CXXI. THE PHOSPHATIDES OF THE WHEAT GERM.

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INVESTIGATIONS of the phosphatides of cabbage leaves have shown that the main constituent is phosphatidic acid, present as its calcium salt, and that lecithin and kephalin are absent [Chibnall and Channon, 1927]. This finding suggested that salts of phosphatidic acid might be the intermediate compounds in the synthesis of lecithin and kephalin from triglycerides, according to the scheme postulated by Trier [1912], and suggested further studies designed to throw light on this question. The work described in this paper was the first part of an investigation planned in order to study the phosphatides of a seed and to trace the changes which occur on germination. While it was in progress investigations along similar lines were reported by Smith and Chibnall [1932] and Jordan and Chibnall [1933]. The former workers identified lecithin, kephalin and the calcium or magnesium salt of phosphatidic acid as constituents of the phosphatide mixture obtained from grass; while the researches of the latter, designed along lines similar to our own, were concerned with an exhaustive study of the distribution of phosphatidic acid, lecithin and kephalin in the runner bean during the different stages of germination and growth. They established for the first time the presence of phosphatidic acid in a seed. Because of the difficulty of obtaining adequate amounts of material for fractionation, we chose wheat germ as our starting material, for it can be obtained commercially and by far the greater part of the phosphatide of the wheat grain is present in the embryo.

EXPERIMENTAL.

Extraction of the wheat germ.

As a result of studies of the phosphatide content of seeds, Schulze and Steiger [1889] recommended extraction by alcohol at 60°, the extract being evaporated to dryness and the residue extracted with ether, because they found that extraction of seeds directly with ether did not remove all the phosphatide. The advantage of the increased phosphatide yield obtained by this method tends to be offset by the fact that the final ethereal extract is contaminated by non-phosphatide substances, some of which may contain phosphorus. Jordan and Chibnall [1933] submitted the cotyledon residues of runner beans which had been already extracted with ether to this process. They found that much of the additional ethereal extract so obtained consisted of substances which readily lost their phosphorus on shaking an ethereal solution with dilute acid, and they consider that the extra amount of true phosphatide extracted by alcohol is not more than one-quarter of the total. With these findings in mind, we carried out an extensive series of extractions with ether, light petroleum, alcohol and acetone, alone and in mixtures, in order to obtain an extract of high phosphatide content and uncontaminated as far as possible. The degree

to which the phosphorus obtained in the extracts existed as non-phosphatide phosphorus was systematically investigated by the methods which were used for the large preparation and are described later, and only the method finally adopted as a result of these experiments need be described. Alcohol-ether (1:1) was found to be the most useful solvent for extraction, and a series of comparative experiments on one sample of wheat germ in which different volumes were employed was carried out. The germ was mixed with the solvent and the mixture heated to boiling. After standing for several hours, at room temperature, the mixture was filtered and the residue washed with successive volumes of ether until the filtrate was colourless (usually 4 volumes). The filtrate was evaporated to dryness *in vacuo* on a water-bath at 60° and the residue dissolved in anhydrous ether. After several hours' standing, the white sediment was removed by filtration and the ether-soluble material obtained by evaporation. The figures recorded below are typical of the results obtained:

Solvent	Volumes	Yield % of germ	P as % of germ
Ether (Soxhlet)	—	7.40	0.019
Alcohol-ether (1:1)	6	7.60	0.028
	9	7.80	0.025
	12	8.25	0.035
	20	8.53	0.036
(mean from 6 different samples)			

Since our object was to obtain the phosphatide fraction in as great a yield as possible, extraction with 12 volumes of alcohol-ether as described above was finally adopted throughout this work. The yield of oil by this method was 8.40 % (mean of seven samples). Its phosphorus content corresponded to its containing about 10 % of phosphatide.

Precipitation of the phosphatide.

The removal of the phosphatide quantitatively from an oil in which it is present in relatively low concentration is difficult without the addition of inorganic salts. Because of a desire to avoid use of the latter, investigations were made of the optimum conditions for precipitation from ethereal solution by acetone. Samples from one preparation of wheat germ oil were dissolved in varying volumes of ether and to these solutions were added 9 volumes of acetone calculated on the volume of oil used. After cooling in the ice-box for several hours, the precipitates were filtered off and washed with acetone. From a phosphorus determination on the precipitate, the recoveries under the varying conditions were calculated. These are recorded in Table I. As a result of this experiment we adopted the method of dissolving the oil in 1.5 volumes of ether followed by precipitation with 9 volumes of acetone.

