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The Inhibition of Enzymes by Beryllium

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1. The action of beryllium on the following enzymes has been examined: alkaline phosphatase (*Escherichia coli* and kidney), acid phosphatase, phosphoprotein phosphatase, apyrase (potato), adenosine triphosphatase (liver nuclei, liver mitochondria, brain microsomes), glucose 6-phosphatase, polysaccharide phosphorylases *a* and *b*, phosphoglucomutase, hexokinase, phosphoglyceromutase, ribonuclease, A-esterase (rabbit serum), cholinesterase (horse serum), chymotrypsin. Alkaline phosphatase and phosphoglucomutase are inhibited by 1μM-beryllium sulphate whereas the other enzymes are largely unaffected by 1mM-beryllium sulphate. 2. Possible mechanisms for the inhibition of phosphoglucomutase and alkaline phosphatase are discussed.

Beryllium salts are toxic to mammals and this toxicity is due to the Be^{2+} ion and not the anions of the salts (Aldridge, Barnes & Denz, 1949; Siem, 1886; Comar, 1935; Sterner & Eisenbud, 1951). After intravenous doses of beryllium salts, e.g. the sulphate, the toxic effect is due to a liver necrosis (Aldridge *et al.* 1949). However, in industry there is much evidence of chronic toxicity (Tepper, Hardy & Chamberlain, 1961) and of the development of a hypersensitive reaction to beryllium (McCord, 1951; Sterner & Eisenbud, 1951; Curtis, 1959; Waksman, 1959), though not everyone accepts this view (Hardy, 1962). In view of the suggestions that an immunological reaction is involved in the chronic human disease, it is important to know for what kind of proteins beryllium has an affinity and how it is combined with them.

It is certain that beryllium has little general affinity for proteins, e.g. those present in blood plasma (Feldman, Havill & Neuman, 1953; Reeves & Vorwald, 1961). Therefore a study of the inhibitory action of beryllium on various enzymes has been made to discover, if possible, the characteristics of those enzymes that have affinity for beryllium. Beryllium inhibits alkaline phosphatase at low concentrations (Grier, Hood & Hoagland, 1949; Aldridge, 1950; Klemperer, Miller & Hill, 1949; DuBois, Cochran & Mazur, 1949) and phosphoglucomutase at higher concentrations (Cochran, Zerivic & DuBois, 1951). The sensitivity of other hydrolases and phosphotransferases has therefore been studied. Only alkaline phosphatase and phosphoglucomutase are inhibited.

METHODS AND MATERIALS

Special chemicals. The following chemicals were obtained from the sources indicated: sodium β-glycerophosphate, dipotassium salt of glucose 1-phosphate, barium and dipotassium salts of glucose 6-phosphate, clupeine, salmine, L-cysteine hydrochloride and $BeSO_4,4H_2O$ from British Drug Houses Ltd., Poole, Dorset; glycylglycine, glycogen and yeast nucleic acid from Roche Products Ltd., Welwyn Garden City, Herts.; casein (Hammerstein) from Hopkin and Williams Ltd., Chadwell Heath, Essex; barium salt of 3-phosphoglyceric acid from Koch-Light Laboratories Ltd., Colnbrook, Bucks.; disodium salts of ATP and AMP from Sigma Chemical Co., St Louis, Mo., U.S.A.; barium salt of 2,3-diphosphoglyceric acid from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany; diethyl *p*-nitrophenyl phosphate from Albright and Wilson Ltd., Birmingham.

Enzyme preparations and determinations

Alkaline phosphatase. The enzyme was purified from bovine kidney by the method of Abul-Fadl & King (1949). The precipitate from acetone (55%, v/v) was dissolved in water to give an approx. 2% solution and dialysed free from phosphate. The activity of this preparation was 6·6μ-moles/min./mg. of protein N at 38°. The enzyme from *Escherichia coli* was also used (Worthington Biochemical Corp., Freehold, N.J., U.S.A.).

