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The Phosphotransferase Activity of Phosphatases

3. COMPARISON OF ENZYMIC CATALYSIS BY ACID PHOSPHATASE WITH NON-ENZYMIC CATALYSIS AT ACID pH VALUES

BY R. K. MORTON*
Department of Biochemistry, University of Cambridge

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In a preceding paper (Morton, 1958*a*) it was shown that highly purified alkaline phosphomonoesterases from cow's milk and from calf intestinal mucosa have associated 'phosphotransferase' (Dixon, 1949; Dixon & Webb, 1953) activity. The kinetics of the phosphorylation of glucose, fructose, glyceraldehyde, dihydroxyacetone and glycerol were investigated with sensitive enzymic methods developed for estimation of the phosphorylated products (Morton, 1958*b*).

This paper describes similar studies with a partially purified acid phosphatase from prostate gland, the phosphotransferase activity of which was first observed by Appleyard (1948). During this investigation it was found that glucose phosphates were formed by phosphate transfer from phosphocreatine in a non-enzymic reaction occurring at the same pH values as used for the studies with the acid phosphatase. A comparison was made of the enzymic and non-enzymic reactions (Morton, 1952*a*).

This work has been briefly reported elsewhere (Morton, 1952*b*, 1953).

MATERIALS AND METHODS

Except for the acid-phosphatase preparation, the materials and methods have been described in a preceding paper (Morton, 1958*a*).

Acid phosphatase. A human prostate gland was finely minced and then ground to a smooth paste in a mortar with fine acid-washed sand and 0·15M-NaCl, pH 7·0 (at 22°). The paste was diluted with 5 vol. of 0·15M-NaCl and the suspension stirred gently for 30 min. and then centrifuged at 2000 *g* for 30 min. The supernatant was then re-centrifuged at 14 000 *g* for 30 min. The clear supernatant was fractionated by addition of $(NH_4)_2SO_4$, the pH being maintained at about 6·8 at 20°. The precipitate obtained between 55 and 65% saturation with $(NH_4)_2SO_4$ was dissolved in water and dialysed in seamless cellophane tubing against water for 24 hr. at 0–4°. A precipitate was removed by centrifuging at 14 000 *g* for 30 min. in a cold-room at 2°, and the clear supernatant was stored at 0°.

RESULTS

Reactions catalysed by acid phosphatase

Phosphorylation of glucose. The reaction mixtures contained acid phosphatase (except in the control tube), 8 mM-phosphocreatine, 0·1M-sodium acetate buffer, pH 5·5, and increasing concentrations of glucose up to 4M. All components except the phosphocreatine were added to the tubes, the volumes were adjusted to 0·4 ml. and the tubes brought to 38°. The reaction was commenced by addition of phosphocreatine, and stopped after 5 min. at 38° by addition of trichloroacetic acid as already described (Morton, 1958*b*). The analyses were carried out as described before (Morton, 1958*a*, *b*), the glucose phosphate being estimated enzymically. Fig. 1 shows that the rate of synthesis of glucose phosphate (curve *C*) increased continuously up to 3·5M-concentration of glucose. The rate of hydrolysis, as indicated by the liberation of inorganic phosphate (curve *B*), declined continuously with increasing concentrations of glucose, whereas the rate of utilization of phosphocreatine (due to both the hydrolysis and transfer reactions) remained fairly constant (curve *A*). Curve *D* shows that the percentage transfer (see Morton, 1958*a*) increased to about 13% with 4M-glucose.

* Present address: Department of Agricultural Chemistry, The Waite Institute, University of Adelaide, South Australia.

Table 1. *Phosphorylation of glycerol and propane-1:2-diol catalysed by acid phosphatase*

The reaction conditions for phosphorylation of propane-1:2-diol were similar to those for Fig. 1, except that the polyol was substituted for glucose. For the experiment with glycerol, 0·02 M-phenyl phosphate replaced the phosphocreatine.

