CXXXVIII. THE PHOSPHORIC ESTERS OF THE PANCREAS: SPHINGOSINE CHOLINE PHOSPHATE

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In a former communication [King, 1932] it was shown that bone phosphatase would completely hydrolyse the phosphoric esters of muscle and of blood and that the optimum *p*H for the hydrolysis of esters from muscle was in the neighbourhood of the optimum for a phosphomonoesterase, while in the case of the esters from blood the optimum for hydrolysis corresponded more closely to that for the action of a diesterase. With complex mixtures of esters such as exist in tissue it is not reasonable to expect a well-defined *p*H optimum, but in the case of muscle the subsequent isolation by various workers of several phosphoric esters showed that most of them are optimally hydrolysed at an alkaline reaction. The isolation from blood of a large amount of diphosphoglyceric acid, an acid which is optimally hydrolysed near the neutrality point, is compatible with the *p*H hydrolysis curves of mixed esters from the blood.

In contrast with the phosphoric acid esters of blood and of muscle, those of some other organs have been found to be only partially hydrolysed by bone phosphatase at any *p*H. This is notably true in the case of pancreas tissue. It was found that only 40–50 % of the phosphorus of this organ is set free when treated with phosphatase even for very long periods. What this phosphoric ester (or esters) so refractory to the action of phosphatase, is, had not been determined. The present paper deals with the isolation of one of the esters.

Experimental

30 lb. of pigs' pancreas, packed in solid CO_2 about 10 min. after the killing of the animals, were treated with 7 % trichloroacetic acid and the extract fractionated as shown in Fig. 1.

The most striking feature of this fractionation is the very large amount of P which is not precipitable by basic lead acetate. Approximately 50 % of the organic P originally present in the trichloroacetic acid extract is not precipitated in the first three procedures. In the case of the phosphoric esters of muscle, blood and yeast fermentation, only very small amounts of P remain in solution after precipitation with basic lead acetate and ammonia [cf. Outhouse, 1935].

Phosphatase hydrolysis of fractions. A study of the phosphatase hydrolysis of the various fractions is shown in Fig. 2. The barium-insoluble fraction, like glycerophosphate, is practically completely hydrolysed at an alkaline reaction in 6 hr. The curve is not smooth, as in the case of glycerophosphoric acid. It shows an optimum *p*H compatible with the presence of primary esters, but there is also some indication of the presence of a secondary ester, or esters, or of some P compound similar to diphosphoglyceric acid.

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The phosphatase hydrolysis of the phosphoric acid esters from the mercury-insoluble and lead-insoluble fractions was similar to that of the barium-insoluble fraction: all esters were completely hydrolysed in 24 hr.

