CLXXXI. FURTHER STUDIES OF AMINO-ETHYL PHOSPHORIC ESTER—A COMPOUND APPARENTLY SPECIFIC TO MALIGNANT TUMOURS

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ONE of the phosphoric esters of malignant tumours was identified by Outhouse [1936] as amino-ethyl phosphoric ester. This ester has never been reported as present in normal tissues. Two questions naturally arise: first, has the occurrence of this ester been overlooked in the isolation of the phosphoric esters of normal tissues, or secondly, is amino-ethyl phosphoric ester peculiar to malignant tumours.

If this ester is peculiar to malignant tumours, it is of fundamental importance that this be definitely established. The only means of establishing this relationship with malignancy is the investigation of the phosphoric esters of normal tissues using a method capable of detecting amino-ethyl phosphoric ester, if present in the tissues.

An attempt to detect the above-mentioned ester in pancreas, liver, placenta and embryo is reported in this paper. These tissues were chosen because it was considered that they would contain this phosphoric ester, if it was to be found in normal tissues. Muscle was not reinvestigated because of the thorough study given to its phosphoric esters by a large number of investigators. The author's experience with the fractionation of the acid-soluble phosphorus of this tissue leads to the belief that it would be impossible to detect amino-ethyl phosphoric ester among the phosphoric esters of muscle. The fractionation of the acid-soluble phosphorus of muscle and of malignant and benign tumours [Outhouse, 1933, 1935] is included in Table I for comparative purposes.

The fractionation of the acid-soluble phosphorus of the normal tissues investigated is given in Table I with the total amount and the number of batches extracted. This information is tabulated to emphasize the fact that sufficient tissue was used to show the presence of amino-ethyl phosphoric ester, if it were present only in very small quantities, and to compare the results obtained from normal tissues with the quantity of this ester previously found in malignant tumours.

EXPERIMENTAL

Method employed in an attempt to detect amino-ethyl phosphoric ester in normal tissues

The 4% trichloroacetic acid extract of the tissue was neutralized with baryta and filtered. To the filtrate was added 5 g. of mercuric acetate per litre. The mercury precipitate was removed by centrifuging. Basic lead acetate was

added to the centrifugate until a maximum precipitation of organic phosphate was obtained. The lead precipitate was centrifuged off and decomposed with hydrogen sulphide. The centrifugate from the removal of lead sulphide was aerated to remove hydrogen sulphide and baryta was added to it until the solution was at pH 10. The solution was filtered into 4 vol. of 95 % ethyl alcohol. The barium precipitate from the approximately 75 % alcohol was filtered off, washed with 95 % alcohol and with ether and dried. The washings were combined with the filtrate and the solution was tested for the presence of organic phosphate to assure that complete precipitation of the organic phosphate present in the lead-insoluble fraction had taken place.

The barium salts were converted into the brucine salts by removing the barium as barium sulphate and adding brucine in ethyl alcohol to the solution until the pH was 9. The brucine salts were fractionally crystallized by evaporating and adding alcohol and acetone until the acetone content of the solution was about 80 %. None of the fractions after drying showed a higher phosphorus content than 2 % P.

The brucine fractions were boiled in methyl alcohol. No amino-ethyl phosphoric ester crystallized from the hot methyl alcohol solution of the brucine salts of the phosphoric esters from the normal tissues.

Table I. *Distribution of acid-soluble phosphorus in mg./100 g.*

Tissue	Trichloroacetic extract			Ba- insol. O.P.	Ba- sol. O.P.	Lead- insol. O.P.	Lead- sol. O.P.	Remarks	
	T.P.	F.P.	O.P.					No. of samples	Total wet wt. tissue kg.
Dark muscle	127	73	54	35	19	16	2	16	12.5
Light muscle	163	90	73	56	17	15	2	13	7.25
Bovine pancreas	87	31	56	12	44	15	29	2	16.24
Bovine liver	126	70	56	11	46	7	37	1	0.9
Human placenta	53	40	13	10	3.0	1.5	1.5	1	2.5
Bovine embryo	138	113	25	15	10	7	3	1	3.3
*Malignant tumour	133	88	45	13	32	13	19	16	42.5
*Benign tumour	19	12	7	2	5	2	3	4	5.6

* These values are the average values from the tables given by Outhouse [1933, 1935].