Acceptor	Concn. of acceptor (M)	Product/tube (μmoles) Creatine or phenol	Inorganic phosphate	Polyol* phosphate	Phosphate transfer (%)
1:2-Propanediol	0	1·25	1·27	—	—
	3	1·47	0·68	0·79	54
	5	0·55	0·25	0·30	55
Glycerol	0	3·75	3·75	—	—
	3	5·91	2·40	3·51	60

* Difference between creatine or phenol, and inorganic phosphate.

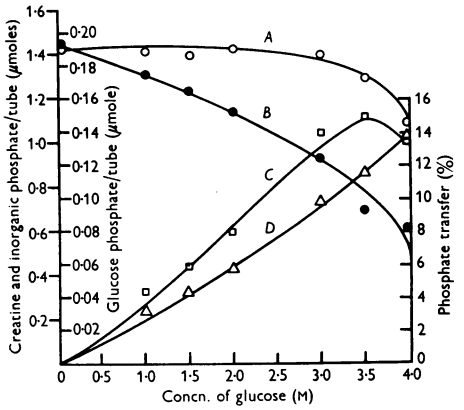

Fig. 1. Phosphorylation of glucose catalysed by acid phosphatase. The reaction mixture (0·5 ml.) contained 0·1 M-sodium acetate buffer, pH 5·5; 8 mM-phosphocreatine; glucose; 0·05 ml. of acid phosphatase. The reaction was commenced by addition of phosphocreatine and was carried out for 5 min. at 38°. *A*, Creatine; *B*, inorganic phosphate; *C*, glucose phosphate determined enzymically; *D*, percentage transfer.

Table 2. *Phosphorylation of glyceraldehyde and of dihydroxyacetone catalysed by acid phosphatase*

Reaction conditions were similar to those for Fig. 1 except that the trioses replaced glucose.

Concn. of acceptor (M) DL-Glyceraldehyde	Dihydroxyacetone	Product/tube (μmole) Inorganic phosphate	Triose phosphate
0	0	1·81	—
1	—	1·72	0·26
—	1	1·70	0·31
—	2	1·63	0·59

With phenyl phosphate (0·01 M) or β-glycerophosphate (0·01 M) as donor and with 4 M-glucose at pH 5·5 as above, the percentage transfer was similar (approx. 14 %).

Phosphorylation of glycerol and propane-1:2-diol. Table 1 shows that both polyols are phosphorylated.

Phosphorylation of glyceraldehyde and dihydroxyacetone. Both these trioses are phosphorylated. Table 2 shows the influence of the concentration of the trioses on the phosphotransferase reaction.

Phosphorylation of nucleosides. The reaction mixture (0·2 ml.) contained 0·05 M-*p*-nitrophenyl phosphate, 0·1 M-sodium acetate buffer, pH 5·2 at 20°, 0·1 M-cytidine and phosphatase. About 4 μmoles of inorganic phosphate were liberated during the reaction (30 min. at 20°). Paper chromatograms were prepared and examined as previously described (Morton, 1958*a*). Papers run in the propan-1-ol–aq. NH_3 soln. solvent of Hanes & Isherwood (1949) showed two new but rather faint spots in the products formed with acid phosphatase. One of these ran in an identical position to authentic cytidine 5′-phosphate. The other spot was probably cytidine 3′-phosphate or cytidine 2′-phosphate, or a mixture of both.

Non-enzymic phosphate transfer

Phosphorylation of glucose. During the investigation of the acceptor specificity of alkaline phosphatase, a mixture containing sucrose (2 M) and phosphocreatine (13 mM) was hydrolysed with 0·1 N-HCl at 60° for 5 min. Determination of glucose phosphate enzymically showed large amounts to be present both in the 'control' and the 'test' (which had been incubated with alkaline phosphatase; see Morton, 1958*a*). The formation of glucose phosphate during acid hydrolysis of phosphocreatine was therefore investigated.

The reaction mixture contained glucose, sodium acetate buffer and phosphocreatine. The reaction was commenced by addition of phosphocreatine and was carried out at pH 4·2 for 10 min. at 38°. Glucose phosphate was estimated enzymically and by difference between the creatine and inorganic phosphate liberated.

