Pyridoxal Phosphate Breakdown by an Alkaline-Phosphatase Preparation

By J. M. TURNER*
Department of Chemistry and Radiation Laboratory, University of California, Berkeley, Calif., U.S.A.

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Pogell (1958) has demonstrated the presence of phosphatase activities for pyridoxal and pyridoxamine phosphates in rabbit-liver fractions. Wada *et al.* (1959) have also demonstrated the breakdown of pyridoxal and pyridoxamine phosphates in rabbit-liver preparations. Roberts, Rothstein & Baxter (1958) have obtained results indicating the presence of pyridoxal phosphate-phosphatase activity in brain tissue, and these results have been confirmed by Begum & Bachhawat (1960), who suggest that a non-specific acid phosphatase is involved. Evidence for pyridoxal phosphate destruction in *Neurospora crassa* extracts has been obtained by Wainwright (1959), and for pyridoxamine phosphate breakdown in the fungus *Ophiostoma multiannulatum* by Wikberg (1960). The latter author has suggested that an acid phosphatase is involved. Turner & Happold (1961) have demonstrated pyridoxal phosphate-phosphatase activity in *Escherichia coli* extracts. By the use of direct spectrophotometric methods for the assay of activity, they suggest that an alkaline phosphatase is involved.

The following report shows that pyridoxal phosphate and pyridoxamine phosphate are readily hydrolysed by purified alkaline-phosphatase preparations.

EXPERIMENTAL

Materials

Alkaline phosphatase. An 'Intestinal phosphatase' preparation, of bovine origin, was obtained from Armour Laboratories, Chicago, Ill., U.S.A. A similar preparation was obtained, for purposes of comparison, from Worthington Biochemical Corp., Freehold, N.J., U.S.A.

Chemical compounds. Pyridoxal phosphate and pyridoxamine phosphate, also pyridoxal and *p*-nitrophenyl phosphate, were obtained from California Corp. for Biochemical Research, Los Angeles, Calif., U.S.A. Versenol (*N*-hydroxyethylethylenediaminetriacetic acid, trisodium salt) was obtained from the Bersworth Chemical Co., Framington, Mass., U.S.A.

Assay of phosphatase activities

Pyridoxal phosphate as substrate. The standard composition of reaction mixtures was: buffer, 0·04 M-veronal, pH 8·9, 2·0 ml.; enzyme preparation, 0·1 mg. of protein, in aqueous solution, 0·1 ml.; pyridoxal phosphate, 0·4 μmole, in 0·4 ml.; water or additions to give a total volume of 4·0 ml. All reactions were carried out at 37° for 4 min. unless otherwise stated. Reactions were stopped by the addition of 0·5 ml. of 2·5 N-NaOH, and absorption values were read at 390 and 300 mμ, i.e. $\lambda_{max.}$ values for substrate and product, against the appropriate blanks. The amount of pyridoxal phosphate and the free base present were calculated as described by Turner & Happold (1961). At the pH of 0·28 N-NaOH, i.e. approx. pH 13, $\epsilon^{300}_{\text{pyridoxal}} = 7000$; $\epsilon^{390}_{\text{pyridoxal}} = 800$; $\epsilon^{300}_{\text{pyridoxal phosphate}} = 1200$; $\epsilon^{390}_{\text{pyridoxal phosphate}} = 6300$. From these measured values, it can be shown that:

$$[\text{pyridoxal phosphate}] = (1{\cdot}63E^{1\,\text{cm.}}_{390} - 0{\cdot}19E^{1\,\text{cm.}}_{300}) \times 0{\cdot}1\ \text{mM}$$
$$[\text{pyridoxal}] = (1{\cdot}48E^{1\,\text{cm.}}_{300} - 0{\cdot}28E^{1\,\text{cm.}}_{390}) \times 0{\cdot}1\ \text{mM}$$

A Cary recording spectrophotometer, model 14, was used to record spectra and to determine the above extinction coefficients. Standard solutions of pyridoxal and its phosphate were diluted to 0·05 mM with buffer and NaOH, and measurements were made in quartz cells of 1 cm. light-path. A Beckman DU spectrophotometer was used for routine absorption measurements at $\lambda_{max.}$ values.

