XXX. AMINO-ETHYL PHOSPHORIC ESTER FROM TUMOURS.

By EDGAR LAURENCE OUTHOUSE.

Department of Medical Research, Banting Institute, University of Toronto.

(*Received November 14th, 1935.*)

THE biochemical importance of phosphoric esters in normal tissues is well recognised, the subject having been discussed fully in the *Annual Reviews of Biochemistry*. The phosphoric esters of malignant tumours have not been as thoroughly investigated and none of them has been positively identified.

The occurrence of an extremely soluble phosphoric ester in the filtrate from the basic lead acetate precipitation of a trichloroacetic acid extract of tumour was reported by Outhouse [1933]. This appears to be similar to the compound obtained by Booth [1935] from kidney, liver and brain. The author [1935] has recently reported the occurrence of this compound in twenty normal tissues as well as in a series of tumours, malignant and non-malignant. In addition, he reported two new phosphoric esters:

(1) a compound whose empirical formula suggests the phosphoric ester of an aminohexahydric alcohol,

(2) a compound which appears to be an amino-ethyl phosphoric ester.

This paper is a report on the isolation, purification and identification of the latter compound.

Isolation and purification.

The material used was bovine malignant tumours, ranging in weight from 500 g. to 10 kg. They were minced and extracted with 3 volumes of ice-cold 4% trichloroacetic acid as soon as possible (generally about 2 hours) after the killing of the animal. The trichloroacetic acid extract was allowed to stand for 1 hour and then filtered. Powdered baryta was added to the filtrate until it was pink to phenolphthalein. The precipitate was centrifuged off and rejected. To the solution 5 g. of mercuric acetate per litre were added. The mercury precipitate was removed by centrifuging and basic lead acetate was added until no further precipitation of organic phosphate occurred.

The lead precipitate was removed by centrifuging, washed with water and decomposed with hydrogen sulphide. After centrifuging, the supernatant liquid was freed from H_2S by aeration, and baryta was added to it until p_H 10 was reached; it was then filtered into 4 volumes of 95% alcohol.

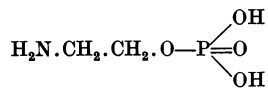
The precipitate was filtered off, washed with alcohol and with ether and dried. It was dissolved in 10 parts of water and twice reprecipitated from 4 volumes of alcohol. The barium salt was converted into the brucine salt and a brucine fractionation was made by crystallising the brucine salts from methyl alcohol to which increasing amounts of acetone were added.

The main fraction (No. 2), which accounts for 60% of the total phosphorus, was boiled in methyl alcohol. Dissociation of the brucine salt occurred and the precipitate obtained from the boiling showed 20·8% phosphorus. It was dissolved in water and reprecipitated from acetone. The phosphorus contents of the

Brucine fractionation.

Fraction	Wt. g.	P %
1	2·55	0·35
2	5·08	4·91
3	3·00	1·19
4	2·85	1·45
5	1·60	1·62
6	1·40	1·21

free compound and of the brucine salt indicated that the organic residue could contain two atoms only of carbon. This, taken in conjunction with the finding of a N : P ratio of 1 : 1 in the free compound, suggested it to be amino-ethyl phosphoric ester:



	% P	
	Free compound	Brucine salt
Calculated for amino-ethyl phosphoric ester	21·98	5·02
Found	20·8	4·91

Identification.

To identify this compound it was considered advisable to prepare amino-ethyl phosphoric ester synthetically and to compare the naturally occurring compound with the synthetic. For further comparison flavianates of the two materials and of their hydrolytic products were prepared.

Preparation of amino-ethyl phosphoric ester. To 9 ml. amino-ethanol were added 3 ml. of orthophosphoric acid. A white crystalline solid (amino-ethanol phosphate) formed, which was ground and dropped slowly into 13 ml. phosphorus oxychloride kept at 0°. After the vigorous reaction had subsided, the flask was removed from the ice-bath and the mixture was refluxed at 100° for 1 hour. The supernatant liquid was poured off and rejected. The gummy mass which remained was dissolved in 400 ml. water and powdered baryta added until the solution was at p_H 10. It was then centrifuged and the supernatant solution poured into 4 volumes of 95 % alcohol. The precipitate, after washing with alcohol and ether and drying, weighed 14·4 g. and contained 7·23 % P. The yield of primary amino-ethyl phosphate was 20 % of the total organic phosphate formed in the reaction. The rest of the organic phosphorus was present as the secondary amino-ethyl phosphate which remained in solution in the 75 % alcohol.

