CLX. THE PHOSPHATIDES OF FORAGE GRASSES.

I. COCKSFOOT.

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A RECENT investigation by Channon and Chibnall [1927] of the phosphatides of cabbage leaves has shown that lecithin and kephalin are absent, and that the main constituent is a new phosphatide, calcium phosphatide. In the preliminary investigations of the grasses the methods of these workers for the preparation and purification of leaf phosphatides were applied, but were not very successful for two reasons. In the first place the grass blades contained a higher proportion of solids, and especially of fibre, than cabbage leaves, consequently the grinding operations necessary to prepare the ether extract from the fresh material were very laborious; further, the percentage of phosphatide in the ether extract was low, so that 20 kg. samples of fresh grass gave only 2–4 g. of crude phosphatide. In the second place the treatment necessary for the preparation of pure phosphatidic acid resulted in the formation of emulsions. These are to be expected when working with any extract containing phosphatides, but in the present case they were far more difficult to break than those which had been encountered during the purification of the cabbage phosphatides. The presence of phosphatidic acid was readily proved, but the yields were disappointingly low, and attempts to separate and purify it by taking advantage of the water-solubility of the sodium salt showed that a large proportion of the crude phosphatide fraction was non-acidic in character, and was therefore essentially different from that found in the cabbage.

This result was to a certain extent unexpected, because in these early stages of the research we were working with products prepared from perennial ryegrass (*Lolium perenne*) and the analysis of the crude material was similar to that obtained from cabbage. (Found: C, 54.5; H, 8.3; ash, 19.8; Ca, 5.4; P, 3.9 %. A typical cabbage preparation gave C, 57.2; H, 8.7; ash, 19.5; Ca, 5.9; P, 4.6 %.) Later batches of material, however, from this and other grasses, often gave lower figures for ash, calcium and phosphorus, and in addition significant amounts of magnesium and nitrogen, *e.g.* batch *R* (Cocksfoot) gave C, 52.8; H, 8.1; ash, 12.5; P, 2.4; Ca, 1.0; Mg, 1.4; N, 1.0 %. These results, coupled with the observation that obstinate emulsions were

always formed when an ethereal solution of crude phosphatide was shaken with hydrochloric acid, led us to suspect the presence of lecithin and kephalin. To separate these two phosphatides from phosphatidic acid successfully demanded a larger quantity of crude phosphatide than we had hitherto been able to prepare by the methods we were then employing, and a change of procedure was necessary.

Now in connection with the general research which is being made in this laboratory into the composition of the ether extract of grasses we had already made numerous experiments to find out the best method of preparation; whether, for instance, the direct extraction of dried grass was preferable to the procedure of Chibnall and Channon [1927]. These results will be reported more fully in another communication, but at the above-mentioned stage in the phosphatide research it had been found that good yields of crude phosphatide could be obtained from grasses that had been dried very rapidly (1-2 hours) in a steam-oven through which a slow current of air was drawn, whereas the yields from grass dried slowly at room temperature or at 40° were poor. But the rapid drying of, say, 150 kg. of grass under these conditions was beyond the equipment of the laboratory, and we could not make use of dried grass as our raw material until we were fortunate enough to have placed at our disposal the new experimental grass-drying plant of the Imperial Chemical Industries at Jealott's Hill, in which the grass, supported on a moving belt, is passed along a chamber through which air heated initially to about 200° is blown. Drying is effected in about 8 minutes without "scorching" of the grass or any apparent chlorophyll breakdown. We were then able to obtain 22 kg. of dried cocksfoot (*Dactylis glomerata*), and from this 56 g. of crude phosphatide (Batch R). Knowing the extreme lability of phosphatides, as evidenced by the history of the "cuorin" and "jecorin" of the earlier workers in this field, we were apprehensive as to the fate of the grass phosphatides under this admittedly drastic drying treatment, and the procedure was adopted more or less under duress because it seemed at the time to be the only one available.

The large batch of crude phosphatide was readily fractionated by the method outlined in Table I, and we had no difficulty in showing that fraction F consisted chiefly of lecithin and kephalin by isolating derivatives of choline and β -aminoethyl alcohol after acid hydrolysis. The elementary analysis of this fraction showed that whereas the ratio N:P was practically 1:1 the actual amounts of N and P present were lower than were to be expected if it consisted only of these two phosphatides. This was found to be due, at any rate in part, to the presence of sugars or substances giving sugars on acid hydrolysis, which, calculated as glucose, were equivalent to 6.1 % of the fraction. This apparent adsorption of sugar by the phosphatide material will be referred to again later.