By using controls in which water replaces substrate, the absolute amounts of pyridoxal and its phosphate also can be calculated. For example, with a standard solution containing pyridoxal phosphate and pyridoxal in 0·28 N-NaOH, where each component was 0·050 mM, $E^{1\,\text{cm.}}$ values at 300 and 390 mμ were found to be 0·405 and 0·358 respectively. By substituting these values in the above equations, the respective concentrations of pyridoxal phosphate and pyridoxal are calculated to be 0·051 and 0·050 mM.

In practice it is convenient to use controls in which substrate or enzyme is added after the NaOH. If absorption values are read at 300 mμ with the control as blank, and the control itself is read at 390 mμ with the stopped reaction mixture as blank, then the changes in both substrate and product concentrations can be calculated. A simple expression can be derived with which it is possible to calculate the mean amount of substrate utilized and product formed. Thus for a stopped reaction mixture having the composition given above:

$$\text{Mean } \Delta \text{ substrate and mean } \Delta \text{ product} = (0{\cdot}304.\Delta E^{1\,\text{cm.}}_{390} + 0{\cdot}290.\Delta E^{1\,\text{cm.}}_{300})\ \mu\text{mole.}$$

Pyridoxamine phosphate and p*-nitrophenyl phosphate as substrates.* Reaction-mixture composition was the same as when pyridoxal phosphate was substrate except that 2·0 μmoles of substrate was used. The higher substrate concentration was necessary owing to greater reaction velocities with the above-mentioned compounds. After incubation at 37°, reactions were stopped by the addition of NaOH. Phosphatase activity was measured by the assay

* Present address: Department of Chemical Pathology, St Mary's Hospital Medical School ,London, W. 2.

of inorganic phosphate, according to the method of Gomori (1941–42). A Beckman DU spectrophotometer was used for absorption measurements at 675 mμ. In a number of experiments with *p*-nitrophenyl phosphate, this compound was directly substituted for pyridoxal phosphate in the procedure previously described but a shorter incubation time was used. The absorption of stopped reaction mixtures was read at 400 mμ, the appropriate blank being used.

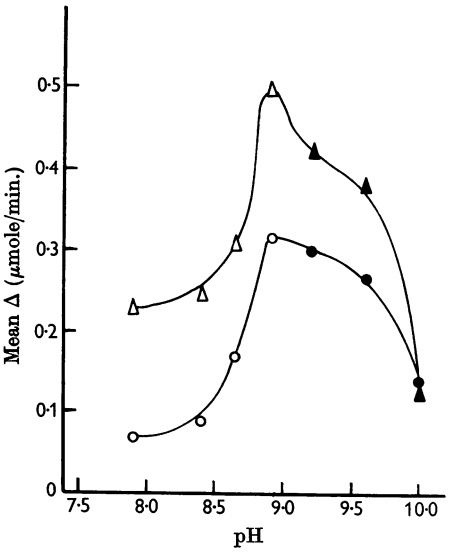

Fig. 1. Effect of pH on pyridoxal phosphate breakdown. Reaction mixtures contained: 2·0 ml. of 0·04M-buffer; 0·1 ml. of enzyme solution (approx. 1 mg./ml.); 0·4 ml. of pyridoxal phosphate solution (containing 0·4 μmole); 1·5 ml. of water or $MgSO_4$ solution. Reactions were carried out at 37° for 4 min. and then stopped by the addition of 0·5 ml. of 2·5N-NaOH. Absorption values were read at 300 and 390 mμ, blanks which contained water in place of substrate being used. Concentrations of pyridoxal and pyridoxal phosphate present were calculated as described in the text. These values are expressed as the mean of pyridoxal phosphate utilized and free pyridoxal formed. Upper curve, pyridoxal phosphate breakdown in the presence of 2·0 mM-$MgSO_4$. ○ and △, Results with veronal buffers; ● and ▲, results with Na_2CO_3–$NaHCO_3$ buffers.

