannot be removed by this treatment. They purified the barium salt by conversion into the lead salt, decomposed the lead salt by means of H_2S and reconverted the glycerophosphoric acid into the lead salt. This operation was repeated until a laevorotatory solution was obtained. From this solution a barium salt was precipitated by means of alcohol; this salt had the normal laevorotation.

Owing to the small amount of barium salt which we obtained it was not possible to purify it by this method, so that the question of its rotation must be left open until such time as we are able to obtain sufficient quantities of material for this purpose. From Levene and Rolf's work it is to be inferred that the barium salt, after the lead treatment, will be laevorotatory.

The original mother-liquor from which the barium glycerophosphate had been precipitated by alcohol was evaporated to dryness *in vacuo*. The residue weighed 0·073 g., the major part of which was barium glycerophosphate. Exhaustive extraction with absolute alcohol dissolved 0·021 g. If the small amount of nitrogen present in the free acid was due to lecithin then we should expect to find evidence of choline in this alcoholic extract. Actually it was found to give no precipitate with either alcoholic $HgCl_2$ or H_2PtCl_6. All the fractions of the barium glycerophosphate mother-liquor were then united and the total nitrogen determined by Kjeldahl. 0·3 cc. of 0·1 N acid were required, equal to 0·00042 g. of nitrogen and equivalent to 0·014 % of the total weight of free acid taken for hydrolysis. The significance of this result is discussed in Appendix 1.

The fatty acids of the diglyceridephosphoric acid. The barium soaps obtained during the baryta hydrolysis just described were decomposed with HCl under ether, the ether extract was thoroughly washed with water and evaporated to dryness. Yield 2·30 g. or 74·9 % of the diglyceridephosphoric acid taken (theory for C_{18} saturated acids 2·48 g. or 80·7 %).

0·1654 g. dissolved in abs. alcohol were neutralised by 0·81 cc. of 5/7 N HCl. Neutralisation value 196.

0·0920 g. absorbed 0·1254 g. iodine (Wijs). Iodine value 136.

These figures for the neutralisation and iodine values are in good agreement with those obtained in earlier (unrecorded) experiments. The amount of the fatty acids at our disposal was far too small for any reliable data to be obtained as to their nature. It seemed worth while however to attempt to determine whether they are in any way parallel to those of lecithin, which Levene has shown, in a large number of papers dealing with the acids of lecithins obtained from various sources, to consist of equal molecular proportions of unsaturated and saturated acids. Further, the work of that author and his colleagues on lyso-lecithins seems to exclude definitely the occurrence either of two saturated or two unsaturated acids in any one molecule of lecithin. The conclusion which Levene draws is that in each lecithin there is one saturated and one unsaturated acid.

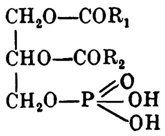
Accordingly 1·8 g. of the fatty acids was converted into the lead soaps, which were extracted with ether. There remained insoluble in ether only a very small amount of material, which yielded on decomposition with acid 0·1 g. of saturated acid, which was hard and white. It would appear therefore that the saturated and unsaturated acids are not present in equivalent proportions, but that the unsaturated acids are present in far greater amount. This result, which will necessarily need confirmation on larger quantities of material, tends to show that this compound differs from lecithin in that in any one molecule there may be two unsaturated acids present.

The fatty acids obtained by decomposing the ether-soluble lead soaps were brominated in anhydrous ether solution. On standing overnight on ice 0·1 g. of solid material separated. This, after careful removal of the excess bromine, had a melting point of 179°. 0·0690 g. was found by Stepanoff's method to contain 0·0432 g. of bromine equivalent to 62·6 %. These results indicate the presence of a C_{18} acid with three double bonds. A consideration of the iodine value of the original mixed acids, together with the amounts of saturated acid and acid with three double bonds obtained, shows that the majority of the remainder must consist of linolic acid and the residue of oleic acid. This is borne out by the fact that the residual brominated acids, which failed to crystallise, contain 47·7 % of bromine.