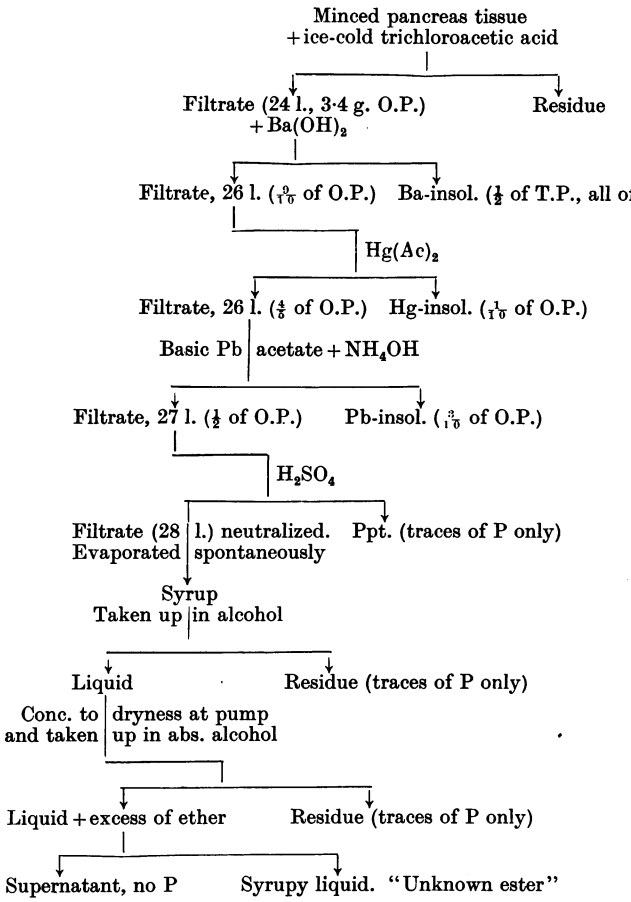

Fig. 1. Separation of phosphorus fractions from the pancreas. T.P. = total phosphorus, O.P. = organic phosphorus, F.P. = free (inorganic) phosphorus. All operations involving a departure from near neutrality in the reaction of the solution, carried out in the cold room.

In contrast is the almost complete absence of hydrolysis in the case of the lead-soluble fraction. It was thought that this phosphorus compound, so refractory to the action of phosphatase, might possibly be a secondary ester. If so, it should be hydrolysed by a secondary esterase. As Gulland [1938] has recently shown, snake venoms are very potent sources of phosphodiesterase. Solutions of the lead-soluble material were treated at different *p*H with a mixture of the venoms of *Crotalus oreganus* and *Naja Naja*, both rich sources of di-esterase. Bone phosphatase was added to each. Considerable hydrolysis occurred during 24 hr. at *p*H values ranging from 7 to 9, the maximum hydrolysis amounting to a little over 60%.

Isolation of an ester from the lead-soluble fraction. The phosphorus-containing substance (or substances) is soluble in absolute alcohol, from which it may be

thrown down as a heavy oil or syrup with ether, ethyl acetate, acetone, light petroleum or chloroform. It is exceedingly soluble in water and because of its extremely hygroscopic nature it has been found difficult to obtain the

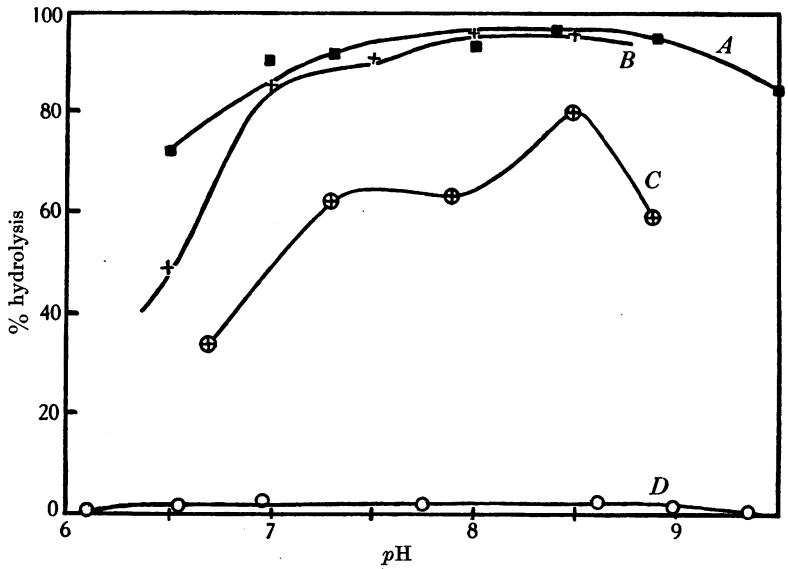

Fig. 2. Hydrolysis by bone phosphatase of phosphoric esters. *A*, glycerophosphate; *B*, esters precipitated by lead; *C*, esters precipitated by barium; *D*, "soluble ester". 6 hours at 37°.

substance in a condition satisfactory for analysis. Nevertheless, repeated precipitation with ether and with ethyl acetate and acetone, failed to change the P content to any appreciable extent.