The barium salt was dissolved in water and the barium was precipitated with sulphuric acid. The volume was reduced and the solution was poured into 4 volumes of acetone. The amino-ethyl phosphate crystallised from the acetone solution.

Analyses.

	C %	H %	N %	P %	
Phosphoric ester from tumour	17·83	5·9	9·65	21·0	$n_D^{22°} = 1{\cdot}497 \pm 0{\cdot}001$
Amino-ethyl phosphate (synthetic)	17·81	5·84	9·46	20·9	$n_D^{22°} = 1{\cdot}497 \pm 0{\cdot}001$
Calculated for $C_2H_8O_4NP$	17·02	5·68	9·93	21·98	

An attempt to compare the melting-point of the phosphoric ester from tumour with that of the synthetic amino-ethyl phosphate showed that both compounds decomposed above 230° with no distinct melting-point.

Preparation of flavianates of the phosphoric ester. The natural and synthetic compounds react similarly with flavianic acid. They form salts which crystallise readily from aqueous butyl alcohol when an excess of flavianic acid is present. The salts are readily dissociated into flavianic acid and the phosphoric ester when dissolved in a hot mixture of 1 part methyl alcohol and 3 parts acetone. From such a mixture the free amino-ethyl phosphate crystallises almost quantitatively.

100 mg. of amino-ethyl phosphate were dissolved in 5 ml. of water; to this were added 400 mg. of flavianic acid (2:4-dinitronaphthol-7-sulphonic acid). 10 ml. of 95% ethyl alcohol and 100 ml. of *n*-butyl alcohol were added to the solution, which was then filtered. The filtrate was evaporated on a boiling water-bath until crystals began to form and was then cooled in a refrigerator. The yield of the flavianate of amino-ethyl phosphate was 90% of the calculated. After filtration the crystals were washed with butyl alcohol and dried in a vacuum desiccator over sulphuric acid.

To the flavianate, dissolved in 2 ml. of water, were added 25 ml. methyl alcohol and 75 ml. of acetone. The mixture was placed on a water-bath and boiled for a few minutes. The flavianate dissociated and the crystals of amino-ethyl phosphate which formed were filtered off, washed with acetone and dried. The yield of free amino-ethyl phosphate from the flavianate was nearly theoretical. The crystals contained about 20% P. These crystals were re-converted to the flavianate as before, using 300 mg. of flavianic acid, and the flavianate, after crystallisation from butyl alcohol, was washed and dried.

The flavianates were analysed for nitrogen (Pregl's micro-Dumas method), phosphorus (method of King [1932]) and for flavianic acid (method of Langley and Albrecht [1935]).

Analysis of the flavianates.

	N %	P %	Flavianic acid
Phosphoric ester from tumour	9·22	6·78	69·6
Amino-ethyl phosphate (synthetic)	9·26	6·81	70·0
Calculated for the flavianate of amino-ethyl phosphate	9·23	6·81	69·1

Flavianate of phosphoric ester from tumour M.P. 223°.
Flavianate of amino-ethyl phosphate (synthetic) M.P. 225°.

Preparation of amino-ethanol flavianate. Flavianates were prepared from the bases produced by hydrolysis of the phosphoric ester from tumour and from the synthetic amino-ethyl phosphate. The natural and synthetic compounds were hydrolysed with a very active phosphatase preparation [Armstrong, 1935].