Sodium β-glycerophosphate or sodium phenyl phosphate was used as substrate and the medium, pH10·0, had the following composition: substrate (12·5mM), Na_2CO_3 (50mM), $NaHCO_3$ (50mM), magnesium acetate (25mM). In a few experiments with bovine kidney alkaline phosphatase EDTA (0·08mM) was added. It was never used with the *E. coli* phosphatase. The enzyme was usually preincubated at 38° for 5min. with the buffer containing magnesium or

beryllium or both, and the determination started by the addition of substrate. The inorganic phosphate liberated during 10 min. was determined by the method of Fiske & Subbarow (1925) after precipitation of protein by $HClO_4$. Liberated phenol was determined as described by King (1951).

Acid phosphatase. Rat-liver mitochondria were isolated as previously described (Aldridge, 1957; Aldridge & Threlfall, 1961), except that the mitochondria were isolated by centrifuging at 6000*g* for 15 min. The mitochondria from 8 g. wet wt. of liver were suspended in 16 ml. of water and dialysed against deionized water to remove sucrose and inorganic phosphate. This procedure causes the lysosomal acid phosphatase to become activated. Enzyme activity was determined as described by Berthet & de Duve (1952), with sodium β-glycerophosphate as substrate.

Phosphoprotein phosphatase. A crude enzyme preparation was obtained by homogenizing the spleens from two rats in 0·3 M-sucrose by using a Potter–Elvehjem-type homogenizer with a Perspex pestle and a smooth glass tube (Aldridge, Emery & Street, 1960). The initial homogenization was made with a homogenizer with total clearance between pestle and tube of 0·02 in., followed by the use of one with a clearance of 0·005 in., both rotating at 1100 rev./min. Debris was removed by centrifugation at 1100*g* for 10 min. The supernatant and washings were dialysed against deionized water at 0° to remove inorganic phosphate. Enzyme activity was measured by the method of Paigen (1958).

Phosphoglucomutase. Enzyme was prepared from rabbit muscle by the method of Jagannathan & Luck (1949) to the stage of heat treatment at 50° at pH 5·0. After cooling, the precipitate was removed and the clear supernatant was dialysed against running distilled water to remove inorganic phosphate. The solution was stored frozen. The activity of this preparation was 0·2 μmole of substrate/min./mg. of protein. From the turnover number of this enzyme (Najjar, 1948) this preparation was 12% pure. Enzyme activity in the presence of cysteine was measured by the method of Najjar (1948). The concentration of reagents were as stated by Najjar (1948), with the addition of a final concentration of 5·2 mM-$KHCO_3$ added as 0·4 ml. of 21 mM-$KHCO_3$ saturated with CO_2+N_2 (5:95). For a few experiments a crystalline preparation was used (C. F. Boehringer und Soehne G.m.b.H.).

Potato apyrase. The enzyme was prepared as described by Lee & Eiler (1951). Enzymic activity was measured in a medium of the following composition: $MgSO_4$ (1 mM), disodium salt of ATP, pH 7·5 (9 mM), 0·4 ml. of $KHCO_3$ (21 mM) saturated with CO_2+N_2 (5:95); the total volume was 1·0 ml. The inorganic phosphate liberated was determined by the method of Fiske & Subbarow (1925), slightly modified to prevent errors due to hydrolysis of ATP (Aldridge, 1962).

Liver-mitochondrial adenosine triphosphatase. Rat-liver mitochondria were isolated (Aldridge, 1957, 1958) and enzymic activity was measured as described by Aldridge & Stoner (1960) after activation by the addition of 30 μM-2,4-dinitrophenol or by lysis in distilled water.

Brain microsomal adenosine triphosphatase. The microsomal fraction from rat brain was isolated and the adenosinetriphosphatase activity determined in the presence of sodium and potassium as described by Aldridge (1962).

Glucose 6-phosphatase. The microsomal fraction from rat liver was isolated as described above and was washed with and suspended in 0·1 M-maleate buffer, pH 5·4, to remove contaminating phosphorylases and phosphoglucomutase. Enzyme activity was measured as described by Swanson (1955).

Muscle phosphorylase. The enzyme was extracted from rabbit muscle and purified to the end of stage 2 as described by Cori, Illingworth & Keller (1955). The precipitated enzyme was dissolved and dialysed for 2 hr. against distilled water. Enzymic activity with and without the addition of AMP was determined as described by Cori *et al.* (1955).