Table I. *Percentage of recovery of phosphatide on precipitation of ethereal solutions by acetone.*

	Ether-soluble P as % of the wheat germ	Phosphatide (P × 25) as % of oil or precipitate	Recovery of ether-soluble P in precipitate %
Original extract	0.0232	7.8	—
Precipitation by acetone (9 vols.) after dissolution in ether:			
Vols. 1	0.0202	54	87
1.5	0.0208	60	90
2	0.0191	60	82
3	0.0171	65	73

Analysis of a series of samples.

Before proceeding with the bulk preparation, a series of extracts from different samples of germ (400–850 g. each) was prepared by these methods in order to study the variations likely to be encountered. Their analyses are recorded in Table II.

Table II. *Analysis of seven samples of wheat germ oil.*

Sample	A	B	C	D	E	F	G	Mean
Age (days)	100	42	1	1	2	1	1	—
	(kept at 4°)	(kept at 0°)						
Yield g./100 g. germ	9.80	8.90	7.7	7.4	8.5	9.1	7.8	8.37
Acid value	—	—	—	17.5	21.7	14.1	16.1	17.6
Saponification value	181	188	190	192	184	180	—	184
i.v.	110	111	115	113	111	107	—	111
P % of extract	0.49	0.55	0.34	0.29	0.32	0.43	0.35	0.39
Fatty acids % of extract	83.2	80.0	82.3	84.7	83.4	82.6	83.8	82.8
i.v.	121	122	112	119	122	124	128	120
Mol. wt.	284	281	282	284	278	278	284	284
Unsaponifiable matter:								
Yield % of extract	6.8	8.0	9.2	5.6	6.7	6.1	6.8	7.03
Sterol (by digitonin)	55	50	47	68	60	65	—	57.5
i.v.	99	109	109	103	108	106	—	105.8

From Table II calculations may be made as to the approximate composition of the extract. Later in this paper it is shown that the phosphatide is present as calcium, magnesium and potassium phosphatides, together with lecithin and kephalin, and that little of the phosphorus of the extract is non-phosphatide-phosphorus. If the mixed phosphatides be regarded as having a mean phosphorus content of 4 %, the approximate composition of the extract calculated on the mean values in Table II is:

Phosphatide $\left(\frac{0.39}{4.0} \times 100\right)$	...	...	...	...	...	...	9.75 %
Free fatty acid $\left(\frac{17.6}{5.6} \times 28.2 \times 100\right)$	...	...	...	...	...	...	8.8 %
Glyceride (total fatty acids – phosphatide acids $(18.2 \times P)$ – free fatty acid) $\times 1.045$	...	...	...	...	...	...	69.9 %
Unsaponifiable matter	...	...	...	...	...	...	7.03 %
Total							95.48 %

Thus about 4.5 % of the extract appears to be contaminating material of non-fatty nature. Analysis of the phosphatide fraction gave the results recorded in Table III.

Table III. *Analysis of the acetone-insoluble fractions from seven samples of wheat germ oil.*

	A	B	C	D	E	F	G	Mean
Yield as % of extract	13.7	15.9	8.0	10.2	10.4	18.7	12.6	12.8
Yield as % of germ	1.3	1.4	0.6	0.8	0.9	1.7	1.0	1.1
i.v.	77	78	74	78	78	75	—	77
Fatty acids %	55.3	52.6	57.2	57.7	53.3	56.4	—	56.2
i.v.	122	121	123	118	125	120	132	123.0
N %	1.8	1.8	1.5	1.5	1.5	1.6	1.5	1.60
P %	2.9	2.9	2.8	2.4	2.6	2.6	2.3	2.65
Ash %	2.4	2.6	—	2.7	3.2	2.6	—	2.70

The figures in Table III show that the mean value of the N:P ratio is 1.34, indicating the presence of contaminating nitrogenous substances which are so often found in the phosphatide-containing ethereal solutions of the residue left after evaporation of an alcoholic extract of tissues. Calculation on the basis of 4 % phosphorus shows that the mean value for the maximum amount of phosphatide possibly present is 66.25 %. This would yield on hydrolysis 48.32 % of fatty acid, calculated as oleic acid ($P \times 18.2$), compared with 56.2 % found. The calculations from Table II, which indicate the presence of 4.5 % of non-lipoid substances in the original extracts, thus seem borne out by the presence of about 30 % of non-phosphatide in the acetone-insoluble fractions. Since these latter constituted 12.7 % of the original extracts, the 4.5 % would become 36 % if all the extraneous material of the extract were precipitated with the phosphatides. The phosphorus recovery in the acetone precipitates corresponds to 86.3 % of that originally present in the extracts. It is to be observed that the i.v. of the acids obtained by hydrolysis is identical with that of those yielded by the original material, which suggests that the composition of the phosphatide acids does not differ markedly from that of the glyceride acids.