T.P. = total phosphorus. F.P. = inorganic phosphorus.
O.P. = organic phosphorus (phosphoric acid esters).

An improved method of synthesis of amino-ethyl phosphoric ester

The method of synthesis described by Outhouse [1936] did not give a very satisfactory yield of amino-ethyl phosphoric ester. The following modification of the method has increased the yield to about 50 % of theoretical. Mix 10 ml. (1 mol.) of mono-ethanolamine with 5.5 ml. (2 mol.) of distilled water; add the ethanolamine solution dropwise from a burette to 15 ml. (1 mol.) phosphorus oxychloride in a 500 ml. suction flask immersed in a bath of cold water. The reaction proceeds vigorously with the generation of hydrogen chloride. When the reaction is complete, remove the flask from the water-bath and evacuate with a water suction pump for 1 hr. at room temperature to remove as much hydrogen chloride as possible. The esterification at this stage is usually about 80 %. Add 500 ml. of distilled water, stir until the gummy mass is dissolved and add hot saturated baryta solution to pH 10. Centrifuge to remove barium phosphate,

wash the precipitate with water and combine the washing with the first centrifugate. To the combined solution add 2 vol. of ethyl alcohol to precipitate the barium amino-ethyl phosphoric ester, place in refrigerator for 1 hr. and centrifuge; wash the precipitate in the centrifuge pot by shaking with twice its bulk of 95% alcohol and again centrifuge. Shake up the precipitate with ether, filter and dry. Recrystallize the barium salt by dissolving in 200 ml. of water, centrifuge and add 2 vol. of alcohol to the centrifugate, wash and dry the barium salt as before. After three recrystallizations the barium salt should be free of contaminating materials.

Amino-ethyl phosphoric ester may be obtained from the barium salt by adding to a solution of the salt the amount of sulphuric acid required to precipitate the barium as barium sulphate. Remove the barium sulphate by centrifuging. Reduce the volume of the centrifugate and add to it sufficient methyl alcohol to make the solution 80% methyl alcohol. The amino-ethyl phosphoric ester crystallizes out from this solution. Filter and wash with methyl alcohol.

The analysis of the amino-ethyl phosphoric ester is given in the table below:

	P %	N %
Found	22.1	9.87
Calculated	21.98	9.93
M.P. 238°, 244° corrected.		

The melting-point obtained on this purified amino-ethyl phosphoric ester is the highest obtained on any preparation, and as the specimen contained no inorganic phosphorus, it is considered to be the true melting-point of the compound. Preparations containing 0.5% inorganic phosphate have the melting-point lowered to 220°. The melting-point, 238°, is that obtained when the bulb of a 300° thermometer is immersed in sulphuric acid. The stem correction brings the value to 244°.

The solubility of amino-ethyl phosphoric ester in water-ethyl alcohol and water-methyl alcohol has been determined.

Temperature 22°	Solvent	g./100 ml.
	Water	17.0
	10% ethyl alcohol	9.7
	20 "	5.3
	40 "	1.93
	60 "	0.37
	80 "	0.085
	Absolute ethyl alcohol	0.003
	10% methyl alcohol	9.5
	20 "	4.32
	40 "	1.44
	60 "	0.35
	80 "	0.084
	100 "	0.003

The barium salt of amino-ethyl phosphoric ester crystallizes from a water-alcohol solution as beautiful white crystals.

The analysis of the barium salt for phosphorus, nitrogen and barium is given below. The calculated value is corrected for the moisture found.

