XXXII. THE PREPARATION AND USE OF THE BONE PHOSPHATASE.

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The phosphatase obtained from the bones of growing animals has proved very useful in the study of the chemical composition of phosphoric esters of biochemical interest. By its aid the phosphoric acid groups may be quantitatively removed under conditions of p_H and temperature which do not involve the rupture of other linkages in the molecule such as would occur during acid hydrolysis.

For such purposes, as well as for the study of the mode of action of the enzyme, it is desirable to have stable preparations of the phosphatase, of high activity and as free as possible from blood pigments and such other impurities as would be difficult to remove from the hydrolysis products. The following is a brief account of experiments carried out with this object in view, and of the methods at present employed.

Extraction of phosphatase.

The bones of young growing rabbits are removed immediately after death, freed from muscle and connective tissue, and split longitudinally. The marrow is removed and the bones with their epiphyses are cut into smaller pieces and placed in a flask with five times their weight of water, to which a few drops of chloroform have been added. The closed flask is kept at room temperature for 7–10 days, shaken each day, and fresh chloroform added if necessary. The extract is then filtered through coarse filter paper. A second extract, much less active, may be obtained by soaking the bones in a fresh quantity of water. The progress of the extraction at 0°, room temperature, and 38° is shown in Table I. *A* represents the amount of hydrolysis reckoned as mg. P effected in 1 cc. 0·2 *M* sodium glycerophosphate by 1 cc. of the filtered extract in 1 hour at 38° at p_H 8·6. *A*/*W* is the above value divided by the weight in mg. of solid matter in 1 cc. of the extract.

Table I.

	Temperature of extraction					
	0°		Room temperature		38°	
Duration of extraction	*A*	*A*/*W*	*A*	*A*/*W*	*A*	*A*/*W*
18 hours	0·16	0·033	0·35	0·075	0·43	0·074
7 days	0·37	0·056	0·57	0·073	0·34	0·044
9 „	0·45	0·060	0·61	0·077	—	—
14 „	0·41	0·051	0·62	0·063	—	—
Second extraction: 5 days	0·14	0·086	0·17	0·076	0·03	0·012

The results show that, while extraction proceeds most rapidly at 38°, some inactivation of enzyme occurs. Other comparative tests have shown that the final yield of enzyme is not greatly increased by preliminary maceration of the bones or by a series of short successive extractions. More highly active extracts are, however, obtained if the rabbits are fed for a month before death on a rickets-producing diet (McCollum's 3143) (see Table III).

Evaporation of the filtered extracts in evacuated desiccators over sulphuric acid yields 5–8 mg. of dry solid per cc., no loss of activity occurring during this process. After 7 months' storage in a dry atmosphere such residues were found to retain 60 % of the original activity of the extract.

Purification.

A very useful degree of purification may be obtained by precipitating the filtered extract with a mixture of alcohol and ether (200 cc. alcohol and 300 cc. ether for each 100 cc. extract). After shaking for a few minutes a flocculent precipitate forms, and is filtered through a Büchner funnel, washed with absolute alcohol and dried in an evacuated desiccator. If the operation is conducted expeditiously, an almost colourless powder is obtained, weighing 3–4 mg. per cc. extract. This preparation possesses the full original activity, which it retains for many weeks when stored in a desiccator. It disperses fairly well in water, and its freedom from alcohol-soluble matter renders it a suitable form of the bone phosphatase for general use.

A further degree of purification can be effected, at the expense of some loss of total activity, by extracting the above preparation with 50 % alcohol. The greater part of the enzyme passes into solution, and may be reprecipitated by a further addition of alcohol and ether. The product is now freely soluble in water and shows a greatly increased activity in relation to its weight (A/W).

Other methods of purification.

Removal of a protein by precipitation at its isoelectric point. On bringing the p_H of the aqueous extract to 5·8 a protein is precipitated, while most of the enzyme remains in solution. The latter should be brought back to the neutral point as quickly as possible after filtration. This method of purification cannot, however, be recommended owing to the risk of inactivation of the enzyme, should the necessary hydrogen ion concentration be momentarily exceeded.