Large scale preparation. 9.5 kg. of wheat germ, received fresh and kept in cold storage for 5 days, were extracted with 114 l. of ether-alcohol (1:1) in six batches. The preparation, carried out as described in 8 days, yielded 770 g. (8.5 %) of a brown oil which gave the following analytical values: acid value, 18.0; fatty acids (i.v. 125, mol. wt. 282), 85.3 %; unsaponifiable matter, 5.4 %; P, 0.39 %; all of which figures correspond closely with those recorded as the mean value in Table III.

Precipitation of the phosphatide.

Acetone (6.5 l.) was added to a solution of 747 g. in ether (1.2 l.). After standing for 15 hours in the refrigerator, the crude phosphatide was filtered off and washed with acetone. It was redissolved in ether (135 ml.) and reprecipitated by the addition of acetone (420 ml.). The product after washing with acetone weighed 68.9 g. (9.2 % of the extract). It analysed as follows: P, 2.97 %; N, 1.52 %; amino-N, obtained by Van Slyke apparatus after hydrolysis for 9 hours with *N* sulphuric acid, removal of the fatty acids and neutralisation after concentration, 0.15 %; the ash, 4.27 %, contained 29 % P and 12 % Mg. In order to ensure the absence of unsaponifiable matter which might have been carried down during the phosphatide precipitation, 0.517 g. was hydrolysed with sodium ethoxide for 4 hours. The yield of unsaponifiable matter was 0.032 g. or 6.20 %. This corresponds to 10.5 % of the unsaponifiable fraction present in the original extract. The fatty acids obtained after removal of the unsaponifiable fraction weighed 0.290 g. (56.1 %). These acids had i.v. 120 and mol. wt. 299.

From these data, and assuming that the ash represents pyrophosphate derived from metallic salts of phosphatidic acid present in the original mixture, an approximate distribution of the phosphorus may be derived as follows:

P present originally as salts of phosphatidic acid $\left(\text{ash } 4.27 \% \times \frac{29}{100} \right)$	...	...	...	...	1.24 %
P present as kephalin $\left(\text{amino-N } 0.15 \% \times \frac{31}{14} \right)$	...	...	...	...	0.33 %
Residual P $(2.97 - 1.24 - 0.33)$	...	...	...	...	1.40 %
The residual P if present as lecithin would require $1.4 \times \frac{14}{31} \% \text{ N}$	...	...	...	...	0.63 %
The non-phosphatide-N, $1.52 - 0.78$	...	...	...	...	0.74 %

On the basis of these figures, the composition of the mixture would be: lecithin, 36.4 %; kephalin, 8 %; phosphatide calculated as the magnesium salt, 28.9 %; unsaponifiable matter, 6.2 %; giving a total of 79.5 %. Hence the fraction contains 20.5 % of extraneous substances which have a mean content of 3.6 % N. A phosphatide mixture such as the above would yield on hydrolysis 54.24 % fatty acids as against 56.1 % found. The phosphorus present in the phosphatide precipitate corresponds to not more than 70 % of that present in the original extract as compared with the mean figure of 86.3 % for samples A-G. We have no explanation to advance for this considerably lower figure.

Evidence was next sought as to the validity of the assumption concerning the nature of the ash and thereby whether the excess nitrogen as shown by the N:P ratio of 1.14:1 was due to extraneous nitrogenous substances present in the fraction or to the presence of a diaminophosphatide. The process finally adopted was that of emulsification of the phosphatide in water followed by aggregation by the addition of acetone and salt, a modification of that used by MacLean [1911]. 1.9 g. of the phosphatide, which will be referred to henceforth as P1, were emulsified in 200 ml. water, and 200 ml. acetone were added. The precipitate P2, 0.5579 g., was removed by filtration; to the filtrate sodium chloride was added until further precipitation ceased. This precipitate P3 weighed 0.976 g. The analyses of P2 and P3 were so similar that they can be grouped together and compared with those of P1.