Having removed the major part of the lecithin and kephalin, and also fraction G, which will be shown later to consist of decomposition products, it

Table I. *Fractionation of crude phosphatide (fraction A).*

was possible to isolate phosphatidic acid by precipitation as the lead salt in the usual way (fractions H and J). We were thus able to obtain definite proof that the three phosphatides lecithin, kephalin and phosphatidic acid (as Ca or Mg salt) were all present in the blades of cocksfoot, which must, therefore, differ materially in their phosphatide metabolism from cabbage leaves, which contained only calcium phosphatide.

While this result was satisfactory we were disappointed to find that the lecithin and kephalin (fraction F) and phosphatidic acid (fractions H and L) accounted for only about 9 % and 15 % respectively of the crude phosphatide (fraction A). An amount equal to 45 % passed into the aqueous phase on shaking with acid and the remainder into the three fractions G, N and P

which we believe to be products of decomposition brought about by the heating necessary to dry the grass and the subsequent treatment with aqueous acid. This conclusion is based partly on the exhaustive researches of Thudichum, MacLean and Levene, who, given adequate quantities of material, have been able to show that crude animal phosphatides contain only lecithin, kephalin and sphingomyelin, and that numerous other so-called individual phosphatides consist of lecithin and kephalin mixed with decomposition products which have been produced by the treatment necessary to prepare and purify the phosphatide material. Were it not for the fact that calcium phosphatide was found by Channon and Chibnall to be the only phosphatide present in cabbage leaves, and that we have evidence, to be published shortly, that the lecithin and kephalin present in runner bean seeds give place first to magnesium and later to calcium phosphatide during germination, we should have hesitated to suggest that fractions H and L represented phosphatidic acid and were not themselves products of kephalin decomposition.

Fraction G was a light grey powder insoluble in all organic solvents other than chloroform, and except that it had a N : P ratio nearly 1 : 1 instead of 1 : 2 resembled in other respects the "cuorin" isolated by Levene and Rolf [1926] from soya bean seed crude phosphatide. After acid hydrolysis it gave 8.1 % of reducing material calculated as glucose and only 20 % of fatty acids soluble in light petroleum. All the nitrogen was in the amino-form, and the fraction appeared to consist of kephalin fragments mixed with other products which had been rendered ether-soluble by the presence of true phosphatides in the original ether extract. Fraction P consisted of glycerides and fraction N of fatty acids. Both these fractions were freely soluble in ether-acetone, and could not have existed as such in the original ether extract; otherwise it is difficult to understand how they could have been precipitated by acetone with the waxes and phosphatides, and in any case have escaped extraction with the waxes during the hot acetone treatment. It seems reasonable to assume that they had been split off by the hydrochloric acid from phosphatide fragments which still retained the acetone-insolubility of the true phosphatides themselves. A study of the component fatty acids (as shown in Table II) lends support to this contention, for the acids from fractions N and P resemble those of the phosphatides F and J more closely than they do those derived from the glycerides of the true fat.

The mixed fatty acids from each of the four fractions F, J, N and P were oxidised under controlled conditions. Tetrahydroxystearic (sativic) acid and hexahydroxystearic (linusic) acid were readily isolated, showing that the original acids contained linoleic and linolenic acids. In a previous paper [Smith and Chibnall, 1932] we discussed at some length our inability to obtain any crystalline dihydroxystearic acid from the oxidation products of the true glyceride fatty acids of cocksfoot and perennial ryegrass and came to the tentative conclusion that if an octadecadienoic acid were present in the glycerides it was not oleic acid. Our experience in the oxidation of the phos-

Table II. *Oxidation of the fatty acids from various fractions of the cocksfoot phosphatides.*

