SUMMARY

1. Latex collected from *Euphorbia lathyris* contains a protease for which the name 'euphorbain' has been proposed.

2. The activity is assessed by measuring the viscosity reduction effect on gelatin or by the milk-clotting method. The gelatinase activity is measured in the digestion flask viscometer or in a specially designed micro-viscometer.

3. A solid preparation which retains its proteolytic activity for at least 1 year is prepared by adding acetone to the latex.

4. Euphorbain acts optimally at pH 8, is heat-labile, is not activated by reducing agents, but is almost completely inactivated by heavy metals and by I_2 at 0·02 M concentration.

5. The activity of the latex is not affected by dialysis and it remains unaffected when O_2, H_2 or N_2 are subsequently bubbled through the solution. Dialysis, however, removes protective materials from the latex and renders the enzyme more readily inactivated.

6. Euphorbain does not fall in the papain class of proteases. Its properties resemble those of the plant proteases hurain and solanain.

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Phosphoric Esters of the Pancreas: Choline Glycerophosphate

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Choline glycerophosphate was found by Karrer & Salomon (1926) among the products of hydrolysis of lecithin by lipase. It was stated to be a sticky, oily substance, intensely hygroscopic. We have found a similar material in the 'lead-soluble' fraction of an extract of commercial dried pancreas powder. After hydrolysis of the ester, choline and glycerophosphate were isolated.

The isolation of choline glycerophosphate resulted from an attempt to prepare choline sphingosine phosphate from the lead acetate-soluble fraction of an extract of commercial dried beef pancreas powder, according to a modification of the method previously described for fresh swine pancreas (King & Small, 1939). As a result there was obtained a fraction, of which the analytical figures were closer

to those of choline glycerophosphate than to those of choline sphingosine phosphate. Although it was not possible to isolate the substance in a pure condition, the material resembled that of Karrer, and had a choline : P ratio = 1. During the decomposition in water of the mercuric chloride compound of the ester with hydrogen sulphide and the subsequent concentration of the aqueous solution, there was a liberation of strong acid, and hydrolysis of the ester took place with a splitting-off of free choline. The resulting organic phosphorus compound was now precipitable by lead acetate. It was isolated as the barium salt (from aqueous ethanol) and proved to be glycerophosphate; the latter had presumably been formed by hydrolysis from lead acetate-soluble choline glycerophosphate.

Repetition of the preparation (twice), with careful attention to the avoidance of hydrolysis, resulted in the final concentration of a syrupy liquid, drying to a sticky hygroscopic mass, which yielded analytical figures suggestive of the possible existence of a tertiary-ester, namely a di-choline glycerophosphate.

EXPERIMENTAL

Analytical methods

Determinations of P were made colorimetrically by the perchloric acid digestion method (King, 1932); nitrogen by the micro-Kjeldahl procedure. Choline estimations were carried out by the method of Roman (1930); free choline was determined on samples freshly dissolved in water, combined choline after hydrolysis in boiling 2 N-HCl for 14 hr. Carbon and hydrogen analyses, on the material thought to be choline glycerophosphate, were by Weiler and Strauss, Oxford; analysis of the barium glycerophosphate was by Schoeller, Berlin.

Preparation of crude lead-soluble ester from pancreas

2 kg. of commercial dried beef pancreas powder were well mixed with 5 l. distilled water and heated at boiling temperature with vigorous stirring for 30 min. The mixture was made acid to litmus by the addition of 60 % perchloric acid.* After having been thoroughly chilled in ice, the mixture was poured on a large Buchner filter and the cake of precipitated protein sucked nearly to dryness.