Table IV. *Recovery after precipitation of the phosphatide emulsion by acetone.*

	P2 and P3	P1	% recovery referred to P1
Yield % of P1	80.7	—	—
Ash	5.23	4.27	98.86
N	0.86	1.52	45.66
P	3.29	2.97	89.19
Fatty acids	63.2	57.0	91.18

Several deductions can be made from Table IV. In the first place the emulsification process has removed 54.34 % of the original nitrogen with a relatively small loss (10.81 %) of the phosphorus and has yielded a product having an N:P ratio of 1:2.61. Further, this mild treatment, which would largely remove water-soluble inorganic material, including inorganic phosphate, and which would not cause any appreciable hydrolysis of the phosphatide, has not removed the original inorganic constituents, for there is a virtually complete recovery of the ash on incineration. The N:P ratio and the nitrogen content (N 0.86 %) definitely show that the phosphatide mixture contains non-nitrogenous phosphatides, for not more than 50 % of lecithin (N 1.74 %) can be present. There is thus strong presumptive evidence for the presence of phosphatidic acid. 0.86 % N, calculated as lecithin, would require P 1.93 % and fatty acids 35.1 %, leaving 1.36 % P and 28.1 % of fatty acids to be accounted for. 1.36 % P would represent 33 % of the fraction if present as the alkaline earth salts of phosphatidic acid and would yield 25 % of fatty acids on hydrolysis. With the 6.2 % of unsaponifiable matter present in P1, 89 % of the fraction can be thus tentatively assigned. Thus this process has left in the product some 10 % of impurity. (Calculation also shows that the 19.3 % of material, removed as water-soluble products, has N 3.78 %, P 1.66 %, fatty acids 26.0 %. Such an amount of phosphorus would correspond to 43 % of it being present as lecithin or similar phosphatide, which would require 0.75 % N and would yield 30.2 % of fatty acids on hydrolysis.)

This experiment suggests therefore that the phosphatide mixture consists of lecithin and kephalin, together with about two-thirds of the sum of their amounts as salts of phosphatidic acid, and the method adopted provides an excellent means for concentrating the phosphatide with relatively little loss.

Before treating the main bulk of material, further preliminary work was carried out with a view to determining the best conditions for the isolation of phosphatidic acid. The original method used by Channon and Chibnall [1927] and also used by Chibnall and his co-workers was to shake the mixed phosphatides in ethereal solution with dilute hydrochloric acid. After washing the solution with water, the residue left after evaporation of the solvent was separated into a lecithin-kephalin fraction and phosphatidic acid by virtue of the solubility of the latter in acetone. The phosphatidic acid was then further purified by conversion into the lead salt. We encountered two difficulties when we attempted to carry out this process with our material. The first of these was the production of persistent emulsions when the mixed phosphatides were shaken in ethereal solution with aqueous acid, due to the lecithin and kephalin present. These were not encountered in the work on cabbage where the latter phosphatides were absent, but are commented on by Smith and Chibnall [1932] in their grass studies. This difficulty which rendered quantitative separations impossible, was overcome by a method which should prove of general use in phosphatide studies. We have used in place of water a mixture of water 100, acetone 40, sodium chloride 5. Acid may be added to the mixture as required and for final washing the salt may be omitted if desired. This has been found an extremely useful device, for separations are usually both rapid and quantitative.

The second difficulty was that we were unable to separate phosphatidic acid from the mixed acid-washed phosphatides by precipitation of the lecithin-kephalin fraction with acetone. Many variations in this procedure were attempted, but studies of the nitrogen and phosphorus percentages in a large number of fractions (an example is fraction P4 given later) showed that little separation was obtainable by this method. We have no suggestion to offer in explanation, save that perhaps the nature of the fatty acids present in the phosphatidic acid, or the position of attachment of the phosphoric acid to the glycerol molecule may be involved. The method adopted to overcome this difficulty has been to shake the phosphatide mixture, after washing with acid, directly with lead acetate. The lecithin fraction was then largely separated from the lead salts by repeated extraction with absolute alcohol. This process was only effective because the percentage of kephalin in our product was low.

In the fractionation which follows, the theoretical analytical figures below will serve for comparison with the results described. Any phosphatide preparation is a mixture of compounds varying in the nature of the fatty acids, and these calculated figures given are based on compounds containing two molecules of oleic acid.