Phosphoglyceromutase. A partial purification was carried out from rabbit muscle by using the method of Sutherland, Posternak & Cori (1949). Enzymic activity was determined essentially by the method of Cowgill & Pizer (1956) by measuring the change in optical rotation when 3-phosphoglyceric acid is converted into 2-phosphoglyceric acid or vice versa. The medium contained: imidazole buffer, pH 7·0 (2·5 mM), 3-phosphoglyceric acid, pH 7·0 (134 mM), or 2-phosphoglyceric acid, pH 7·0 (20 mM), and 2,3-diphosphoglyceric acid, pH 7·0 (0·02 mM). Samples were taken at zero time and at 10 min. and added to 1·7 ml. of 25% (w/v) ammonium molybdate, and the change in optical rotation was measured with 5 cm. polarimeter tubes. Phosphoglyceromutase preparations from Koch–Light Laboratories Ltd. and C. F. Boehringer und Soehne G.m.b.H. were also used.

Liver nuclear adenosine triphosphatase. Nuclei were isolated from rat liver as described by Stirpe & Aldridge (1961) and enzymic activity was determined as described by Aldridge & Stoner (1960).

A-esterase. Rabbit serum was used and was incubated with 30 μM-diethyl *p*-nitrophenyl phosphate at 37° to inhibit any B-esterases present. Enzyme activity was measured by a manometric technique with phenyl acetate as substrate (Aldridge, 1953*b*).

Yeast ribonuclease. A commercial enzyme preparation (Koch–Light Laboratories Ltd.) was used and enzymic activity determined by a manometric method (Bain & Rusch, 1944). Yeast nucleic acid was purified by the method of Smith & Markham (1950).

Cholinesterase. Horse serum was used and enzymic activity determined by a manometric technique with butyrylcholine (30 mM) as substrate (Aldridge, 1953*a*).

Chymotrypsin. Crystalline chymotrypsin (Armour and Co. Ltd., Eastbourne, Sussex) was used and enzymic activity measured with *N*-acetyl-L-phenylalanine ethyl ester as substrate by a manometric technique (Parks & Plaut, 1953).

Hexokinase. This enzyme was prepared from yeast by Mr V. H. Parker by a modification of the method of Berger, Slein, Colowick & Cori (1946), and activity was measured by a modification of the method of Berger *et al.* (1946). The medium contained $NaHCO_3$ (20 mM), glucose (31 mM), $MgCl_2$ (6·3 mM), disodium ATP, pH 7·4 (5 mM), 1·5 ml. of enzyme diluted with 1% (w/v) glucose; the total volume was 3·2 ml. The solution was gassed with CO_2+N_2 (5:95) at 37° and the reaction started by the addition of the ATP and $MgCl_2$ from side arm. Readings were taken at 2 min. intervals for 10 min.

RESULTS

Inhibition of enzymes by beryllium. Table 1 shows the results of the treatment of a variety of enzymes with beryllium. Of these enzymes only

two are inhibited by beryllium sulphate at concentrations of 10 μM or lower. Of the eight phosphohydrolases tested only alkaline phosphatase (from both kidney and *E. coli*) is inhibited at low concentrations (1 μM or less) of beryllium sulphate, and of the phosphotransferases only phosphoglucomutase is inhibited, hexokinase and phosphoglyceromutase being unaffected by much higher concentrations. Other enzymes tested include muscle phosphorylase *a*, ribonuclease, A-esterase, cholinesterase (serum) and chymotrypsin. All are unaffected by concentrations of beryllium sulphate up to 0·4 mM.