The acid extract of the pancreas was neutralized with KOH and the precipitated $KClO_4$ removed. Basic lead acetate and ammonia were added till no further precipitate was obtained and the insoluble lead salts were filtered off. The 4 l. of filtrate were freed of lead by treatment with H_2S. The filtrate from the PbS was freed of H_2S, adjusted to pH 7, and carefully evaporated to dryness in a large balloon-flask *in vacuo*. A waxy mass remained which was only partially soluble in absolute ethanol. On concentration of the filtered ethanolic solution and addition of excess of ice-cold ether, a heavy precipitate was obtained which settled as two distinct layers. The lower yellow layer contained most of the P; the upper white layer was almost P-free. By carefully skimming off the white layer with a spatula, and repeating the process several times—solution in ethanol, precipitation with ether, settling or centrifuging in 250 ml. pots, followed by mechanical separation—a large mass of non-P containing material was eliminated. The final ethanolic solution contained 0·5 g. of organic P.

* Perchloric acid is a useful deproteinizing agent for blood (King, 1939), and is preferable to trichloroacetic acid for the preparation of acid extracts of tissue because of the great ease of disposal of excess acid in the extract through the insolubility of its potassium salt.

Isolation of choline glycerophosphate and glycerophosphate

The crude ester was submitted to the fractionation outlined in Fig. 1.

The mercuric chloride precipitates were amorphous white solids; the residues left on evaporation of the aqueous extracts after decomposition of the mercury precipitates were sticky gums. On adding ether to the ethanolic solutions of these gums oily precipitates were obtained which settled well on standing or centrifuging. The gummy residues and oily precipitates could be dried *in vacuo* over H_2SO_4 or P_2O_5 to clear yellow scaly solids, but these were very hygroscopic and some difficulty was encountered in weighing samples for analysis.

The various steps in the fractionation, outlined in Fig. 1, were followed by estimations of N, free and combined P, and free and combined choline. These estimations were performed on the solutions and on samples of the dried residues and precipitates. Precipitations of the ester with organic solvents (ether, etc.) from its solution in absolute ethanol were nearly complete and not much combined P was lost in these steps of the preparation. But in the later stages of the fractionation, when much of the contaminating material had been eliminated, the precipitation with ether tended to be less efficient, particularly if traces of water were present. It will be seen that the mercuric chloride precipitations were followed by considerable losses of combined P, N and free choline. It was not realized until several $HgCl_2$ precipitations and H_2S decompositions had been used, thattfree HCl was being formed and hydrolysis of the ester was taking place. This hydrolysis appears to have been responsible for the loss of large amounts of the ester through liberation of choline and formation of primary phosphoric ester not precipitable by $HgCl_2$. Whether the ester lost in this way was choline glycerophosphate, or whether some of it was choline sphingosine phosphate (isolated in the former preparation from fresh pig pancreas) cannot now be said.