Table 3. *Non-enzymic synthesis of glucose phosphates*

In Expt. *A*, the reaction mixture (0·5 ml.) contained 0·2 M-sodium acetate buffer, pH 5·8, 0·5% ammonium molybdate, 26 mM-phosphocreatine and glucose. The reaction was commenced by addition of phosphocreatine and was continued for 5 min. at 38°.

In Expt. *B*, the reaction mixture (0·5 ml.) contained 26 mM-phosphocreatine and M-glucose in 0·1 N-HCl. The reaction was carried out for 7 min. at 100°.

Glucose phosphate was estimated enzymically.

Experiment	Concn. of glucose (M)	Products/tube (μmoles) Creatine	Inorganic phosphate	Glucose phosphate	Phosphate transfer (%)
A	0	2·00	1·96	—	—
	2	1·91	1·65	0·08	4
	4	1·93	1·47	0·14	7
B	1	13·2	11·9	0·59	4

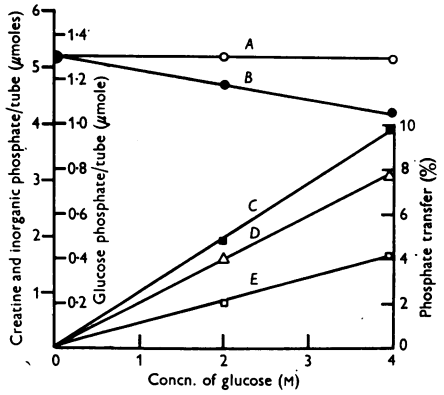

Fig. 2. Non-enzymic synthesis of glucose phosphate. The reaction mixture (0·5 ml.) contained 0·1 M-sodium acetate buffer, pH 4·2; 16 mM-phosphocreatine; glucose. The reaction was commenced by addition of phosphocreatine, and was carried out for 10 min. at 38°. *A*, Creatine; *B*, inorganic phosphate; *C*, glucose phosphate as estimated from the difference between creatine and inorganic phosphate; *D*, percentage transfer; *E*, glucose phosphate, estimated enzymically.

Table 4. *Non-enzymic phosphorylation of dihydroxyacetone*

The reaction mixture (0·5 ml.) contained 0·2 M-sodium acetate buffer, pH 4·2, 26 mM-phosphocreatine and dihydroxyacetone. The reaction was commenced by the addition of phosphocreatine and was continued for 5 min. at 38°.

Concn. of dihydroxyacetone (M)	Product/tube (μmoles) Inorganic phosphate	Triose phosphate
0	5·02	—
1	4·29	0·48
2	3·47	0·89

Fig. 2 shows that the rate of formation of both total glucose phosphate (curve *C*) and of enzymically estimated glucose phosphate (curve *E*) increases linearly with glucose concentration. The rate of hydrolysis (as indicated by curve *B*) declines linearly with increasing concentration of glucose, so that the rate of creatine liberation (curve *A*) remains constant. The percentage transfer based on enzymically estimated glucose phosphate (curve *D*) increases continuously up to the maximum concentration of glucose.

Table 3 shows that essentially similar results were obtained at pH 5·8 with 0·5% ammonium molybdate in the reaction mixture. This was added as it was thought that it catalysed the hydrolysis of phosphocreatine. The compound, however, is not essential for the reaction. Table 3 also shows that glucose phosphates are formed from phosphocreatine and glucose at 100° in 0·1 N-HCl.

Phosphorylation of dihydroxyacetone. Table 4 shows that the rate of synthesis of dihydroxyacetone phosphate (as estimated enzymically) increases with the acceptor concentration.

DISCUSSION

Appleyard (1948) used a crude extract of prostate gland with phenolphthalein phosphate as donor and a number of aliphatic alcohols and glycols as acceptors. The discrepancy between the phenolphthalein and the inorganic phosphate found in the reaction products was attributed to the activity of a 'transphosphorylase'. However, comparison of the kinetic behaviour of the acid phosphatase with glucose, trioses and polyols as acceptors with that of the purified alkaline phosphatases (Morton, 1958*a*) with similar acceptors indicates that the transferase activity is a property of the phosphatase itself. In crude and highly purified preparations of prostate-gland phosphatase the ratio of transferase to hydrolase activity is the same, and the heat-denaturation constants for the two activities are similar (London & Hudson, 1955). Axelrod (1948*a*) established that inorganic phosphate is not an intermediate in the phosphate transfer catalysed by acid phosphatases.