Fraction	F (Lecithin- kephalin) %	J (Phospha- tidic acid) %	N (Free fatty acids) %	P (Glyceride material) %	Fatty acids from glycerides of true fat [Smith and Chibnall, 1932] %	Fatty acids from linseed oil %
Saturated acids	19	33	28	34	14	14
Dihydroxystearic acid fraction	3.3 (gum)	2.8 (gum)	5.4 (gum)	4.6 (gum)	3.6 (gum)	12.7 (crystalline M.P. 131°)
Tetrahydroxystearic acid	12.1	17.2	10.0	7.0	4.1	5.4
Hexahydroxystearic acid	4.3	6.7	9.0	10.0	3.4	5.9
Wt. of acids taken for oxidation (g.)	1.16	1.8	2.59	1.48	11.0	2.05
i.v. of acids taken for oxidation	144	128	137	125	185	176

phatide fatty acids again leads to the same conclusion. In each case the dihydroxystearic acid fraction consisted of a small amount of gum from which no crystalline material other than minute amounts of tetrahydroxystearic acid could be isolated. To obtain definite evidence that our technique was not at fault we oxidised a similar quantity of fatty acids derived from linseed oil, and isolated dihydroxystearic acid, M.P. 131°, without any difficulty. We therefore conclude that oleic acid is not present in the cocksfoot phosphatide fatty acids. It is interesting to note that Levene and Rolf [1925; 1926] in their analyses of the constituent fatty acids of soya bean lecithin and "cuorin" were unable to identify oleic acid.

The low proportion of saturated acids in the mixed fatty acids from cocksfoot phosphatides is in keeping with the results of Levene and Rolf for soya bean and of Channon and Chibnall for cabbage leaves, and emphasises the fact that plant lecithin and kephalin differ from those derived from animal sources in that they do not contain saturated and unsaturated acids in equimolar proportions.

The persistence with which most of these phosphatide fractions retain reducing sugars recalls the similar observations of Schulze and his co-workers, who worked with crude phosphatide preparations from seeds. Levene and Rolf [1925; 1926] were able to show, however, that in the case of the soya bean phosphatides this sugar was an impurity which could be removed by drastic purification, showing that no cerebroside or sugar-containing phosphatide was present. Sufficient evidence has been collected to suggest that this is also the case with the grass phosphatides.

When the crude phosphatide (fraction A) was shaken in ethereal solution with hydrochloric acid, material equivalent to 45 %, including reducing sugars equivalent to 8.1 %, passed into the aqueous phase. Fraction G on acid

hydrolysis gave 11 % and fraction F 6 % of reducing sugars, equivalent to 1.6 % and 0.5 % of fraction A respectively. Fraction A therefore contained at least 10 % of reducing sugars. The values for the sugar content of other batches of cocksfoot examined during the course of this work are collected in Table III. It will be observed that in the two cases in which the crude

Table III. *Sugar content of certain cocksfoot phosphatide fractions.*

Batch number	Treatment of grass	Solvent used for extraction	Reducing sugars in crude phosphatide (calculated as glucose) %
Q 1930	Fresh method of Chibnall and Channon [1927]	Ether	0.9
	Rapidly dried	Ether	3.1
A 1931	Fresh method of Chibnall and Channon [1927]	Ether	0.7
	Rapidly dried	Ether	3.0
	Rapidly dried	Light petroleum B.P. 40–60°	3.7

phosphatide was prepared by the procedure of Channon and Chibnall the sugar content was low, and when it is remembered that this method removes most of the water-soluble products, and therefore most of the leaf sugars, before the "coagulum" and residual leaf material are extracted with ether to prepare the crude phosphatide, it seems legitimate to assume that in the case of the dried grasses the sugar has been taken into ethereal solution through the agency of the lecithin and kephalin, which are known to possess unique solubility properties.

To summarise the results of our investigations on the crude phosphatide of rapidly dried grass, we have been able to show that it contains lecithin, kephalin and a salt of phosphatidic acid. Considerable amounts of decomposition products, probably derived from kephalin are also present, and for this reason it does not seem to us profitable to discuss either the relationship which the amount of crude phosphatide isolated bears to the amount of true phosphatide originally present in the fresh grass, or whether the phosphatidic acid was present as calcium or magnesium salt.

EXPERIMENTAL.

Before describing the fractionation of the phosphatides prepared from cocksfoot it is necessary to discuss briefly the theoretical analytical figures which are to be assumed for the lecithin, kephalin and phosphatidic acid present.