	P %	N %	Ba %	Moisture
Found	10.20	4.64	45.89	7.8
Calculated	10.26	4.63	45.44	7.8

The solubility of barium amino-ethyl phosphate

Temperature 22°	Solvent	g./100 ml.
	Water	12.5
	10 % ethyl alcohol	4.57
	20 ,,	2.20
	40 ,,	0.54
	60 ,,	0.06
	95 ,,	0.006
	Absolute ethyl alcohol	0.002
	10 % methyl alcohol	4.20
	20 ,,	2.54
	40 ,,	0.81
	60 ,,	0.162
	100 ,,	0.018

The brucine and quinine salts were studied to determine their usefulness in isolating amino-ethyl phosphate from tissues.

The brucine salt does not crystallize from ethyl or methyl alcohol, but crystallizes from acetone, or a mixture of alcohol, water and acetone when the acetone content of the solution is about 80 %, in which case some free amino-ethyl phosphate comes down with the precipitate.

The phosphorus content of the brucine precipitate was found to be 6.35 %, the calculated is 5.02 %. The brucine salt of amino-ethyl phosphoric ester when dissolved in methyl alcohol dissociates into amino-ethyl phosphoric ester and brucine even when kept at 0°, and with excess brucine present. The recovery of the ester from the dissociation of the brucine salt is nearly quantitative.

The quinine salt of amino-ethyl phosphoric ester is more stable than the brucine salt and can be crystallized from ethyl alcohol with the phosphorus content, 6.85 % P.

DISCUSSION

The brucine salts of the barium-soluble esters, precipitated by basic lead acetate, of muscle, pancreas, liver, placenta and embryo have been found to be soluble in hot methyl alcohol and recrystallize from it on cooling, no crystalline precipitate being formed in the hot solution. The brucine salts from the same fraction of the acid soluble phosphoric esters of malignant tumours have been found to differ from those of normal tissues. 60 % of the organic phosphorus of this fraction [Outhouse, 1936] is present as amino-ethyl phosphoric ester whose brucine salt on dissolving in hot methyl alcohol dissociates into amino-ethyl phosphoric ester and brucine. The amino-ethyl phosphoric ester separates out almost quantitatively as a crystalline precipitate. The investigation of the properties of the brucine salt of this ester has shown that the dissociation occurs in either hot or cold methyl alcohol even in the presence of excess brucine.

Since no trace of free amino-ethyl phosphoric ester was found on boiling the methyl alcohol solution of the brucine fractions from the lead-insoluble fraction of the organic phosphates from the normal tissues investigated, it is concluded that amino-ethyl phosphate, if present in normal tissues, occurs in an amount that would correspond to less than 1 mg. per 100 g. wet weight. If the ester is present in such small amounts, it will be necessary to develop a micro-method for its detection. At present such a method is not available. Amino-ethyl phosphoric ester occurs in considerable quantity in malignant tumours. At least 60 % of the lead insoluble fraction shown in Table I is amino-ethyl phosphate. The average amount of this ester found in malignant tumours is 36 mg./100 g. This expressed as phosphorus is 8 mg./100 g. wet weight. None of the ester was found in benign tumours. The phosphorus content of the lead-

insoluble fraction from these tumours was so low that it would be impossible to isolate it, if traces were present.

The results of this investigation suggest that amino-ethyl phosphoric ester is peculiar to malignant tumours. If this is true, a new field is opened for the investigation of tumour metabolism, which may lead to a better understanding of the problem of malignancy.

The data on the solubility of amino-ethyl phosphoric ester and its salts have given added assurance that a rational method has been used in the attempt to detect this ester in the normal tissues investigated.

SUMMARY

1. Amino-ethyl phosphoric ester could not be found in pancreas, liver, placenta and embryo. In malignant tumours the average content of amino-ethyl phosphoric ester was previously found to be 36 mg./100 g.

2. If any of this ester was present in the normal tissues investigated, the amount present was less than 1 mg./100 g.

3. Amino-ethyl phosphoric ester appears to be peculiar to malignant tumours.

4. A satisfactory method of synthesis of this ester has been described.

5. The solubilities of amino-ethyl phosphoric ester and its barium salt are given for water-ethyl alcohol and water-methyl alcohol.

6. The brucine and quinine salts of the ester were prepared and some of their properties described.

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