Like the alkaline phosphatases (Morton, 1955*a*), prostate-gland acid phosphatase has a wide substrate specificity (Delory, Wiberg & Hetherington, 1955) and hydrolyses compounds having an N–P linkage, such as phosphocreatine (Morton, 1952*a*, 1955*a*; Møller, 1955), as well as typical orthophosphomonoesters. The rate of hydrolysis of phosphocreatine by this enzyme is markedly dependent on the substrate concentration. Relative initial rates of hydrolysis were 100 and 20 μg. of P liberated/min./0·1 ml. of enzyme at 5 and 50 mM-phosphocreatine respectively. Hence the choice of the relatively low (8 mM) substrate concentration in the transferase studies (Fig. 1).

Although the kinetic results presented here are limited as compared with those obtained with alkaline phosphatases (Morton, 1958*a*), certain similarities in the transferase activities of the acid and alkaline phosphatases are apparent. With three donors (phenyl phosphate, glycerophosphate and phosphocreatine), the transferase activity was parallel with hydrolytic activity, and the percentage transfer with 4M-glucose was essentially the same. Thus the efficiency of phosphorylation is independent of the standard free energy of hydrolysis of the donor (Morton, 1953). With two concentrations (8 and 50 mM) of phosphocreatine, the same percentage transfer to 2M-glucose was obtained, although very much less glucose phosphate was formed at the higher substrate concentration, owing to inhibition of the enzyme (see above). Tsuboi & Hudson (1953) found that the acid phosphatase of haemolysed human red cells catalysed transfers from phenyl phosphate to glycerol and methanol, and that the ratio of phosphate transferred to phosphate hydrolysed was independent of the concentration of phenyl phosphate. Axelrod (1948*b*) reported a similar finding with citrus-fruit acid phosphatase.

As with intestinal alkaline phosphatase (Morton, 1958*a*), so with prostate acid phosphatase, the relative distribution of the transferred phosphate groups among the water and acceptor molecules varies with the concentration of acceptor (Fig. 1 and Tables 1 and 2). However, the rate of glucose phosphate synthesis increases almost linearly with increasing concentration of glucose up to between 3·5 and 4M, whereas with alkaline phosphatase a well-defined maximum appears at between 1·5 and 2·0M-glucose (cf. this paper, Fig. 1, and Morton, 1958*a*, fig. 3). The percentage transfer also increases up to the maximum concentration of glucose (Fig. 1).

With the trioses (Table 2) and polyols (Table 1) as phosphate acceptors, the rate of utilization of the donor is higher as compared with the rate in the absence of acceptor, as also observed with acid phosphatases from human red cells (Tsuboi & Hudson, 1953) and from citrus fruits (Axelrod, 1948*b*).

Acceptor specificity of phosphatases and nature of synthesized products

The studies reported here extend and supplement those of Appleyard (1948). The two investigations show that the prostate-gland acid phosphatase has a wide acceptor specificity. The percentage transfer to these different acceptors varies according to the nature of the acceptor, as was shown for citrus-fruit acid phosphatases by Axelrod (1948*b*) and for two alkaline phosphatases by Morton (1958*a*).

As with alkaline phosphatase (Morton, 1958*a*), so with the prostate enzyme, the newly formed ester probably accumulates because of the relatively low rate of hydrolysis of the newly formed ester as compared with that of the donor compound. The optimum pH of hydrolysis of esters by prostate acid phosphatase varies with the nature of the ester (Delory *et al.* 1955; Lundquist, 1947). Conditions chosen for optimum rate of utilization of the donor will generally be grossly suboptimum for hydrolysis of the newly formed ester. With the usual reaction conditions, at equilibrium there would be negligible amounts of donor compound or of newly formed ester.