Investigation of the constituent fatty acids from each of the products isolated has shown that the i.v. was 125–145 and that approximately one-third was composed of saturated fatty acids M.P. 53–55°, the analytical figures for which in every case fall between those of palmitic acid $C_{16}H_{32}O_2$ (C, 74.9;

H, 12.6 %; mol. wt. 256), and stearic acid $C_{18}H_{36}O_2$ (C, 76.0; H, 12.8 %; mol. wt. 284). The saturated acids from the glycerides of the true fat [Smith and Chibnall, 1932] were similar and were shown to consist of about 70 % palmitic, 20 % stearic and 10 % higher acids. If therefore 25 % of the total acids from the three phosphatides be taken as palmitic acid, then it is probable that the remaining 75 % consists of C_{18} acids whose mean unsaturation is equivalent to three double bonds in two molecules, and it is on this basis that the following analytical figures have been calculated.

	C %	H %	N %	P %
Lecithin	65.3	10.9	1.77	3.91
Kephalin	65.6	10.6	1.91	4.24
Phosphatidic acid	66.5	10.4	—	4.52
Lead phosphatide	51.2	7.7	—	3.48

Lead phosphatide having this composition should give an ash of lead pyrophosphate equal to 33 %.

During the present research more than one hundred micro-combustions have been carried out on products obtained at various stages in the fractionation, and we have found it unusually difficult to obtain reproducible figures for carbon, hydrogen and ash. In almost all cases the carbon figure was liable to vary between 1 and 2 units in 50 or 60, while the ash showed a similar but not necessarily corresponding difference.

The following are quoted as typical examples:

		C %	H %	Ash %
Crude lead phosphatide (Batch P)	1	50.3	7.8	30.5
	2	48.2	7.1	29.5
The same, after purification by precipitation from ethereal solution by alcohol	1	47.9	7.6	32.9
	2	47.0	7.6	32.6
	3	48.3	7.4	32.6
Theory requires		51.2	7.7	33.0

Throughout the investigation reported below we have not been able to handle sufficient material to enable us to prepare chemically pure lecithin, kephalin or phosphatidic acid. We have, therefore, been dealing throughout with mixtures, and the errors may be due, in large part, to the difficulty of obtaining a representative sample of such gummy substances in the small amount required for a micro-combustion. Loss of ash may also be caused by the creeping of the molten material from the boat before it is completely burnt.

Fractionation of the crude phosphatide from dried cocksfoot.

22 kg. of the dried cocksfoot (*Dactylis glomerata*) were exhaustively extracted with ether by British Drug Houses, Ltd. and gave 770 g. of ether extract. This was dissolved in 2 litres of warm ether and 4 litres of hot acetone were added with stirring. After standing overnight at 0° the precipitate of

wax and phosphatide was filtered off, redissolved in a similar quantity of warm ether and again precipitated with acetone. The resulting mixture was then extracted six times with hot acetone to dissolve out the wax fraction, each acetone extract being filtered hot. As the crude phosphatide was a sticky gum which might well retain some of the wax, it was thought advisable, after the second and fourth extractions with hot acetone, to dissolve the residue in ether and remove the solvent by evaporation, thereby exposing a new surface of the phosphatide to the action of the hot acetone. No appreciable amount of phosphatide passed into the hot acetone, although, as will be shown later, it contained both lecithin and kephalin. The crude phosphatide thus obtained (fraction A, Table I) weighed 56 g., equivalent to 7.3 % of the ether extract. It analysed as follows: C, 52.8; H, 8.1; P, 2.44; Ca, 1.02; Mg, 1.36; N, 1.0; ash, 12.5 %. The subsequent treatment of the material is outlined in Table I.

Treatment of the crude phosphatide with dilute hydrochloric acid.

55 g. of "A" were dissolved in ether, shaken four times successively with 500 cc. of dilute hydrochloric acid, and then well washed with water. Emulsification at this stage caused considerable trouble, but by varying the relative amounts of ether and of water present, by the judicious addition of small amounts of alcohol, and by the aid of the centrifuge, this difficulty was largely overcome. 55 % of the original material remained soluble in ether, so that by difference 45 % had passed into the acid and aqueous washings. These latter were collected and analysed. They contained 79 % of the calcium, 76 % of the magnesium, 41.5 % of the phosphorus and 26 % of the nitrogen originally present in the crude phosphatide. In addition, reducing substances, estimated as glucose by the method of MacLean [1919] were present to the extent of 8.1 % of the crude phosphatide.