0·1347 g. substance absorbed 0·0644 g. Br.

	Br
Found	47·7
Theory for C_{18} acids with (A) one double bond	35·9
„ „ (B) two double bonds	53·3

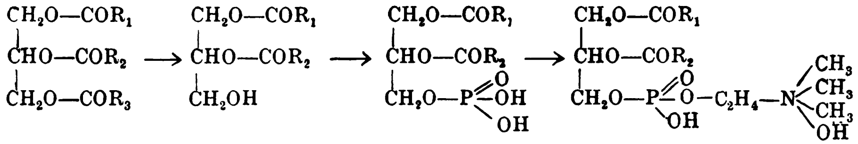
Structure of the diglyceridephosphoric acid. Sufficient evidence has been adduced to enable a structural formula to be ascribed to the diglyceridephosphoric acid. From it there has been isolated an optically active barium glycerophosphate and hence the phosphoric acid is linked to the glycerol in the α-position. The yield of fatty acids has proved that there are two molecules of fatty acid to one of glycerophosphoric acid, and it would seem reasonable to suggest that the structural formula for this substance is similar to that accepted for lecithin, and is



A substance of this type, namely αβ-distearylglycerophosphoric acid, was synthesised by Hundeshagen [1883] and such compounds have been frequently used since, as intermediate products in attempts to synthesise lecithin itself. So far as we are aware their occurrence has not been reported in natural products.

It is of interest to note however that a preparation of a phosphatide-like substance was obtained from the leaf by Winterstein and Stegmann [1909]. These workers extracted 4 kg. of air-dried leaves of *Ricinus* with ether for two or three days at 30°. The essential point of their subsequent procedure was to evaporate the ether extract to dryness and extract with hot alcohol. The insoluble residue, after solution in ether, was precipitated with alcohol. The substance, after drying in a vacuum desiccator, contained 5·27 % of P and 6·74 % of CaO. Beyond showing that it did not contain any sugar these workers went no further. This substance would appear to be a crude calcium salt similar to that described in this paper. Also Stern and Thierfelder [1907] reported that a phosphatide fraction from egg-yolk contained 3·96 % P, 1·37 % N and 1·03 % Ca. They were convinced that the calcium was present in organic combination.

Although the natural occurrence of a diglyceridephosphoric acid had not been reported, it has been suggested by Trier [1912] that the successive stages in the synthesis of plant lecithins are as follows:



Triglyceride   αβ-Diglyceride   αβ-Diglyceride-phosphoric acid   Lecithin

If the work reported in this paper be substantiated it may prove to be a link in the above scheme.

One further point needs discussion. It will be remembered that in the preparation of the original coagulum, the expressed juice is heated to 70° to coagulate the cytoplasm, and the possibility of this process having caused the partial hydrolysis, and the substitution of the choline present in lecithin by calcium, remains to be considered. The p_H of the expressed juice was about 6, and it seemed improbable that heating a suspension of lecithin to 70° for a few minutes in a solution of such low effective acidity would cause such a reaction to occur, and to proceed to completion.

To prove this point a batch of 7 kg. of leaves was minced in the usual way and the green solution containing the colloidal cytoplasm filtered instead of being warmed to 70° to coagulate the cytoplasm. Part of the protein and all the ether-soluble material was retained in the upper region of the filter pad. This green slime was scraped off and pressed in the Buchner press to a hard cake. This material, which had thus been prepared without the application of heat, was directly extracted with ether; the ether extract weighed 14·5 g. and yielded 2·6 g. of crude calcium salt.

0·0972 g. substance : 0·2090 g. CO_2, 0·0759 g. H_2O, 0·0182 g. ash.
0·2596 g. „ 0·0503 g. $CaSO_4$, 0·0032 g. Fe_2O_3.
0·2596 g. „ 0·0414 g. $Mg_2P_2O_7$.