The use of beryllium salts presents some difficulties because of a tendency to form insoluble compounds (hydroxide, phosphates etc.) or complexes (pyrophosphate, citrate etc.). We have tried therefore to check that the reagents used in the enzyme assays listed in Table 1 do not prevent inhibition of alkaline phosphatase. It was found that most of the reagents (imidazole, ATP, glycylglycine, sodium maleate, sodium acetate, albumin, 2-phosphoglyceric acid, 2,3-diphosphoglyceric acid) are unlikely to prevent inhibition by beryllium of enzymes intrinsically as sensitive as phosphoglucomutase or alkaline phosphatase. Alkaline phosphatase is inhibited by beryllium when β-glycerophosphate, glucose 1-phosphate and glucose 6-phosphate are used as substrates in bicarbonate–carbonate buffers, and phosphoglucomutase is inhibited in the presence of high concentrations of cysteine. However, there is evidence that a substrate for phosphoglyceromutase (3-phosphoglyceric acid) does form a complex with beryllium. Colour formation between aurinetricarboxylic acid and beryllium is decreased and inhibition of *E. coli* phosphatase by beryllium is less when one of the above substrates is added. *E. coli* phosphatase was assayed at pH 8·2 (by measuring *p*-nitrophenol liberation from *p*-nitrophenyl phosphate) in the presence and absence of those concentrations of 2-phosphoglyceric acid and 2,3-diphosphoglyceric acid used in the assay of phosphoglyceromutase. With both of the above conditions for the determination of enzyme activity the inhibition after preincubation of the enzyme with 1 mM-beryllium sulphate was the same. It is therefore considered unlikely that phosphoglyceromutase can be as intrinsically sensitive to beryllium as either *E. coli* phosphatase or phosphoglucomutase; preincubation of phosphoglyceromutase with 1 mM-beryllium sulphate for 5 min. at 37° leads to 15% inhibition; to produce the same inhibition of phosphoglucomutase would require only 0·4 μM-beryllium sulphate (Aldridge & Thomas, 1964).

DISCUSSION

Alkaline phosphatase from ox kidney and *E. coli* is inhibited by beryllium sulphate at micromolar concentrations. It has been known for some time

Table 1. *Effect of beryllium on various enzymes*

Beryllium sulphate was used and in each case the enzyme was preincubated with beryllium for at least 10 min. in the absence of substrate. At pH above 7 precipitates were obtained with concentrations of $BeSO_4$ of 1 mM and above. Inhibition at these concentrations may be non-specific.

Enzyme	Activated by Mg^{2+}	pH of assay	Effect of $BeSO_4$ at the concn. indicated
Alkaline phosphatase (kidney)	+	9·4	50% inhibition, 1 μM
Alkaline phosphatase (*E. coli*)	+	8·1	50% inhibition, 0·5–1 μM
Acid phosphatase	−	5·0	No inhibition, 0·6 mM
Phosphoprotein phosphatase	−	6·0	No inhibition, 0·1 mM
Apyrase (potato)	+	7·5	No inhibition, 4·0 mM
Adenosine triphosphatase (liver nuclei)	+	6·8	No inhibition, 0·5 mM; 97% inhibition, 5 mM
Adenosine triphosphatase (liver mitochondria)	+	6·8	No inhibition, 0·2 mM; 40% inhibition, 2·1 mM
Adenosine triphosphatase (brain microsomes)	+	7·4	20% inhibition, 0·64 mM
Glucose 6-phosphatase	−	6·5	No inhibition, 0·8 mM
Polysaccharide phosphorylase *a*	−	6·0	No inhibition, 0·64 mM; 91% inhibition, 6·4 mM
Phosphoglucomutase	+	7·5	50% inhibition, 5 μM
Hexokinase	+	7·4	45% inhibition, 1·5 mM; no inhibition, 0·15 mM
Phosphoglyceromutase	−	7·0	No inhibition, 2·0 mM*; 15% inhibition, 1·0 mM†
Ribonuclease	−	7·5	No inhibition, 1·0 mM
A-esterase (rabbit serum)	−	7·6	No inhibition, 1·0 mM
Cholinesterase (horse serum)	−	7·6	No inhibition, 1·0 mM
Chymotrypsin	−	7·0	10% inhibition, 1·0 mM

* 134 mM-3-Phosphoglyceric acid as substrate.
† 20 mM-2-Phosphoglyceric acid as substrate.

that mammalian alkaline phosphatase of various tissues is inhibited by beryllium [calf intestinal mucosa (Morton, 1955); rat intestine, kidney and bone (Grier *et al.* 1949); hog and rabbit kidney (Klemperer *et al.* 1949; Aldridge, 1950); rat and guinea-pig serum (Cochran *et al.* 1951; Schubert & Lindenbaum, 1954; DuBois *et al.* 1949)]. The alkaline phosphatase present in *E. coli* grown on orthophosphate-deficient media but containing β-glycerophosphate (Garen & Levinthal, 1960) is also inhibited by beryllium (Plocke, Levinthal & Vallee, 1962). Although the alkaline phosphatases from the mammalian sources listed above are activated by Mg^{2+} among other ions (Roche & Thoai, 1950; Cloetens, 1941; Clark & Porteous, 1963), there is little or no activation of the enzyme from *E. coli* by magnesium salts (Plocke & Vallee, 1962; Plocke *et al.* 1962; Garen & Levinthal, 1960).