	N	P	C	H	Mol. wt.	Fatty acids yielded by hy- drolysis %	Ash as pyro- phos- phate %
Lecithin	1.74	3.86	65.75	10.71	803	70.3	—
Kephalin	1.88	4.17	66.08	10.48	743	75.9	—
Calcium phosphatidate	—	4.20	63.33	9.88	739	76.3	17.18
Lead phosphatidate	—	3.42	51.66	8.06	906	62.3	32.45
Magnesium phosphatidate	—	4.29	64.71	10.09	723	78.1	15.36
Potassium phosphatidate	—	4.00	60.31	9.15	775	72.7	21.28
Phosphatidic acid	—	4.42	66.76	10.41	701	80.5	—

Fractionation of the main preparation.

1. *Treatment by emulsification in water and precipitation by acetone.* 30.8 g. P1 (N, 1.52; P, 2.97 %) were emulsified in 500 ml. water and, after adding acetone (500 ml.) and 12.5 g. sodium chloride, the mixture was chilled for 15 hours. The precipitate P2, 27.1 g. (N, 0.94 %; P, 3.28 %), was treated in similar manner with half the quantities of water, salt and acetone. The precipitated phosphatide P3 weighed 25.38 g. (N, 0.87; P, 3.26 %). The first treatment thus removed 45.36 % of the nitrogen; the small amount removed by the second treatment, 7.28 %, was taken as evidence that further repetition of the procedure would not materially improve the product. The total loss of phosphorus into the aqueous phase was 11.5 %.

2. *Treatment with acid.* 25.1 g. P3 dissolved in 300 ml. ether were shaken successively with 300, 150 and 150 ml. of a mixture of water 400 ml., acetone 200 ml., sodium chloride 40 g., with sufficient hydrochloric acid added to make the solution *N*/20. The ethereal solution was washed five times with the acetone-brine solution without added acid, 700 ml. in all, and yielded on evaporation 24.4 g.

3. *Separation by ether-acetone.* 24.3 g. dissolved in ether (80 ml.) were precipitated with acetone (480 ml.). The process was repeated three times. The combined mother-liquors were evaporated to dryness and, by chilling the product after dissolution in hot acetone, a further precipitate was obtained. The total acetone-insoluble fraction P4 was 22.65 g.; the soluble fraction P5 weighed 1.64 g.

The yields and the analyses of these four products and that of P1 were:

	Yield g.	C %	H %	N %	P %
P1	30.8	60.88	9.56	1.52	2.97
P2	27.1	62.93	9.69	0.94	3.28
P3	25.38	60.42	9.31	0.87	3.26
P4	22.65	58.82	9.39	0.93	3.54
P5	1.64	71.25	11.32	0.25	1.53

4. *Treatment with lead acetate.* (a) *Acetone-soluble fraction P5.* 1.56 g. dissolved in 70 ml. ether were shaken with 20 ml. aqueous lead acetate solution containing a little acetic acid. The ethereal solution after four washings with water yielded 1.66 g. This was dissolved in 10 ml. ether and the lead salt precipitated by adding 30 ml. alcohol. The lead salt was again dissolved in ether and reprecipitated by alcohol. The twice precipitated lead salt P6 weighed 0.57 g. The soluble residue 1.09 g. was discarded.

(b) *Acetone-insoluble fraction P4.* In an attempt to avoid the emulsions encountered under (a), a saturated lead acetate solution in 50 % aqueous acetone was employed. 17.8 g. P4 dissolved in 180 ml. ether were shaken with 180 ml. of this lead acetate solution and the mixture was centrifuged. The ethereal solution was washed in the centrifuge-cups four times with 40 % aqueous acetone. On evaporation this yielded 19.5 g. while from the emulsion 2.43 g. were obtained; total yield 21.93 g. The product was extracted three times with hot absolute alcohol, 350 ml. in all, each extract being cooled to room temperature before filtration. The insoluble residue P8 weighed 7.80 g., the soluble fraction P9 12.2 g. The analyses of the lead compounds P6 and P8, together with the lecithin-kephalin fraction P9, were as follows:

	Yield g.	C %	H %	N %	P %	Pb %	Ash %
P6	0.57	52.02	7.74	0.12	3.25	21.6	30.7
P8	7.80	46.79	7.06	0.13	3.69	23.3	34.8
P9	12.20	62.18	10.11	1.30	2.45	—	2.2