The ethereal solution on evaporation gave 30 g. of material which analysed as follows: C, 63.0; H, 9.3; P, 2.73; Ca, 0.28; Mg, 0.12; N, 0.63; ash 3.9 %. This was dissolved in the minimum amount of ether and several volumes of acetone added. After standing overnight at 0° the precipitate was centrifuged and washed with acetone. The ether-acetone solution and the acetone washings were collected, the solvents removed, the resulting material again dissolved in the minimum amount of ether and precipitated with acetone. The material insoluble in ether-acetone constitutes the impure lecithin-kephalin fraction D, and the soluble material the crude phosphatidic acid mixture E.

Fraction D. This fraction represented 23 % of fraction A, and analysed as follows: C, 53.4; H, 8.8; ash, 9.3; P, 2.82; N, 1.5 %. It was extracted three times with boiling acetic acid, the extracts united, reduced to a small volume and allowed to stand at 0° for some hours. The material soluble in the cold acetic acid represents the lecithin-kephalin fraction F, and the material insoluble in both hot and cold acetic acid constitutes fraction G, which will be shown later to consist of decomposition products.

Lecithin-kephalin (fraction F). This fraction, representing 8.7 % of the original fraction A, was a black sticky gum which analysed as follows: C, 63.5; H, 10.1; P, 3.0; N, 1.4; ash, 6.0 %; N : P ratio 1.03 : 1. It was shown to consist chiefly of lecithin and kephalin by identification of the products obtained on acid hydrolysis.

The material (2.3 g.) was hydrolysed by boiling with 5 % sulphuric acid for 8 hours. The cooled mixture was then exhaustively extracted with ether, which removed 1.59 g. of fatty acids. The aqueous solution and washings were reduced *in vacuo* to 100 cc. and analysed for nitrogen and reducing sugars. The total N was 25 mg., the amino-N was 8 mg. and the non-amino-N (by difference) was 17 mg. Reducing substances, estimated as glucose by the method of MacLean [1919] were 140 mg. or 6.1 %. Glucose, and especially fructose, when submitted to the hydrolysis mentioned above would be partially destroyed, so that the low values for phosphorus and nitrogen in this lecithin-kephalin preparation are due, at any rate in large part, to the presence of carbohydrate.

Identification of β -aminoethyl alcohol. A sample of synthetic β -aminoethyl alcohol, kindly provided by Dr Dudley, was found to be quantitatively precipitated by the mercury carbamate method employed by Neuberg and Kerb [1912] for amino-acids, and the aqueous hydrolysis products of fraction F were accordingly treated in the following way.

Sulphuric acid was removed from solution with baryta, the barium sulphate being centrifuged and well washed with hot water. Lead acetate was then added, the small precipitate centrifuged, and lead removed from the clear liquid by means of hydrogen sulphide. The water-clear solution thus obtained was concentrated to 100 cc. and treated alternately with small amounts of 20 % aqueous sodium carbonate and 20 % aqueous mercuric acetate. The reaction was kept just alkaline to litmus and the reagents added until the addition of either produced a permanent orange precipitate. An equal volume of alcohol was then added and the mixture allowed to stand overnight. The precipitate was centrifuged off, washed with a little 50 % alcohol, suspended in water and mercury removed with hydrogen sulphide. The mercuric sulphide was well washed with hot water, the clear liquid and washings acidified with a little hydrochloric acid and evaporated to dryness *in vacuo*. The hydrochloride of β -aminoethyl alcohol was then extracted from the residue of inorganic salts with alcohol. The alcoholic extract was run into a small beaker, evaporated to dryness on the water-bath, the residue taken up in a few cc. of dilute hydrochloric acid and gold chloride added. The mixture was allowed to evaporate slowly in a vacuum desiccator. Brown cubic crystals came out during the course of a few days, and long orange needles were obtained on recrystallisation from water. The melting-point alone, and when mixed with authentic β -aminoethyl alcohol chloroaurate was 189–191° with previous softening at 187°. (Found: Au, 48.7. $C_2H_7ON.HAuCl_4$ requires Au, 49.2 %.)