	C	H	Ca	P	Ash	(Fe)
Found	58·63	8·67	5·70	4·65	18·73	0·86

This result would make it appear certain that the calcium salt of the diglyceridephosphoric acid pre-existed in the leaf cell, and that it was not a product of phospholipin decomposition.

Properties of the diglyceridephosphoric acid, and the preparation and properties of some of its salts.

Free acid. Prepared by evaporation of the ether solution at a temperature not exceeding 30°, it is a pale brown sticky mass, which does not readily absorb moisture if exposed to the air, but rapidly darkens due to oxidation. Thus prepared it is readily soluble in the usual organic solvents, especially ether. An alcohol or acetone solution can be considerably diluted with water without the formation of an emulsion. Slow decomposition occurs if the free acid or any of its salts are dried *in vacuo* at 100°, giving rise to products that are no longer soluble in ether.

Lead salt. Two analyses of this salt have already been quoted. A third preparation made from Batch G by the methods described above gave the following analysis:

0·0946 g. substance : 0·1735 g. CO_2, 0·0608 g. H_2O, 0·0293 g. ash.
0·1642 g. „ 0·0524 g. $PbSO_4$ and 0·0204 g. $Mg_2P_2O_7$.

	C	H	Pb	P	Ash
Found (Prep. G)	50·01	7·14	21·80	3·46	30·86
„ („ H)	51·07	7·38	22·02	3·43	31·60
„ („ M)	50·06	7·49	—	—	30·00
Theory	51·47	8·14	22·80	3·41	32·38

When precipitated by absolute alcohol from a solution in anhydrous ether and dried *in vacuo* at 30° it is obtained as brown flakes resembling gelatin, which become sticky under pressure. It is readily soluble in ether, from which it is almost completely precipitated by two volumes of alcohol or less completely by four volumes of acetone. The dry product darkens rapidly when exposed to the air.

Barium salt. Prepared from an ether solution of lead salt H by converting into the free acid and shaking with barium acetate.

0·2456 g. substance : 0·0322 g. $Mg_2P_2O_7$.
0·1228 g. „ 0·0352 g. $BaSO_4$.

	Ba	P
Found	16·80	3·65
Theory	16·37	3·69

Calcium salt. Prepared from an ether solution of lead salt G by converting into the free acid and shaking with calcium acetate.

0·0488 g. substance : 0·1154 g. CO_2, 0·0397 g. H_2O, 0·0089 g. ash.

	C	H	Ash
Found	64·47	9·04	18·03
Theory	63·10	10·10	17·17

The barium and calcium salts have properties similar to those of the lead salt.

APPENDIX 1.

On the possible presence of lecithin.

It will have been noticed that in all the preparations there has been present a small amount of nitrogen (0·15 to 0·3 %). This nitrogen, which persists and is not readily removed by the many purification treatments, presumably cannot be present in the molecule itself, as this would entail a molecular weight of 7 or 8 thousand. The presence of nitrogen in the phospholipin fraction is of course to be expected, and we thought naturally that this nitrogen represented lecithin. Throughout the work, however, we have failed to obtain any evidence of the presence of that substance. On no occasion has a precipitate been obtained when treating an alcoholic solution of the free acid with alcoholic $CdCl_2$. (This test cannot be carried out on the salts which are insoluble in alcohol.) Further this free acid, containing nitrogen sufficient to account for, say, 10 % of lecithin, is very soluble in cold acetone. Another impurity has also persisted. The small quantity of iron present in the crude calcium salt is not removed when the latter is converted into the free acid. Again, after the hydrolysis with baryta in the preparation of the barium glycerophosphate the barium soaps formed a precipitate as they are insoluble in water. The fatty acids which were prepared by acidification of these soaps under ether contained practically all the nitrogen and iron present in the original substance. Hence, as most of the nitrogen was not water-soluble after hydrolysis, it cannot be present as choline, and this is again evidence of the absence of lecithin. Another interesting point arises here, namely, that the iron and nitrogen have appeared together in the same fraction of the hydrolysis products and this may indicate the presence of ether-soluble iron- and nitrogen-containing substances which had been unaffected by the hydrolysis, and which, being water-insoluble, have precipitated along with the barium soaps. The evidence thus seems to negative the possibility of lecithin being present in this ether extract of leaf cytoplasm—a very interesting result.