The analytical figures for fractions P6 and P8 are in fair agreement with those required by lead phosphatidate, although these products are not pure. Our experience bears out that of Smith and Chibnall [1932]. These workers comment on the difficulty of obtaining reproducible figures for carbon and hydrogen on similar lead salts. Thus their crude lead phosphatidate preparation gave C, 50.3; 48.2 %; and on purification by precipitation by alcohol from a solution in ether these salts contained 47.0 to 48.3 % of carbon, as against their theoretical content of 51.2 %. Hence not too much reliance can be placed on these carbon figures, which are usually some units below the theoretical value. Our two fractions contain also about 0.1 % N, which we failed to remove in spite of various treatments attempted for this purpose. This same difficulty was experienced by Chibnall and Channon [1927] and Channon and Chibnall [1927], who were unable to remove the 0.11 % N from the free phosphatidic acid prepared from cabbage leaves. Further evidence that these products are mixtures was obtained in the preparation of phosphatidic acid from the lead salt. 0.39 g. P6 was suspended in 40 ml. of hot benzene and decomposed with hydrogen sulphide. Considerable difficulty was encountered in dealing with the colloidal solution which resulted. It was finally evaporated to dryness and the residue extracted with acetone. After filtration, the acetone solution was cooled to 0° for 24 hours and again filtered. On evaporation there was obtained 0.284 g. of a viscous oil, which represented 73 % of P6 as against the 78.4 % required by theory. This contained 3.9 % P (theoretical P 4.42 %) and had i.v. 108. Similar difficulties with colloidal solutions were encountered in the decomposition of 1 g. of fraction P8. This was treated similarly and, after standing in the ice-chest, the final acetone solution yielded after filtration 0.70 g. (P, 4.25 %; N, 0.15 %), whereas the theoretical requirement is 0.77 g. Further the mol. wt. of the product 718, determined by titration in alcohol, shows the presence of non-acidic impurities. Similar findings were recorded by Smith and Chibnall [1932] whose product had mol. wt. 746. This was confirmed by hydrolysis of 1.09 g. of P8 by boiling for 20 hours with 2.5 *N* hydrochloric acid. The yield of fatty acids so obtained, 0.542 g., was 49.6 % of P8 which is no more than 79.8 % of theory, an experience again similar to that of Smith and Chibnall, who obtained 77 % from the lead phosphatidate prepared from grass. These fatty acids had i.v. 107, and contained 24.4 % of saturated and 75.6 % of unsaturated acids determined by the Twitchell method. This i.v. is greater than that of the acids from the lecithin-kephalin fraction, 90.2, but no significance can be attached to this difference, because considerable oxidation of the acids must have taken place during the two years throughout which this work was being carried out, since the i.v. of the acids of the starting material was 120. It seems to us not improbable that the low yields of fatty acids from the lead salts is due in part to oxidation of the more unsaturated constituents and their consequent insolubility in light petroleum on liberation by hydrolysis. This might, in part, also explain the low carbon figures which are generally obtained in these preparations.

The lecithin-kephalin fraction P9.

The N:P ratio in this fraction was 1:0.9, and the low nitrogen and phosphorus values showed that not more than two-thirds of this fraction could be lecithin. The excess of nitrogen over phosphorus, together with the demonstration of the presence of carbohydrate, which is mentioned below, show that, in spite of the treatment by emulsification in water twice and shaking the ethereal solution with aqueous acid, this fraction still contains much non-lipoid material.

Calculation suggests that about 20 % of this fraction (3.1 g.) is not lipoid, which shows that the impurities still present after the double emulsification process, about 15 % of P3, have accumulated in this fraction. The difficulty of removing carbohydrates from plant phosphatide preparations has been encountered by many workers [Levene and Rolf, 1925; Schulze and Winterstein, 1903-04; Winterstein and Smolenski, 1908-09; Smolenski, 1908-09; Trier, 1913]. No attempt could be made to isolate lecithin or kephalin from so small an amount of material, and we accordingly contented ourselves with carrying out various experiments, designed to throw further light on the composition of this fraction.

(a) *Presence of carbohydrate.* Acid hydrolysis sufficiently prolonged to ensure the complete breakdown of the phosphatide resulted in the presence in the aqueous filtrate of reducing substances to the extent of 3 % when calculated as glucose. This contamination of plant phosphatide preparations with sugars is a general phenomenon, and Smith and Chibnall [1932] point out that the conditions used for acid hydrolysis of phosphatides are such as to reduce, possibly seriously, the yield of reducing sugar found. Hence this figure of 3 % is probably considerably too low and carbohydrates may thus account for a considerable part of the non-phosphatide material.