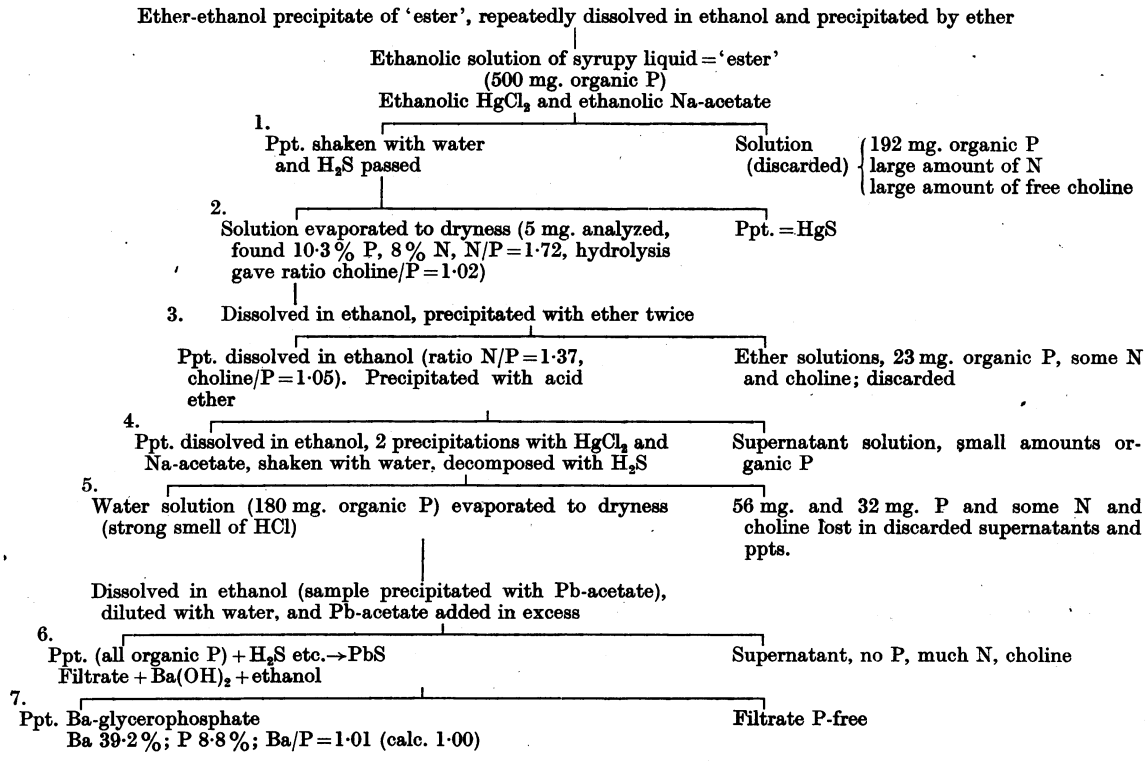

Fig. 1. Separation of lead acetate-soluble fraction from acid extract of dried pancreas.
Choline glycerophosphate, $C_8H_{22}O_7NP$ = 11·3 % P; 5·1 % N; 44·0 % choline; ratios: N/P = 1·0; choline/P = 1·0.
Choline sphingosine phosphate, $C_{23}H_{51}O_6N_2P$ = 6·43 % P; 5·8 % N; 25·1 % choline; ratios: N/P = 2·0; choline/P = 1·0.

At an early stage in the fractionation it was realized that the analytical figures being obtained, i.e. the percentages P, N and choline, and their ratios were nearer to those required by theory for choline glycerophosphate than for choline sphingosine phosphate, and the results obtained for the analyses suggested that we were almost certainly dealing with the former substance. But it was not certain that choline glycerophosphate had been present from the beginning of the process. The large amount of free choline formed and lost at step 1 could have been derived from some other ester, or from a precursor of choline glycerophosphate which contained a greater proportion of choline. (Figures suggestive of the latter possibility are presented below.)

The material thought to be choline glycerophosphate (ppt. stages 2 and 3, Fig. 1), which suffered complete hydrolysis to glycerophosphate at steps 4 and 5, was analyzed. Found: C, 35·2; H, 7·3; N, 6·8; P, 11·0 %. Calc. for choline glycerophosphate, $C_8H_{22}O_7NP$: C, 34·8; H, 7·99; N, 5·09; P, 11·3 %. The many N and P determinations performed on dried samples of the several precipitates gave figures of which the above are fairly representative; the % N was always higher, and the % P lower, than was demanded by theory, i.e. the N/P ratio was too high.

Choline was isolated from a sample of the above material, after it had been hydrolyzed with HCl, by precipitation as the platinic chloride salt, and as the periodide according to the procedure described by Booth (1935). Parts of the hydrolysis product were acetylated with acetic anhydride and with acetyl chloride, and gave typical acetyl choline responses when tested with the isolated frog's heart, and with the rectus abdominis muscle.

After the processes outlined in steps 4 and 5 had been carried out, and it was apparent that free HCl was present in considerable amount, a sample of the aqueous solution of the material was tested with lead acetate, and all the P was found to be precipitated. The whole product was thereupon treated with excess of lead acetate and ammonia, and the precipitate purified by transforming it into the barium salt and by repeated solution of the latter in water followed by precipitation with 2 vol. of ethanol. The glycerophosphate thus isolated had no optical activity when viewed in 2·1 % aqueous solution in a 4 dm. tube. Its pH titration curve showed a pK of 6·45 (found for authentic specimens of α-glycerophosphate pK 6·44, β-glycerophosphate

pK 6·34). The colour reaction of Hovey & Hodgins (1937) for glycerol was positive. Found: C, 11·9; H, 3·0; P, 9·6; Ba, 42·1%. Calc. for barium glycerophosphate, $C_3H_7O_6PBa.H_2O$: C, 11·1; H, 2·8; P, 9·5; Ba, 42·2%.