Units of activity. Activities are expressed in terms of μmoles/mg. of protein/min. Protein concentrations were determined by a quantitative biuret procedure. In cases where the protein concentration was not accurately measured, activities are expressed as amount of change (μmole) under the conditions described.

RESULTS

pH *optimum for pyridoxal phosphate breakdown.* Preliminary experiments showed the pH optimum to be in the range 8–10. Veronal buffers (Britton & Robinson, 1931) were used in the range 8–9 and sodium carbonate–sodium hydrogen carbonate buffers (Delory & King, 1945) between pH 9 and 10. The pH optimum was found to be approx. 8·9. Results are illustrated in Fig. 1. It was concluded that ammediol (2-amino-2-methyl propane-1:3-diol) buffers (Holmes, 1943) should not be used in the range pH 8–10, owing to differences observed in the absorption spectra of both pyridoxal and pyridoxal phosphate when these are plotted at pH 10 with ammediol rather than sodium carbonate–sodium hydrogen carbonate buffer.

Effect of substrate and enzyme concentrations. With the standard reaction-mixture composition, initial rates of pyridoxal phosphate breakdown at 37° were found to be proportional to the amount of enzyme up to a value of at least 0·16 mg./4 ml. Under similar conditions, with 0·10 mg. of enzyme, substrate utilization was linear with time up to approx. 8 min., and reaction rates remained constant at substrate concentrations above approx. 0·05 mM, i.e. 0·2 μmole/4 ml. (see Table 1). At an enzyme concentration of 0·3 mg./4 ml. the variation of substrate concentration between 0·02 and 0·17 mM gave a double-reciprocal plot (Lineweaver & Burk, 1934), indicating approx. K_m 0·05 mM (see Fig. 2). With *p*-nitrophenyl phosphate as substrate, with the same conditions except for an incubation time decreased from 4 min. to 2·75 min., and a similar spectrophotometric-assay procedure, approx. K_m 0·06 mM was obtained. K_m values were calculated from visually drawn straight lines.

Table 1. *Effect of substrate concentration on pyridoxal phosphate breakdown*

Reaction mixtures had the standard composition described in the Experimental section, except that substrate concentration was varied as indicated. In each case, 0·1 mg. of enzyme was present (cf. Fig. 2), and incubation was for 4 min. at 37°.

10^4 × Initial substrate concn. (M)	$\Delta E^{1\,cm.}_{390\,m\mu}$	$\Delta E^{1\,cm.}_{300\,m\mu}$	Substrate utilized (μmole/mg. of protein/min.)	Mean Δ substrate and product (μmole/mg. of protein/min.)
0·28	0·070	0·073	0·115	0·108
0·50	0·090	0·092	0·143	0·135
0·75	0·095	0·100	0·153	0·145
1·00	0·092	0·094	0·148	0·138
1·25	0·085	0·092	0·135	0·133
2·25	0·083	0·103	0·130	0·138

Effect of magnesium ions and N-*hydroxyethylethylenediaminetriacetate.* Magnesium ions were found to increase phosphatase activity with pyridoxal phosphate as substrate. The optimum Mg^{2+} ion concentration was found to be approx. 2 mM (see Table 2). At this concentration, the rate of pyridoxal phosphate breakdown was increased by 56%. The pH optimum for phosphatase activity, measured in the presence of Mg^{2+} ions, was the same as that observed in their absence (see Fig. 1).

The metal-chelating agent trisodium *N*-hydroxyethylethylenediaminetriacetate inhibited pyridoxal phosphate breakdown at all concentrations used, without preincubation with the enzyme (see Table 2).