(b) *Fatty acid content.* By alkaline hydrolysis with alcoholic potassium hydroxide, the fraction was found to contain 6.8 % of unsaponifiable matter and 57.39 % of fatty acids. If present as lecithin, the phosphorus content would yield about 45 % of fatty acids on hydrolysis. The excess 12.0 % of fatty acids (about 1.5 g.) may have been produced by slight hydrolysis during the very many fractionation processes. The fatty acids had i.v. 90.2 and yielded by the Twitchell process 58 % of unsaturated acids and 42 % of saturated acids.

(c) *Amino-N content.* Determinations of total and amino-N on aliquot parts of the aqueous filtrate from an acid hydrolysis of 2 g. were carried out. The amino-N 0.30 % represented 25.7 % of the total N present in the hydrolysate, or 46.6 mg. which was the amount present in the original material P1. The remainder of this hydrolysate was used for the base separation.

(d) *Isolation of choline and colamine as gold salts.* The methods used by Smith and Chibnall [1932] were employed for the separation. The aliquot of the hydrolysate employed contained 3.53 mg. of amino-N and yielded 65.3 mg. of colamine chloroaurate, which was 65 % of that likely to be present from the amino-N content. It melted at 190° and contained 48.1 % Au (required 189-191°, Au 49.2 %). The filtrate from the mercuric acetate precipitation of the colamine was made acid and concentrated, and the mercury was removed as sulphide. After evaporation to dryness, the residue was dissolved in alcohol and an excess of alcoholic mercuric chloride added. The choline mercurichloride which crystallised was obtained in poor yield in very impure state. The mercury was therefore removed by means of hydrogen sulphide and after concentration of the filtrate a slight excess of gold chloride was added. The 10.29 mg. of non-amino-N present in the original aliquot gave 292 mg. of gold salt (M.P. 240-241°; Au 42.9 %). This product was equally impure, for choline chloroaurate melts at 269° and contains 44.5 % Au. From several preparations of the choline fraction of the phosphatide base, the highest melting-point for the gold salt was 248-249°.

Investigation of the inorganic constituents.

5.19 g. P1 were hydrolysed by boiling for 15 hours with *N* HCl. The fatty acids were removed by extraction with ether. The combined aqueous acid washings were concentrated to small volume and contaminating organic matter

removed by oxidising with nitric acid and perhydrol. Estimations of calcium as oxalate, magnesium colorimetrically and potassium as sulphate on suitable aliquot parts of the solution gave: Ca, 23.4 mg.; Mg, 28.4 mg.; K, 11.1 mg. The amounts of the metals found correspond to 59.22 mg. P, when calculated in ratios Ca/P, Mg/P, K/P, of 1, 1 and 2 respectively. If these metals were originally present as phosphatides, the amounts in the 5.19 g. of P1 would be Ca phosphatide, 0.432 g. (8.3 %); Mg phosphatide, 0.8553 g. (16.5 %); K phosphatide, 0.1103 g. (2.12 %). In the absence of other contaminating inorganic constituents these percentages of salts of phosphatidic acid would cause the original preparation to give an ash (as pyrophosphates) of 4.41 % (found 4.27 %).

DISCUSSION.

For ease of discussion of the results, a summary of the fractionation is given in outline below. The percentages of N and P in each fraction are recorded, together with the total N and P contents, and these are expressed as a percentage of the corresponding figures on the original material. The weights of fractions have been corrected for the aliquots used for analysis.

Diagram 1. Balance sheet showing the corrected weights, nitrogen and phosphorus percentages and percentage recovery.

In this discussion of the relative amounts of the various constituents present, the calculations will refer to the phosphorus content and not to the gross weight of the individual substances, as it appears more informative to discuss the distribution of the phosphatides on this basis. The diagram shows that the total lead phosphatide isolated in fractions P6 and P8 contains 42.1 % of the total phosphorus originally present. Since choline and colamine were isolated as gold salts from the lecithin-kephalin fraction in amounts which compared favourably with the content of total and amino-N, it is reasonable to regard