Cochran *et al.* (1951) state that, in the absence of magnesium salts, 59 μM-beryllium chloride inhibits phosphoglucomutase by 64, 45, 93 and 48% in homogenates of rat skeletal muscle, rat liver, guinea-pig skeletal muscle and guinea-pig liver respectively. They further state that, in the presence of 1·5mM-magnesium chloride, 0·59mM-beryllium chloride only produces 5% inhibition. The purified phosphoglucomutase preparation from rabbit muscle is much more sensitive to inhibition by beryllium. The inhibitory process shows some rather unusual characteristics; it is competitive with respect to magnesium but is progressive, and when the inhibition is established it is no longer reversed by adding magnesium sulphate (Aldridge & Thomas, 1964). The inhibitory process is reminiscent of the activation process (Robinson & Najjar, 1961).

Of the enzymes examined in this paper (Table 1) or by other workers (Table 2) there are only two, alkaline phosphatase and phosphoglucomutase, for which there is clear evidence of a direct action of beryllium on the enzyme. For the others reported in the literature to be inhibited by higher concentrations of beryllium there is evidence that this is due to combination of beryllium with the substrate, depleting the enzyme of its usual magnesium–substrate complex [yeast hexokinase (Gutfreund & Hammond, 1963); enolase (Malmström, 1955); yeast pyrophosphatase (Bailey & Webb, 1944)]. The inhibition of phosphatidic acid phosphatase may be of the same type (Hokin, Hokin & Mathison, 1963). The only remaining report is the inhibition of amylase (McGeachin *et al.* 1962), but, in addition to the requirement for rather high concentrations of beryllium sulphate, inhibition was much less when beryllium acetate was used, making it uncertain whether the inhibition was due to Be^{2+} ion. Inhibition by beryllium sulphate at micromolar concentrations seems therefore to be confined to two enzymes. Is there, then, any common feature between alkaline phosphatase and phosphoglucomutase? There is good evidence that they both form phosphoryl-enzyme intermediates. Phosphoglucomutase is normally isolated as the phosphoryl-enzyme (Jagannathan & Luck, 1949; Najjar & Pullman, 1954; Milstein & Sanger, 1961; Sidbury & Najjar, 1957; Koshland &

Table 2. *Reported action of beryllium on enzymes and enzyme systems*

References: 1, Klemperer (1950); 2, Gutfreund & Hammond (1963); 3, Ashmore, Hastings & Nesbett (1954); 4, Axelrod, Saltman, Bandurski & Baker (1952); 5, Malmström (1955); 6, McGeachin, Pavord & Pavord (1962); 7, Bailey & Webb (1944); 8, Hurst, Little & Butler (1951); 9, Klemperer *et al.* (1949); 10, Hokin *et al.* (1963).

Enzyme	Activating ion present	Inhibition	Concn. of $BeSO_4$ (mM)	Reference
Adenosine triphosphatase (myosin)	Ca^{2+}	−	1·0	1
Hexokinase (yeast)	Mg^{2+}	+		2
Succinate-oxidase system	Al^{3+}	Activation		1
Carboxylase (yeast)	Mg^{2+}	−		1
Arginase	Mn^{2+}	−	2·6	1
Carbonic anhydrase	−	−	1·0	1
Uricase	−	−	1·0	1
Glycolysis (hexose diphosphate to lactic acid)	Mg^{2+}	−	1·0	1
Glucose 6-phosphatase	−	−	1·0	3
Phosphohexokinase (pea)	Mg^{2+}	−	5·0	4
Enolase	Mg^{2+}	+	0·5	5
Amylase	Ca^{2+}	+	0·1–1·0	6
Pyrophosphatase (yeast)	Mg^{2+}	+		7
5-Nucleotidase	Mg^{2+}	−	1·0	8
Acid phosphatase (hog kidney)	−	−	0·1	9
Phosphatidic acid phosphatase (erythrocyte membrane)	Na^{+}, Mg^{2+}	+	0·1	10