Effect of various compounds on pyridoxal phosphate-phosphatase activity. Pyridoxamine phosphate was found to behave as a competitive inhibitor of pyridoxal phosphate breakdown. With variable inhibitor concentrations and with two different substrate concentrations, the breakdown of pyridoxal phosphate was measured spectrophotometrically by the previously described method. Control experiments showed that the possible simultaneous breakdown of inhibitor did not cause any change in absorption at the wavelengths used for the assay procedure. The simple graphical method of Dixon (1953) was used to obtain the inhibition constant directly, a value of K_i 0·15 mM being found (see Fig. 3). K_m values were also obtained from Fig. 3, by virtue of the fact that the extrapolation of each linear plot cuts the base line at a value of i equal to $-K_i\left(\frac{s}{K_m}+1\right)$ (Dixon & Webb, 1958). K_m values thus derived were 0·08 and 0·06 mM. At the higher substrate

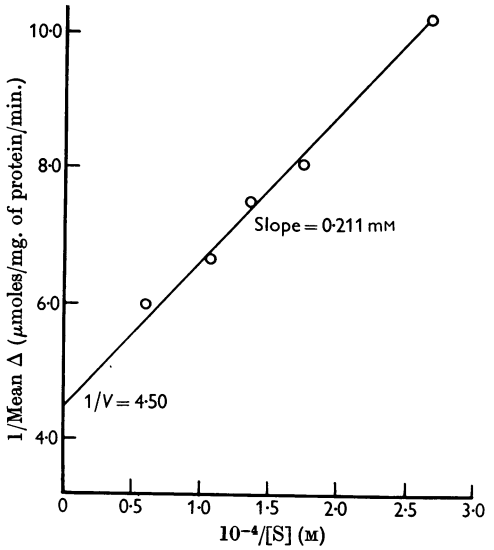

Fig. 2. Effect of substrate concentration on pyridoxal phosphate breakdown. Reaction-mixture composition was as described in Fig. 1, except that 0·3 mg. of enzyme was present, and initial substrate concentration varied as indicated. No metal ions were added. Reaction rates were calculated as described in the text.

Table 2. *Effect of magnesium ions and* N-*hydroxyethylethylenediaminetriacetate on the hydrolysis of pyridoxal phosphate*

Standard reaction-mixture composition and conditions were used, and results were calculated as described in the text. In the Mg^{2+} ion experiments, reaction was started by the addition of substrate, $MgSO_4$ solution and enzyme being allowed to preincubate in buffer for approx. 30 min. With *N*-hydroxyethylethylenediaminetriacetate (Versenol) reactions were started by the addition of enzyme and no preincubation of these two components occurred. The Mg^{2+} ion and Versenol experiments were carried out on separate occasions.

Addition	Concn. of addition (mM)	Mean Δ substrate and product (μmole/4 min.)	Activity (%)
	0	0·108	100
	2·0	0·144	133
$MgSO_4$	2·0	0·168	156
	0·2	0·148	137
	0·02	0·134	124
	0	0·073	100
	0·27	0·044	60
Versenol	0·47	0·035	48
	0·67	0·012	17
	2·0	0·006	8

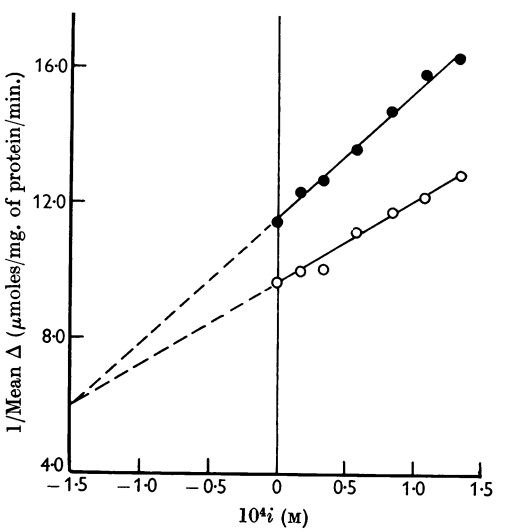

Fig. 3. Effect of pyridoxamine phosphate on pyridoxal phosphate breakdown. Reactions were carried out under the standard conditions previously described, with inhibitor concentrations shown. The two substrate concentrations used were 0·067 mM (●) and 0·133 mM (○).

concentration, an equivalent concentration of pyridoxamine phosphate caused 25% inhibition, at the lower concentration, 18% inhibition.