Identification of choline. The aqueous-alcoholic filtrate from the mercuric

acetate precipitation mentioned above was concentrated *in vacuo* to 20 cc. and treated with a hot concentrated alcoholic solution of mercuric chloride. A white crystalline precipitate appeared almost at once, and increased on cooling. The mother-liquor on concentration gave another crop of crystals, and the total yield was 1.3 g. The crystals were typical of choline mercuric chloride and melted at 252° with previous softening. For confirmation 0.5 g. of the material was dissolved in a few cc. of hot water, mercury removed with hydrogen sulphide and the calculated amount of gold chloride added to the clarified liquid. Fine yellow needles of choline chloroaurate appeared on standing, and were filtered off. The mother-liquor on concentration gave a further yield of beautiful yellow plates. Both needles and plates melted at $269\text{--}270^{\circ}$ with decomposition. (Found: C, 13.9; H, 3.2; Au, 44.6. $\text{C}_5\text{H}_{14}\text{ON} \cdot \text{AuCl}_4$ requires C, 13.5; H, 3.2; Au, 44.5 %.) Allowing for aliquots withdrawn for analysis the yield of choline mercuric chloride represented 70 % of the non-amino-N present in the hydrolysis products of fraction F.

Identification of the fatty acids. The 1.59 g. of fatty acids mentioned above (i.v. 144; mol. wt. 290) represented a yield of 69 %. The theoretical yields for lecithin and kephalin are 69 % and 75 % respectively. For further identification of the constituent acids of this and other mixed fatty acids derived from the crude phosphatide the acids were oxidised with dilute alkaline permanganate below 10° as described by Lapworth and Mottram [1925]. The oxidation was allowed to proceed for 5 minutes when it was stopped by decolorising with sodium bisulphite followed by a little concentrated hydrochloric acid. The oxidation products insoluble in water were filtered off, dried in a vacuum desiccator, and the saturated acids extracted with light petroleum (B.P. $80\text{--}100^{\circ}$). These were then subjected to the magnesium soap treatment of Bertram [1927], to give an approximate value for the amount of saturated acid present in the original mixed fatty acids. The residue insoluble in light petroleum was next extracted three times with boiling ether to remove any dihydroxystearic acid. The material thus obtained in this and other similar fractions from the crude phosphatide was a brown gum, and attempts to prepare crystalline material resulted in the isolation of minute amounts of tetrahydroxystearic acid, whereas in the case of linseed oil, crystalline dihydroxystearic acid, M.P. 131° , was obtained without difficulty. The material insoluble in ether was recrystallised from alcohol and in each case was found to consist of mixed α - and β -sativic acids. The oxidation products which remained soluble in water were obtained by evaporating the solution to dryness *in vacuo* and extraction with alcohol. In all cases either α - or β - or both linusic acids were obtained.

In the case of the mixed fatty acids from fraction F, 1.16 g. were oxidised and gave (1) 0.21 g. of saturated acids which was recrystallised from alcohol. (Found: C, 74.5; H, 12.6 %; mol. wt. 282; M.P. $53\text{--}55^{\circ}$.) (2) 0.04 g. of dark gum representing the dihydroxy-derivatives. (3) 0.09 g. of tetrahydroxystearic acid M.P. $155\text{--}165^{\circ}$. (Found: C, 62.2; H, 10.7. $\text{C}_{18}\text{H}_{36}\text{O}_6$ requires C,

62.0; H, 10.4 %.) Fractionation from ethyl acetate gave α - and β -sativic acids of m.p. 157–159° and 172–173° respectively. (4) 0.05 g. of hexahydroxystearic acid. m.p. 185–190°. By repeated recrystallisation from water α -linusic acid, m.p. 203–204°, was obtained. (Found: C, 56.9; H, 9.7. $C_{18}H_{36}O_8$ requires C, 56.8; H, 9.5 %.)

Phosphatidic acid (fraction J).