With intestinal alkaline phosphatase (Morton, 1958*a*) the enzymically estimated glucose phosphates accounted for between 80 and 100% of the phosphate which was apparently transferred to glucose, and DL-α-glycerophosphate for most of the phosphate which was apparently transferred to glycerol. With prostate-gland acid phosphatase, however, in different experiments the enzymically estimated glucose phosphate was between 30 and 65% of the total phosphate apparently transferred to glucose, and, in one experiment, DL-α-glycerophosphate was about 60% of the phosphate apparently transferred to glycerol. Brawerman & Chargaff (1954*a*) found that about equal amounts of 5′- and 3′-cytidylic acid, and less of the 2′-cytidylic acid, were formed with cytidine. As previously pointed out (Morton, 1955*b*), these results indicate that prostate acid phosphatase is less specific in the position of phosphorylation of sugars and polyols than intestinal alkaline phosphatase.

Non-enzymic phosphorylation at acid pH *values*

The finding of a non-enzymic phosphate transfer from phosphocreatine to glucose (Morton, 1952*a*, *b*) was entirely unexpected. The reaction proceeds simultaneously with hydrolysis of this acid-labile compound, and occurs under similar conditions to those used with prostate acid phosphatase, namely at pH 5·8 and 38° (Table 3). Here the catalysis can be attributed only to H^+ or OH^- ions, and no

surfaces are involved. Hence the linear relationships for the rate of synthesis of glucose phosphate (Fig. 2, curve *B*) and rate of hydrolysis (curve *C*) with increase of glucose concentration is to be expected. The results indicate a direct competition of glucose molecules with water molecules, since the rate of liberation of creatine (curve *D*) remains quite constant at the different glucose concentrations.

Table 3 indicates a possible source of error in the estimation of phosphocreatine as 'acid-labile phosphate'. If the reaction mixture also contains glucose (or other suitable hydroxy compounds) under the usual conditions for estimation of 'acid-labile phosphate' (digestion for 7 min. at 100° in 0·1 N-HCl), some of the phosphate will be transferred from phosphocreatine to glucose and will fail to appear as inorganic phosphate. Under the conditions used in Table 3 (M-glucose and 26 mM-phosphocreatine) approx. 10% of the phosphate was transferred to glucose and approx. 4% appeared as glucose 6-phosphate.

Only 25–40% of the phosphate apparently transferred to glucose is estimated by the enzymic method of Slater (1953), indicating the synthesis of other esters in addition to glucose 1- and 6-phosphate. A negligible amount of the acid-labile glycosidic ester would be expected under the reaction conditions. Seegmiller & Horecker (1951) found that all hydroxyls of glucose except that of C-1 were phosphorylated by polyphosphoric acid, and the results suggest that the acid-catalysed transfer from phosphocreatine may also produce random phosphorylation. With dihydroxyacetone, for example, some triose diphosphate may be synthesized as well as the monophosphate, but only the latter would be estimated enzymically (Table 4). It is probable that a wide variety of other hydroxyl-containing compounds would act as phosphate acceptors in this non-enzymic-transfer reaction. The synthesis of histidine phosphate by non-enzymic transfer from amino phosphate was recently reported by Müller, Rathlev & Rosenberg (1956).

Biological significance of the phosphotransferase reaction

The variation in activity of alkaline phosphatase during growth and development of several organs indicates that this type of enzyme is associated with growth processes (see reviews by Moog, 1946, and by Bradfield, 1949). Reactions with compounds concerned in the metabolism of nucleic acids or of proteins could explain this relationship. Morton (1952*a*, 1953) showed that nucleotides could act as phosphate donors for the phosphotransferases, and Brawerman & Chargaff (1954*a*, *b*) that various nucleosides could act as phosphate acceptors. These latter workers used the name 'nucleoside transferases' for enzymes catalysing nucleoside phosphorylation. Their results are complicated by the use of crude tissue extracts and long incubation periods (17–30 hr. at 30°). Tunis & Chargaff (1956) have used adsorption techniques to separate hydrolytic and transferase activities of a carrot extract which catalyses phosphate transfer from phenyl phosphate. Plant extracts contain a number of so-called 'isodynamic' acid phosphatases (see Roche, 1950) which may be separated by adsorption methods (Boroughs, 1954). These may well differ quantitatively in acceptor specificity, and this could explain the results of Tunis & Chargaff (1956).