Isolation of a di-choline ester

The preparation was now repeated on a new lot of dried pancreas powder. Similar processes were followed in the fractionation, but great care was given to the maintenance at or near neutrality of all solutions. Purification was accomplished by repeated precipitation with ethanolic $HgCl_2$ and Na-acetate, extraction with water, decomposition with H_2S, concentration to dryness, solution in ethanol and precipitation with ether. Very little hydrolysis of phosphoric ester occurred, as was evidenced by the very small amounts of organic P and of free choline, in the supernatants from the $HgCl_2$ precipitations and ethanol-ether separations. The substance was finally concentrated as a syrupy liquid, which dried to a scaly, light yellow, transparent solid; it was hygroscopic and became sticky even on brief exposure to the air.

For analysis, about 24 mg. of wet material were taken. This was dried to constant weight, and then dissolved in 2N-HCl and analyzed for N, P and choline (Table 1, first fractionation). There was only a trace of free choline, and hydrolysis showed that almost all the N was represented by combined choline. But it was not certain (*a*) that the sample was completely hydrolyzed, or (*b*) that no destruction of choline had taken place during the prolonged boiling with 2N-HCl. Calculated as percentages, the figures are much closer to those demanded by theory for a di-choline glycerophosphoric ester than for the monocholine ester. The ratios of N/P and of choline/P are particularly suggestive of the existence of a tertiary ester.

Table 1. *Analyses of a 'lead-soluble' phosphoric ester (di-choline glycerophosphate?) from beef pancreas*

Fractionation of semi-pure ester	Analyses			Atomic (or molar) ratios	
	N (%)	P (%)	Choline (%)	N/P	Choline/P
First	6·1	6·8	46	1·98	1·73
Second	6·1	6·9	52	1·97	1·93
Third	6·1	6·9	45	1·98	1·67
Third recalc. to a chloride-free basis	7·0	7·8	51	—	—
Calc. for di-choline glycerophosphate	7·4	8·2	64	2·00	2·00

After a further precipitation with $HgCl_2$, etc., the material was carefully dried and another sample taken for analysis and hydrolysis (second fractionation, Table 1). The conditions used for hydrolysis in this experiment were 2N-HCl at 100° for 8 hr.

One more fractionation of the material was carried out, and the largest fraction re-analyzed. Its composition remained practically unaltered (third fractionation, Table 1). When the small weight of contained chloride was subtracted from that of the sample taken and the results recalculated on a chloride-free basis, then figures approximating to the theoretical for a di-choline glycerophosphate were obtained.

DISCUSSION

This material is still obviously impure, but the constancy of its composition on reprecipitation indicates that further elimination of contaminating substances is not likely to be attained by the type of fractionation we have employed. Furthermore, the amount of ester surviving the manipulations is too small to allow more work to be done on it. It is hoped to return to the problem of characterizing the Pb-soluble phosphoric esters of the pancreas and other tissues at a later date, when circumstances permit, and when larger batches of material can be worked up and studied. At present it appears permissible to conclude that there is probably a tertiary phosphoric ester in beef pancreas, a di-choline glycerophosphate, but proof of this must await further work.

SUMMARY

1. Glycerophosphoric acid has been isolated from the lead acetate-soluble fraction of an acid extract of commercial dried beef pancreas.

2. The glycerophosphate is thought to be derived from di-choline glycerophosphate, which is easily hydrolyzed to free choline and monocholine glycerophosphate. The latter further breaks down to choline and glycerophosphoric acid.

3. Substances approximating to the composition of both di- and monocholine glycerophosphate have been concentrated. They are intensely hygroscopic and difficult to obtain in a pure state.

The work described here was carried out in 1938–9 at the British Postgraduate Medical School and in 1939–40 at the Instituto di Pathologia, Rome. It has only now become possible to assemble the results and embody them in this communication.

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