APPENDIX 2.

The possible presence of the calcium salt of a monoglyceridephosphoric acid.

The point which will now be discussed is the significance of the fact that when the crude calcium salt in ether solution is shaken with acid, part of the phosphorus originally present passes into the aqueous solution. This fraction was usually 20–40 %. As to the nature of the substance from which it is derived, we have no positive evidence, but a number of isolated observations seem to throw some light on the question. Firstly, if the regenerated calcium salt is again shaken out with acid there is no further loss of phosphorus, which would suggest that the phosphorus which passes into the aqueous solution is derived from a second phosphorus-containing body and not from the calcium salt of the diglyceridephosphoric acid. Secondly, the regenerated calcium salt obtained by decomposing the crude salt with acid, and shaking the free acid so obtained with lime water always gives a high value for calcium and a low value for phosphorus. We may surmise from this that whatever has been hydrolysed by the shaking with acid and thereby has lost its phosphoric acid, must have given rise at the same time to other products of a fatty nature, which are able to unite with the lime. We suspected that these acidic substances might be fatty acids, and this was confirmed to some degree as follows. When the free acid in ether solution is shaken with aqueous lead acetate the lead salt of the diglyceridephosphoric acid is formed, and can be precipitated by the addition of excess of alcohol to the ether solution. If it be filtered off, the mother-liquors on evaporation leave a residue consisting of small amounts of lead salt together with another substance. This latter can be removed by solution in cold alcohol, and can be titrated in that solvent with NaOH. From the neutralised product there was obtained from batch M, discussed above, 1·22 g. of crude fatty acid having a neutralisation value of 177. Hence the second substance, which breaks down on treatment with acid, contains calcium and phosphorus, and gives rise to fatty acids. Further, since the calcium and phosphorus in the original crude calcium salt are both above the theoretical for the calcium salt of a diglyceridephosphoric acid the other substance present must contain a high percentage of calcium and phosphorus. Since this latter gives rise to fatty acids on hydrolysis, it seems possible that the second substance may be the calcium salt of a monoglyceridephosphoric acid, which would contain Ca 8·42 %, P 6·53 %. The presence of this substance in the crude Ca salt would raise both the calcium and phosphorus, and it seems reasonable to suggest that if such a substance in ether solution were treated with aqueous acid, it would tend to undergo hydrolysis much more easily than would the compound containing two fatty acid radicles in the molecule. No figures have been quoted for the amount of phosphorus passing into the aqueous solution, for, when the calcium salt has been shaken with mineral acid and the ether solution of the free acid is being shaken with water to remove the last traces of mineral acid, emulsions always occur. These emulsions are difficult to break, and it has been our practice to do this by adding small quantities of alcohol. There is little doubt that some of the loss of phosphorus is due to the removal of small quantities of free acid owing to its solubility in water containing alcohol. This fact has made it seem useless to quote figures for the phosphorus of the aqueous extract.

We hope to investigate this calcium salt of a monoglyceridephosphoric acid in greater detail at a later date. It is possible that this supposed salt did not pre-exist in the leaf cell, but was formed from the calcium salt of the diglyceridephosphoric acid by the action of enzymes or heat.

SUMMARY.

The so-called phospholipin fraction—obtained by adding acetone to an ether solution of a fat—contains no phospholipins in the case of the ether-soluble substances of the cytoplasm of the cabbage leaf. All the phosphorus is present in combination with calcium, glycerol and fatty acids. Nitrogen is virtually absent.

The main constituent of the fraction is the calcium salt of a diglyceridephosphoric acid. The preparation and analysis of this acid, and the preparation and properties of some of its salts are given.

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