In addition to P the material was found to contain N, half of which was in the amino-form. Repeated precipitations did not alter the P : N ratio. A very low reduction was given with the ferricyanide sugar reagent of Hagedorn and Jensen. Acid hydrolysis caused no increase in the reducing power. The iodine reduction (method of MacLeod & Robison [1929]) was much higher, probably owing to the presence of the amino group. There was no optical rotation. Comparative figures for several other phosphoric esters are shown in Table I.

Table I. *Comparison of soluble phosphoric ester from pancreas with other phosphoric esters*

	Reducing power as glucose (%)			Hydrolysis constant $k \times 10^{-3}$
	H. and J.	Iodine	$[\alpha]_D$	
Pancreas ester	6	27	0	0·060
Sphingosine choline phosphate	"No reducing carbohydrate"		0	Nearly equal to glycerophosphate*
Aminohexahydric alcohol phosphate	2	2	0	Nearly equal to glucose phosphate†
Aminoethanol phosphate	—	—	—	0·067‡
Choline phosphate	—	—	—	0·23‡
Ba glucose-6-phosphate	35	45	21° $[\alpha]_{5461}$	0·13§
Ba β-glycerophosphate	0	0	0	0·05

* Booth [1935].
† Outhouse & King [1935].
‡ Calculated from data of Plimmer & Burch [1937].
§ Robison & King [1931].

The properties of this very soluble P compound of the pancreas are in part similar to those of an ester from malignant tissue described by Outhouse [1933], and to the P-containing compounds shown to be present in embryonic tissue by Needham *et al.* [1937]; but its chief points of similarity seem to be with the water-soluble sphingomyelin-like substance of liver [Strack *et al.* 1933], and with the very soluble ester isolated from the kidney by Booth [1935] and thought to be the sphingosine phosphoric ester of choline. Booth isolated this ester by mercuric chloride precipitation from the alcoholic solution of the residue obtained on evaporation of the basic lead acetate filtrate. Precipitation of the present compound from alcoholic solution with mercury, followed by decomposition with H_2S in aqueous solution, evaporation and re-solution in alcohol did not alter the P or N content. Cadmium chloride likewise precipitates it from alcoholic solution; barium hydroxide gives no precipitate even when added to *p*H 12. The properties of the compound appear to be identical with those given by Booth for his ester.

The rate of hydrolysis for the phosphate group was determined in *N* HCl at 100°. It is difficultly hydrolysable like that of Booth's ester. The choline is split off much more easily than the phosphate group.

An examination of the products of prolonged hydrolysis with HCl showed the presence of choline, which was precipitated as the double salt with gold chloride. Its amount was estimated in the hydrolysate by the periodide method of Roman [1930], after heating a solution of the ester containing 2*N* HCl at 100° for 14 hr.

The analytical figures were as follows:

	P %	N %	Choline %
Found	6·40	5·80	25·5, 25·2
Calc. for sphingosine choline phosphate $C_{23}H_{51}O_6N_2P$	6·43	5·80	25·1

From the products of hydrolysis with hot barium hydroxide solution the barium salt of an ester was obtained which contained P and N in the ratio of 1 : 1. This is believed to be barium sphingosine phosphate. The amount of material finally available was insufficient for a satisfactory analysis.

The investigation of the esters contained in the barium- and lead-insoluble fractions is being continued, as well as the possibility of there being other esters in the lead-soluble fraction.

Summary

Approximately one half of the acid-soluble phosphorus of the pancreas is unprecipitated by barium hydroxide, mercuric acetate and basic lead acetate. The ester-phosphorus of this very soluble fraction is hydrolysed with difficulty by acids and by bone phosphatase, but is hydrolysed by the combined action of phosphomonoesterase and phosphodiesterase. A substance, thought to be sphingosine choline phosphoric ester, has been isolated from this fraction.

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