Under similar conditions pyridoxal had no inhibitory effect on pyridoxal phosphate breakdown when used at concentrations between 0·25-fold and 1·66-fold substrate concentration, i.e. up to 0·166 mM.

Inorganic orthophosphate inhibited pyridoxal phosphate hydrolysis competitively, the value of K_i being determined by the method of Dixon (1953) as approx. 0·06 mM. Under standard reaction conditions, with substrate at a concentration of 0·1 mM, an equivalent amount of inorganic phosphate caused 43% inhibition.

No effect of NH_4^+ ions on pyridoxal phosphate-phosphatase activity could be detected at concentrations between approx. 0·17-fold and 1·66-fold substrate concentration, i.e. 0·1 mM.

Pyridoxamine phosphate as substrate of alkaline phosphatase. It was not possible to investigate the breakdown of pyridoxamine phosphate spectrophotometrically. The spectra of the free base and its phosphate ester are similar at all pH values. Accordingly, rates of hydrolysis were measured by assay of the inorganic phosphate produced. The composition of reaction mixtures is described in the Experimental section.

Preliminary experiments showed that rapid hydrolysis of pyridoxamine phosphate was catalysed by the alkaline-phosphatase preparation, and that the pH optimum was approx. pH 8·9. A direct comparison of the rates of hydrolysis of pyridoxamine and pyridoxal phosphates showed that the former was approx. 1·5-fold that of the latter. Similar ratios were observed by Pogell (1958). These results, together with those obtained with *p*-nitrophenyl phosphate as a standard, are given as Expt. 1 in Table 3.

Specificity of phosphatase action. To determine whether the same species of enzyme was responsible for the hydrolysis of pyridoxal, pyridoxamine

Table 3. *Comparative rates of hydrolysis of pyridoxal, pyridoxamine and* p-*nitrophenyl phosphates*

In Expt. 1, the reaction-mixture composition was that specified for the assay of phosphatase activity with pyridoxamine phosphate as described in the Experimental section. In Expt. 2, however, only 1·5 μmoles of each substrate was used. Approx. 0·1 mg. of protein was used in each case. To each 4·5 ml. of stopped reaction mixture was added 1·25 ml. of approx. N-H_2SO_4, and water to 8·0 ml. Inorganic phosphate was then measured by the method of Gomori (1941–42). The colorimetric blanks were the controls, in which NaOH was added to reaction mixtures before the substrate. A solution of KH_2PO_4 was used as standard.

	Inorganic phosphate formed (μmole)			Relative reaction velocity (initial)
Time interval (min.)	2·5	5	10	
Expt. 1				
Substrate				
Pyridoxamine phosphate	0·07	0·14	0·22	0·64
Pyridoxal phosphate	0·045	0·09	0·14	0·41
p-Nitrophenyl phosphate	0·11	0·19	0·31	1·00
Expt. 2				
Pyridoxamine phosphate	0·10	—	—	0·67
Pyridoxal phosphate	0·06	—	—	0·40
p-Nitrophenyl phosphate	0·15	—	—	1·00
Pyridoxamine and pyridoxal phosphates	0·06	—	—	0·40
Pyridoxal and *p*-nitrophenyl phosphates	0·06	—	—	0·40
Pyridoxamine and *p*-nitrophenyl phosphates	0·12	—	—	0·80

Table 4. *Relative reaction rates for pyridoxal, pyridoxamine and* p-*nitrophenyl phosphates with different enzyme preparations*

Reaction-mixture composition and assay procedure used were the same as described for Table 3. Approx. 0·1 mg. of enzyme protein and 2·0 μmoles of substrate were used and the concentration of $MgSO_4$ was 2·0 mM in each case. Enzyme A was prepared by Armour Laboratories, and enzyme B by Worthington Biochemical Corp.