Erwin, 1957). Alkaline phosphatase from *E. coli* is phosphorylated on incubation with [^{32}P]orthophosphate in the range pH 5–6, when the enzyme is not active (Engström, 1961, 1962; Schwartz & Lipmann, 1961; Milstein, 1963; Schwartz, Crestfield & Lipmann, 1963; Schwartz, 1963); at pH10, when the enzyme is fully active, little or no labelling can be found. The rate of dephosphorylation of the phosphoryl-phosphatase under conditions when the enzyme is active has been determined and has been shown to be at least 3–4 times the maximum turnover rate (determined with *p*-nitrophenyl phosphate) (Aldridge, Barman & Gutfreund, 1964). The phosphoryl-enzyme therefore has properties that are consistent with its being an intermediate in the enzymic mechanism. For none of the enzymes studied in this paper is there certain evidence of a phosphoryl-enzyme intermediate. For some of the enzymes examined there is no evidence. For others there are either positive results against [hexokinase (Hass, Boyer & Reynard, 1961; Trayser & Colowick, 1961; Crane, 1962)] or results of kinetic measurements or exchange experiments that are consistent with the formation of a phosphoryl-enzyme intermediate. However, in most cases the authors indicate that other mechanisms are possible [myosin (Levy & Koshland, 1958; Koshland, Budenstein & Kowalsky, 1954); glucose 6-phosphatase (Hass & Byrne, 1960; Nordlie & Arion, 1964)]. Phosphorylase *a* is a phosphoprotein but does not catalyse any exchange between ^{32}P-labelled enzyme and orthophosphate (Krebs, Kent & Fischer, 1958); neither does the phosphate of AMP interchange in a reaction containing [^{32}P]-orthophosphate, glucose 1-phosphate, glycogen and phosphorylase *b* (Cohn & Cori, 1948). For this enzyme the bound phosphoryl group does not appear to be intimately involved in the catalytic process (Brown & Cori, 1961). An interesting case is phosphoglyceromutase from muscle, which might be expected to operate by a similar mechanism. Although early work seemed to indicate that a phosphoryl-enzyme intermediate was involved (Pizer, 1958, 1960; Grisolia, Joyce & Fernandez, 1961), there have always been doubts about whether this was qualitatively similar to the phosphoglucomutase in view of the different stability of the intermediate to acid and lack of exchange of the label with substrate (Pizer, 1962). Zwaig & Milstein (1963) have presented evidence that the acid-stable component of labelling (phosphorylserine) is entirely due to contamination of the enzyme preparation with phosphoglucomutase, and that the component stable under milder conditions is an enzyme–2,3-diphosphoglycerate complex. Beryllium does not inhibit this enzyme. Alkaline phosphatase and phosphoglucomutase have two things in common: a phosphoryl-enzyme intermediate step in their mechanisms, with the phosphate after acid treatment attached to serine, and sensitivity to beryllium. Enzymes, such as cholinesterase or chymotrypsin, that are phosphorylated on serine by organophosphorus compounds are not inhibited by beryllium (Table 1). It is tempting to suggest that beryllium attaches itself to alkaline phosphatase via the phosphate group; this seems unlikely since a given concentration of beryllium sulphate inhibits less when it is added to the enzyme after the substrate instead of before (W. N. Aldridge, unpublished work). It seems probable therefore that beryllium combines with the unphosphorylated enzyme in a way so that magnesium cannot compete for the unphosphorylated enzyme. Beryllium also seems to combine with phosphoglucomutase in such a way that the inhibition cannot be reversed by magnesium (Aldridge & Thomas, 1964). The common factors in the mechanism of the sensitivity of these two enzymes remain to be elucidated, but, in view of the increasing evidence for conformational changes in the protein during the catalytic process (Koshland, 1964), the possibility that beryllium either initiates unfavourable changes or prevents essential changes must be considered.

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