Whether the phosphomonoesterases function *in vivo* as transferases, or as hydrolases, may depend on the concentration of available water. The increased rate of utilization of the phosphate donor and the decreased rate of hydrolysis by acid phosphatases with increasing concentrations of suitable phosphate acceptors is in sharp contrast with the behaviour of the substrate-specific phosphatases (Morton, 1953, 1955*b*). The alkaline phosphatases of calf intestine and of cow mammary gland are probably associated with the lipoprotein structure of the endoplasmic reticulum, which gives rise to microsomes on cell rupture (Morton, 1954*a*, *b*; Hodge & Morton, 1956; Hodge *et al.* 1956). Liver acid phosphatase is bound to lipoprotein particles called lyosomes (Appelmans, Wattiaux & de Duve, 1955). The association of these enzymes with lipoprotein structures may restrict hydrolysis and favour transferase action *in vivo* (Morton, 1952*a*). However, in view of the wide specificity towards donors and acceptors shown by both the acid and alkaline phosphatases, the nature of the substrates of these enzymes *in vivo* is a matter for speculation at the present time.

SUMMARY

1. The transferase reaction catalysed by acid phosphatase of human prostate gland was studied in relation to the specificity towards donors and acceptors, and the influence of the concentration of donor and acceptor on the transfer reaction.

2. Non-enzymic phosphorylation of glucose with phosphocreatine as donor occurs at pH 4·2 and at 38°.

3. The results indicate that certain hydroxyl-containing compounds can compete with water in both the enzymic- and non-enzymic-transfer reactions. The possible biological significance of the transferase activity of phosphatases is discussed.

I wish to thank Dr M. Dixon, F.R.S., for his stimulating interest and advice in this work, which was carried out in

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Cell-wall Polysaccharides of Myrobalans

By D. E. HATHWAY and J. W. T. SEAKINS
The British Leather Manufacturers Research Association, Milton Park, Egham, Surrey

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Myrobalans are the dried fruit of an Indian tree, *Terminalia chebula* Retz.; they contain 43–53 % of tannin.

The present study was undertaken as there was no information relating to the cell-wall components, particularly as this is the first combretaceous plant to be investigated in this way. However, since the present work was carried out with dried-plant material, changes may have occurred in the polysaccharides during drying (cf. the rapid depletion of hemicelluloses in ripening banana; Barnell, 1943). This limitation applies with less emphasis to such polysaccharides as cellulose and polygalacturonic acid, at least some proportion of which would be expected to survive the changes occurring during the rapid drying process.

MATERIALS AND METHODS

Paper chromatography was effected in an all-glass apparatus at a constant temperature of 25°. Chromatograms were dried at room temperature, unless otherwise stated, and all solutions were evaporated in N_2 under reduced pressure at <35°.

Whole myrobalans, the dried fruit of *Terminalia chebula* Retz, grown in the Jubbulpore district of the Madhya Pradesh, were split open, the kernels were removed and the remaining tissue was pulverized (Hathway, 1956).

Preparation of cell-wall material

Pulverized myrobalans (100 g.) were extracted with several changes of fresh methanol in a Soxhlet apparatus until successive extracts were colourless. The residual material was then air-dried and ground to 60-mesh in a Wiley mill. The ground meal was then extracted with 89 % ethanol in a Soxhlet apparatus until sugars could not be detected in the last extract when treated with the anthrone reagent (Hathway, 1956). The final product was dried successively in a current of air, and over P_2O_5 at 20° *in vacuo* for 4 days. Yield, 18–19 g.

Cell-wall material prepared in this way contained polysaccharides (chiefly pectic material) originally present in the cell sap of the ripe fruit.

Fractionation of cell-wall material

The results of the analysis to which duplicate samples of cell-wall material were submitted are presented in Table 1. Water-soluble polysaccharides, equivalent to 'pectin', were removed with hot water, and, after chlorite delignification, the remaining 'holocellulose' was fractionated by