The crude phosphatidic acid (fraction E) mentioned above represented 32 % of the crude phosphatide (fraction A). The analysis, C, 68.5; H, 10.3; P, 2.02; ash, 2.6 %, showed that some other fatty material, possibly glyceride, must have been present. This mixture, dissolved in ether, was shaken four times with a strong solution of lead acetate. The ethereal solution was well washed with water, concentrated to a small bulk and three volumes of alcohol added. The precipitate of lead phosphatide was centrifuged, redissolved in a similar volume of ether and again precipitated with alcohol. The weight of lead salt (fraction H) so obtained, allowing for the lead present, represented 11 % of the crude phosphatide (fraction A). (Found: C, 50.7; H, 7.5; ash, 31.4. Lead phosphatide requires C, 51.2; H, 7.7; ash, 33.0 %.) An ethereal solution of the salt was shaken with dilute hydrochloric acid to remove the lead, washed with water, and evaporated to dryness *in vacuo*. The amount of phosphatidic acid (fraction J) was equivalent to 10 % of the crude phosphatide (fraction A). (Found: C, 63.9; H, 9.8; ash, 6.6; P, 4.24; N, 0.6. Phosphatidic acid requires C, 66.5; H, 10.4; P, 4.52, N, 0 %; mol. wt. 686.) The i.v. was 98 and the mol. wt. by titration 746, showing that the preparation still contained some non-acidic material. This was confirmed when 3.46 g. were saponified with strong alcoholic potassium hydroxide for 2½ hours. 1.91 g. of mixed fatty acid, representing only 77 % of the theoretical yield, were obtained. The i.v. was 128 and the mol. wt. 298.

Identification of the fatty acids. 1.80 g. of mixed fatty acids were oxidised by the methods outlined above, and gave (1) 0.60 g. of saturated acids, m.p. 52.5–54.5°. (Found: C, 75.6; H, 13.0 %.) (2) a gum weighing 0.06 g. representing the dihydroxystearic acid fraction. (3) 0.31 g. of mixed α - and β -sativic acids, which were separated by means of ethyl acetate. (4) 0.12 g. of hexahydroxystearic acid. Several recrystallisations from water and alcohol gave products melting at 197° and 167° respectively. (Found, for the latter: C, 56.8; H, 9.8 %.) The melting-points of α - and β -linusic acids are stated to be 203° and 173° respectively and in view of the small amount of material available it is probable that the fractionation had not been carried far enough.

Phosphatide decomposition products.

Under this heading are described the three fractions which appear to be products of phosphatide decomposition brought about by heat during the drying of the cocksfoot.

Fraction G. This fraction was insoluble in glacial acetic acid, and represented 14 % of the original crude phosphatide (fraction A). It was a light grey powder which absorbed chloroform to form a gel but which did not readily disperse into solution. Otherwise it was insoluble in the usual organic solvents. (Found: C, 51.0; H, 8.3; ash, 3.5; P, 2.15; N, 1.1 %.) The product resembled in many respects the so-called "cuorin" fraction which Levene and Rolf [1926] isolated from soya bean seed phosphatides, which was soluble only in chloroform, had a ratio N : P of 1 : 2 and which they considered to be a mixture of kephalin decomposition products. In the present case the decomposition had been more extensive and the product was contaminated with substances giving reducing sugars on acid hydrolysis.

4.4 g. of material were hydrolysed by boiling with 10 % hydrochloric acid for 8 hours, the mixture cooled and extracted with ether. 0.25 g. of a black powder remained insoluble in both ether and water, while the ether extracted 1.31 g. of material which was molten at 100° and semi-solid at room temperature. This latter gave 0.92 g. of fatty acids soluble in light petroleum, a yield of only 21 %, showing that the phosphatide fragments had lost part of their complement of fatty acids. The aqueous acid contained reducing substances equivalent to 0.48 g. of glucose and also 0.043 g. of nitrogen, all of which was in the amino-form.