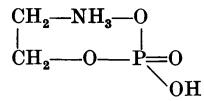
Dissolve 100 mg. of amino-ethyl phosphate in 20 ml. of water, add 0·8 ml. 0·1 *M* $MgCl_2$ and 10 mg. of the phosphatase preparation. Shake thoroughly and keep at 37·5°. Add slowly, with shaking, 5 ml. of a solution containing 1 part of 0·5 *N* $BaCl_2$ to 2 parts of 0·5 *N* $Ba(OH)_2$. An instantaneous hydrolysis takes place and barium phosphate is precipitated. If the hydrolysis is not complete the addition of a few mg. of additional phosphatase may be advisable or the solution may be kept for an hour or two at 37·5°. Centrifuge to remove barium phosphate, and to the centrifugate add *N* H_2SO_4 until the barium is quantitatively precipitated. Remove the barium sulphate by centrifuging. To the solution add 400 mg. of flavianic acid and 1 volume of 95% ethyl alcohol. Reduce the volume to 20 ml., add 20 ml. of ethyl alcohol and 100 ml. of butyl alcohol. Place on a boiling water-bath and reduce the volume to about 100 ml., cool and filter. Then reduce the volume until crystals begin to form, cool in refrigerator, filter and wash the crystals with butyl alcohol and dry in a desiccator. Recrystallise by dissolving the amino-ethanol flavianate in 95% alcohol, filter, add butyl alcohol, reduce the volume and crystallise in the refrigerator. The yield is usually about 80% of theoretical.

	N%	M.P. (°)
Amino-ethanol flavianate (from tumour phosphate)	11·21	198
,, (from synthetic amino-ethyl phosphate)	11·18	198
,, (from pure amino-ethanol)	11·18	198 (212)
Calculated for amino-ethanol flavianate	11·19	

When amino-ethanol flavianate was prepared from amino-ethanol, two types of crystals were obtained, one melting at 212°, the other at 198°. Usually either one type or the other was formed, but in one experiment both were formed. The crystals with melting-point 212° correspond with those described by Langley and Albrecht [1935]. The form melting at 198° was less frequently encountered when using pure amino-ethanol than when the flavianates were prepared from the products of hydrolysis. It was found that very small amounts of contaminating material cause a marked depression of the melting-point, *e.g.* an amino-ethanol flavianate preparation containing 2% of the flavianate of amino-ethyl phosphate melted at 192°. The purified amino-ethanol flavianate from the hydrolysis of amino-ethyl phosphate contained 0·4% of the flavianate of amino-ethyl phosphate (calculated from the phosphorus content) and it is conceivable that this, or some other persistent impurity, is responsible for the melting-point of 198°. The influence of the phosphatase preparation and of various solvents on the crystal form and melting-point of amino-ethanol flavianate is being studied. The fact that the crystals melting at 198° have a different refractive index from those melting at 212° suggests that two distinct crystal forms are involved and that the 198° value is not a depressed melting-point due to impurities.

Discussion.

Probably the open chain formula given on p. 198 does not give the most accurate picture of the compound under discussion. There is evidence that an inner salt of the following nature:



is the form in which the compound exists in solutions at p_H values between 5 and 9.

This formulation is supported by the observations that: (*a*) the barium salt does not form, or at least cannot be precipitated from alcohol, unless the solution has a p_H of at least 10; (*b*) the brucine salt contains only one formula weight of brucine; (*c*) the flavianate of amino-ethyl phosphate cannot be formed from one equivalent of flavianic acid unless a p_H of about 3 is produced by the addition of some other acid (*e.g.* acetic) to free the amino-group from its inner salt linkage with phosphoric acid; a sufficient excess of flavianic acid will bring about the same result; (*d*) the brucine and flavianic acid derivatives dissociate completely on boiling in non-aqueous solvents.

Now that amino-ethyl phosphate has been identified as one of the phosphoric esters occurring in all malignant tissues studied to date, the question arises, "Does it occur in normal tissues?" At the present time the author is engaged in working up corresponding fractions from normal tissues in an attempt to isolate amino-ethyl phosphate from pancreas, liver and other organs.

SUMMARY.

One of the phosphoric esters from tumour has been shown to be identical with synthetic amino-ethyl phosphate.

The author wishes to thank Dr E. J. King, at whose suggestion the investigation of the phosphoric esters in malignant tumours was undertaken, for his encouragement and advice, and Mr C. C. Lucas for valuable criticism during the latter part of the work. The writer is also indebted to Sir F. G. Banting for his continued interest in the problem.

REFERENCES.

Armstrong (1935). *Biochem. J.* **29**, 2020.
Booth (1935). *Biochem. J.* **29**, 2071.
King (1932). *Biochem. J.* **26**, 292.
Langley and Albrecht (1935). *J. Biol. Chem.* **108**, 729.
Outhouse (1933). *Trans. Roy. Soc. Can.* Sec. V, 123.
—— (1935). *Trans. Roy. Soc. Can.* Sec. V, 77.