the amino-N of this fraction as originating from kephalin, and to assess the remainder of the P after subtracting the kephalin-P as representing lecithin. Fraction P9 thus contains 2.48 g. kephalin and 7.2 g. lecithin, corresponding to 11.3 % and 30.4 % of the P of the original material. It remains to account for the phosphorus in the aqueous mother-liquors resulting from the emulsification of fractions P1 and P2. We made no attempt to investigate these further, save to show that the phosphorus present in them was not in inorganic form. Bearing in mind the impossibility of precipitating lecithin quantitatively by the means adopted, it is probable that the greater part of this phosphorus was present as lecithin. If this be assumed and the possibility that the original material contained substances containing phosphorus other than phosphatides be disregarded, a balance sheet to show the lipin-P distribution may be drawn up. We recognise of course the limited value of attempting to assess the proportions of different phosphatides in a mixture in the light of the limited knowledge of the constituents and the deficient methods of separation at present available, but such a distribution table is of some value in this particular instance, where attempts to maintain all separations as quantitative as possible have been made. On this basis, the total P of the original mixture had the distribution: lecithin, 46.6 %; kephalin, 11.3 %; phosphatidic acid, 42.1 %.

It remains to correlate these findings with the percentage of ash and its composition. It is shown in a preceding section that calculation of the amounts of the Ca, Mg and K in fraction P1 corresponded to a theoretical ash value of 4.41 % as against 4.27 % found if the metals were present as salts of phosphatidic acid. These amounts would account for 1.141 % of the P of P1, or 38.41 % of the total. The excellent agreement between this figure and that derived from the amount of crude phosphatidic acid, 42.1 %, must however be regarded with caution. In the first place the literature shows repeatedly that preparations of pure lecithin almost always give some figure for ash, and in our own experience two such preparations gave varying figures of 1 to 3 %. Our phosphatide preparation P1 with an ash of 4.27 % therefore was giving a relatively low value, and this percentage is no more than is required were its metallic constituents entirely present as phosphatides. Secondly, it seems rather doubtful if the potassium were present as phosphatide, for the alkali salts of phosphatidic acid are water-soluble. It must not be overlooked, however, that ethereal solutions of phosphatides will readily dissolve many substances which are insoluble in ether though soluble in water.

Previous work on the phosphatides of wheat germ has been confined to determinations of ether-soluble P or the recognition of the presence of lecithin from a study of the hydrolysis products. In regard to these and other findings, reference may be made to the work of Schulze and Steiger [1889], Winterstein and Hiestand [1907-08], Schulze [1908], Andrews and Bailey [1932], Sullivan and Near [1933], Knowles and Watkin [1932].

The function which salts of phosphatidic acid are playing in the plant cannot be assessed on the present limited evidence. In leaves, Channon and Chibnall found calcium phosphatide to be the only phosphatide present in the case of the cabbage. Smith and Chibnall [1932], who encountered very great difficulty in extracting the phosphatides of grass, were finally compelled to use grass dried for 8 minutes at 200°. This material contained many phosphatide decomposition products but the investigations showed that in this leaf lecithin and kephalin were also present. Jordan and Chibnall [1933] obtained 0.4 g. of crude phosphatide from the embryo axes of 5000 seeds of the runner bean and, so far as could be achieved on this very limited amount of material, the results

suggested that the major part of the mixture consisted of a salt of phosphatidic acid, together with lecithin-kephalin in the proportions of 1:2. Our results on wheat germ give a corresponding ratio of 1:1.5. These authors found that on germination the disappearance of the cotyledon phosphatides was much more rapid than the appearance of phosphatides in the growing organs and hence they deduced that seed phosphatides must function chiefly as reserve food.

SUMMARY.

1. Before fractionation of a preparation of the phosphatides of wheat germ, investigations were made of the best methods for extraction of the germ and of precipitation of the phosphatide from the extract.

2. Analyses of the oil prepared from seven samples of germ are recorded. The phosphatide fractions from these extracts were analysed for their contents of nitrogen, phosphorus and ash, together with the yields of fatty acids obtained by hydrolysis.

3. The deductions made from these analyses were confirmed by a detailed investigation of the phosphatide fraction prepared from 9.5 kg. of wheat germ. This fractionation showed the presence of phosphatidic acid (as calcium, magnesium and potassium salts), lecithin and kephalin. These substances occur in the approximate proportions of 4:4:1 when referred to the phosphorus content; at least 42 % of the total phosphatide being present as phosphatidic acid.

4. A method of working ethereal solutions of phosphatides with aqueous solutions, which largely overcomes the persistent emulsions usually resulting, is described.

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