Dialysis and ultrafiltration. Dialysis experiments were carried out with the object of removing the traces of inorganic phosphate which are always present in the aqueous extract, and also to investigate the possible presence of a co-enzyme. It was found that a collodion membrane, immersed for 24 hours in 95 % alcohol, was impermeable to the enzyme, which could in this way be separated from phosphates and other salts. The method of ultrafiltration in a Bechhold filter through discs of collodion of similar porosity

proved a more satisfactory procedure, the filtrate being entirely inactive and containing the whole of the phosphate. The residue on the filter may be taken up in water and precipitated with alcohol and ether as described above. The complete freedom from inorganic phosphate, while usually of no great importance, was of value in certain experiments in which it was desired to eliminate the inhibitory effect of phosphate on the activity of the enzyme.

No evidence of the presence of a co-enzyme was obtained by dialysis or ultrafiltration. A measured volume of the aqueous extract was filtered through a collodion membrane in a Bechhold filter, the residue was washed with an equal volume of water and finally dispersed in the same volume, so that 1 cc. of the residue and 2 cc. of the dialysate corresponded with 1 cc. of the original extract (Table II).

Table II.

	Inorganic P (mg. in 1 cc.)	*Activity*
1 cc. residue	0·0	0·107
2 cc. dialysate	0·052	0·0
1 cc. residue + 2 cc. dialysate	0·054	0·090
1 cc. residue + 10 cc. dialysate	0·253	0·077

The activity of the residue is actually diminished by the addition of dialysate, this being due to the inhibitory effect of the inorganic phosphate thereby introduced. If a co-enzyme exists it must therefore have been still present in excess in the washed residue.

Specific adsorption. Attempts have been made to purify the enzyme by specific adsorption, using kaolin, calcium phosphate and aluminium hydroxide (type B) prepared by the method of Willstätter and Kraut [1923]. Up to the present the results have been unsatisfactory. Where adsorption has appeared to take place, as judged from the loss of activity of the solution, attempts to recover the enzyme by elution have proved unsuccessful.

Table III.

Description of preparation	*A*	*A/W*
1. 20 % aqueous extract of bones of young normal rabbits	0·61	0·077
2. „ „ „ rachitic rabbits	1·15	0·120
3. Alcohol-ether precipitate of (2)	—	0·180
4. (3), extracted with 50 % alcohol, reprecipitated with alcohol-ether	—	0·570
5. Residue from ultra-filtration of aqueous extract taken up in water and precipitated with alcohol-ether	—	0·240

Table III gives the values A and A/W for various enzyme extracts and preparations purified by the above methods.

It is interesting to calculate from these values of A the amount of inorganic phosphate which could be produced in 24 hours by the phosphatase in 1 g. of bone, assuming that this phosphate is immediately precipitated and the rate of hydrolysis consequently maintained. These amounts in terms of calcium phosphate $Ca_3(PO_4)_2$, are, at p_H 8·6, 0·37 g. and 0·66 g. per g. of normal and rachitic bone respectively, while at the p_H of the blood the values

would be about 0·08 g. and 0·15 g. These figures have, of course, only limited significance, since the conditions of the test (degree of extraction of enzyme, concentration of phosphoric ester, etc.) are not those which obtain in living cartilage, but they show by their magnitude that the activity of this enzyme is quite adequate for the rôle which we have ascribed to it in the process of ossification.

Use of the enzyme as a biochemical reagent.

The following methods have been found satisfactory for the removal of phosphoric acid groups from such esters as can be hydrolysed by this enzyme.

(*a*) 1 g. of the barium salt of the phosphoric ester is placed in a small flask with 15 cc. water and 50–100 mg. of the enzyme preparation. The flask is kept at 40°; the p_H is adjusted to 8·6–8·8 and maintained approximately within that range by the addition at frequent intervals of a cold saturated solution of baryta from a burette. The use of a p_H higher than 9·0 invites the risk of rapid inactivation of the enzyme. Measurements of p_H are made on minute amounts (0·01 cc.) by means of a capillator.

The baryta required gives a rough indication of the amount of hydrolysis, but usually falls short of the equivalent of the phosphate set free. This is probably due to the precipitation of the phosphate as a mixture of $Ba_3(PO_4)_2$ and $BaHPO_4$. By this precipitation the inhibitory effect on the enzyme of an increasing concentration of inorganic phosphate [Martland and Robison, 1927] is avoided, but the rate of hydrolysis nevertheless falls, and from other experiments it is evident that the enzyme is partially adsorbed on to the precipitated phosphate. For this reason the addition of a further quantity of the enzyme preparation may be necessary in order to complete the reaction in reasonable time (4–8 hours).