Fraction N (free fatty acids). Fraction K, the mother-liquor from the precipitation of the lead phosphatide (fraction H), was concentrated to a small volume and three volumes of alcohol added. A further quantity of crude lead phosphatide (fraction L) was precipitated. (Found: C, 55.1; H, 8.3; ash, 23.5 %.) The clear alcoholic solution (fraction M) represented 14 % of the crude phosphatide (fraction A). (Found: C, 73.8; H, 11.1; ash, 1.2 %.) The analysis suggested that the product was a mixture of fatty acids or glycerides. 5.20 g. were accordingly dissolved in ether and shaken with aqueous sodium hydroxide. 2.25 g. of neutral material (fraction P) remained in the ether and 2.76 g. of fatty acids (fraction N) were recovered from the alkaline soap solution. 2.59 g. of these acids were oxidised and gave (1) 0.72 g. of saturated acids (Found: C, 75.4; H, 12.4 %; mol. wt., 284; m.p. 53.5–55.5°); (2) 0.14 g. of dark gum representing the dihydroxystearic acid fraction; (3) 0.21 g. of tetrahydroxystearic acid, m.p. 165° indefinite (Found: C, 61.8; H, 10.5. $C_{18}H_{36}O_6$ requires C, 62.0; H, 10.4 %); fractionation from ethyl acetate and alcohol gave α - and β -sativic acids, of m.p. 158–160° and 173° respectively; (4) 0.27 g. of crude hexahydroxystearic acids; m.p. 184° with previous softening at 165°.

Fraction P (mixed glycerides). The neutral material from the above separation represented 6 % of fraction A. (Found: C, 74.9; H, 11.8 %.) On mild saponification 2.1 g. gave 1.63 g. of mixed fatty acids (i.v. 125; mol. wt. 280) and 0.25 g. of unsaponifiable material, probably a wax alcohol. (Found: C, 81.3; H, 12.8 %.) The saponification value was 167, or, allowing for the small amount of unsaponifiable material, 185. These results suggest that the

saponifiable material consisted chiefly of diglyceride fragments split off from the phosphatide. A monoglyceride requires C, 70.7; H, 11.3 %; sap. val. 156: a diglyceride C, 75.4; H, 11.7 %; sap. val. 180: and a triglyceride C, 77.1; H, 12.3 %; sap. val. 189.

Oxidation of 1.49 g. of the fatty acids gave (1) 0.51 g. of saturated acids (Found: C, 75.1; H, 12.8 %; M.P. 59–60°; mol. wt. 259); (2) 0.07 g. of gum representing the dihydroxystearic acid fraction; (3) 0.105 g. of mixed α - and β -sativic acids, M.P. 157–163°, (4) 0.145 g. of mixed α - and β -linusic acids, M.P. 173°. The composition of these glyceride fatty acids, and also of the free fatty acids of fraction N, more closely resembles that of the phosphatides than that of the glyceride components of the cocksfoot fat (compare Table II).

Oxidation of fatty acids from linseed oil. 2.35 g. of linseed oil gave on saponification 2.17 g. of mixed fatty acids of i.v. 176. 2.05 g. of these acids were oxidised and gave (1) 0.29 g. of saturated acids, M.P. 52–53°, (2) 0.26 g. of dihydroxystearic acid; recrystallised repeatedly from alcohol it melted at 130.5–131° (Found: C, 68.3; H, 11.4; $C_{18}H_{36}O_4$ requires C, 68.5; H, 11.5 %); (3) 0.11 g. of mixed α - and β -sativic acids, M.P. 168–170°; (4) 0.12 g. of mixed α - and β -linusic acids, M.P. 192°.

SUMMARY.

The phosphatide fraction prepared from a sample of rapidly dried cocksfoot has been investigated in some detail.

Considerable phosphatide decomposition appears to have occurred during the drying, but lecithin, kephalin and the Mg or Ca salt of phosphatidic acid have been definitely identified.

The constituent fatty acids of the phosphatides have been investigated by controlled oxidation. Saturated acids, linoleic acid and linolenic acid were present. As in the case of the glyceride fatty acids of cocksfoot, no evidence for the presence of oleic acid could be obtained.

The ratio of saturated to unsaturated acids was less than unity, as appears to be usual in the case of plant phosphatides.

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REFERENCES.

- Bertram (1927). *Chem. Weekblad.* **24**, 226, 319.
Channon and Chibnall (1927). *Biochem. J.* **21**, 1112.
Chibnall and Channon (1927). *Biochem. J.* **21**, 226.
Lapworth and Mottram (1925). *J. Chem. Soc.* **127**, 1628.
Levene and Rolf (1925). *J. Biol. Chem.* **62**, 759.
—— (1926). *J. Biol. Chem.* **68**, 285.
MacLean (1919). *Biochem. J.* **13**, 135.
Neuberg and Kerb (1912). *Biochem. Z.* **40**, 498.
Smith and Chibnall (1932). *Biochem. J.* **26**, 218.