Should the barium salt of the ester be sparingly soluble in water, the method is still applicable, but the flask must be frequently shaken to keep the solution saturated. When the hydrolysis approaches 100 % the enzyme, together with any barium salt left in solution, is precipitated by the addition of alcohol, and the filtered solution evaporated to dryness in an evacuated desiccator. The residue may be purified by extraction with water, evaporation of the filtered solution and re-extraction with alcohol. The product should now contain not more than a few mg. of material derived from the enzyme, this being without appreciable optical rotation or reducing power, as shown by control experiments omitting the substrate.

Should the dephosphated product be insoluble in alcohol, a different method must be employed to separate it from the enzyme material. After completion of the hydrolysis the protein may be coagulated by boiling, and the aqueous filtrate evaporated or poured into alcohol according to the properties of the dissolved substance.

(*b*) The chief disadvantage of method (*a*) lies in the alkaline reaction, which is undesirable when dealing with reducing sugars. It has been found

possible to carry out the hydrolysis at a p_H as low as 7·0 although, owing to the greatly reduced activity of the enzyme, several days may be required for the completion of the reaction. The soluble sodium salts are used and, as the liberated phosphate is not precipitated, no further addition of alkali is necessary. The isolation of the product is carried out in the same manner as described under (*a*), although the precipitation of sodium phosphate by alcohol is not so complete as that of the barium salt.

Method (*a*) as applied to esters of non-reducing sugars or sugar derivatives has given excellent results. Thus α- and β-methyl-γ-fructosides have been obtained from the corresponding derivatives of hexosediphosphoric acid [Morgan and Robison, 1928], pure crystalline trehalose has been isolated from trehalosemonophosphoric ester, which is a product of the fermentation of hexoses by dried yeast [Robison and Morgan, 1928], and gluconic acid has been obtained from the oxidation product of hexosemonophosphoric acid [Robison and King, 1929]. In all these cases a very good yield of the hydrolysis product was obtained without difficulty.

When applied to the reducing hexosephosphoric esters, both methods gave results less easy of interpretation, some of which are discussed below.

Hydrolysis of hexosediphosphoric ester.

The specific rotations of a number of the sugar products obtained by the action of the bone phosphatase on the barium and sodium salts of hexosediphosphoric acid are set forth in Table IV. The amount of ester used for each experiment was equivalent to 50–100 mg. P, the degree of hydrolysis varying from 63 to 95 %. The rotations are calculated on the amounts of sugar in the syrups, dried over sulphuric acid, as determined by the Hagedorn-Jensen method.

Table IV.

Salt used	p_H	Duration of hydrolysis	$[\alpha]_{5461}$ of sugar product
Ba	8·8	4 hours	−82°
Ba	8·8	6 ,,	−60°
Na	8·8	24 ,,	−48°
Na	8·8	24 ,,	−63°
Na	7·7	5 days	−64°
Na	7·4	4 ,,	−64°
Na	7·0	5 ,,	−76°

Hexosediphosphoric acid is believed to be a derivative of γ-fructose, which on removal of the phosphoric acid groups should change into the ordinary $\alpha\beta$-fructose. Young [1909] found, however, that the solution obtained by acid hydrolysis was less laevorotatory than a solution of pure fructose of the same reducing power. The product obtained by the action of the bone phosphatase has, likewise, a laevorotation much lower than that of fructose ($[\alpha]_{5461} - 111°$), and the values obtained vary within rather wide limits. For the products of hydrolysis at p_H 8·6 this could be explained by assuming that the Lobry de Bruyn transformation had taken place during

the course of the reaction. It is surprising, however, that the product obtained by hydrolysis at p_H 7·0 should also have a similar low specific rotation. The possibility arises that the synthesis and hydrolysis of phosphoric esters, which must be considered as occurring simultaneously during the whole course of the experiment (even though the final equilibrium represents almost complete hydrolysis) are causatively involved in the intramolecular transformation of the hexoses. Neuberg and Leibowitz [1928] have found that the partial hydrolysis of fructose diphosphate by various phosphatases may give rise to either of the two hexosemonophosphates, which are probably derivatives of glucose and fructose respectively. The theoretical suggestion put forward by Robinson [1927], namely, that the hydrolysis of a phosphoric ester might be accompanied by a Walden inversion, is of interest in this connection, although the transformation of fructose to glucose cannot be simply explained in this way. It is hoped that more evidence will be obtained on this problem from experiments now in progress.

SUMMARY.

Methods for the extraction and purification of the bone phosphatase and its use as a biochemical reagent are described.

The application of this method to the hydrolysis of hexosediphosphoric acid and the properties of the hexose so obtained are discussed.

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