	Inorganic phosphate formed (μmole)				Relative reaction velocity (initial)	
	Enzyme A		Enzyme B			
Time interval (min.) ...	2·5	5	2·5	5	Enzyme A	Enzyme B
Substrate						
Pyridoxamine phosphate	0·10	0·23	0·11	0·25	0·67	0·73
Pyridoxal phosphate	0·06	0·14	0·05	0·12	0·40	0·33
p-Nitrophenyl phosphate	0·15	0·33	0·15	0·34	1·00	1·00

and *p*-nitrophenyl phosphates, experiments were carried out with all three substrates, both singly and in pairs. The results are given as Expt. 2 in Table 3. Relative reaction velocities for the three substrates were also determined with an alternative preparation of alkaline phosphatase (Worthington Biochemical Corp.). Preliminary experiments showed that this sample of the enzyme had an absolute requirement for Mg^{2+} ions, and also suggested a lower pyridoxal phosphate-phosphatase activity when compared with activity with *p*-nitrophenyl phosphate. The results of an experiment to determine relative reaction velocities with each enzyme preparation in the presence of Mg^{2+} ions are given in Table 4.

DISCUSSION

In schemes summarizing vitamin B_6 metabolism (Snell, 1958; Braunstein, 1960), the hydrolysis of both pyridoxal and pyridoxamine phosphates by phosphatase action has been postulated. No evidence, however, has been published about the nature and specificity of the phosphatases involved. Whereas most enzyme reactions involved in vitamin B_6 metabolism appear to be specific for the heterocyclic-ring portion of the vitamin, the oxidation of pyridoxal to 4-pyridoxic acid has been shown to be catalysed by the broadly specific mammalian enzyme aldehyde oxidase (Schwartz & Kjeldgaard, 1951). From the results presented here, it appears that alkaline phosphatases of apparently broad specificity are involved in vitamin B_6 metabolism. Results obtained with pairs of mixed substrates (Table 3), where the observed activity was in all cases less than the sum of the individual activities, suggests that a mixture of narrowly specific phosphatases is not involved. The competitive inhibition by pyridoxamine phosphate of pyridoxal phosphate breakdown also supports this view. The slightly different relative reaction velocities observed with different sources of phosphatase preparation may mean that each preparation is a mixture of enzymes, each with comparatively broad but quantitatively different specificity.

K_m 0·05 mM for pyridoxal phosphate, compared with K_m 0·06 mM for *p*-nitrophenyl phosphate measured under identical conditions, appears to be of the same order as K_m values for other naturally occurring substrates as reported in the literature, although such comparison is difficult because of the influence of pH, buffers etc. (Roche, 1950). Activation by Mg^{2+} ions and inhibition by metal-chelating agents and orthophosphate are common phenomena with mammalian alkaline phosphatases (Roche, 1950). The apparent activation of pyridoxal phosphate hydrolysis by NH_4^+ ions, observed by Pogell (1958) with *Escherichia coli* extracts, could not be demonstrated in the present case; nor could the inhibition of phosphatase activity by NH_4^+ ions as reported by Aebi (1949) and Motzok & Wynne (1950).

The possible role of a phosphatase for the coenzyme pyridoxal phosphate in the control of amino acid metabolism has previously been pointed out (Turner & Happold, 1961).

SUMMARY

1. An intestinal-phosphatase preparation of bovine origin was found to hydrolyse pyridoxal phosphate. At a substrate concentration of 0·1 mM, the pH optimum is approx. 8·9, and at this pH value K_m is approx. 0·05 mM. K_m for *p*-nitrophenyl phosphate, a commonly used substrate for alkaline-phosphatase assays, was found to be 0·06 mM when measured under identical conditions.

2. Phosphatase activity was increased by Mg^{2+} ions, the optimum concentration being approx. 2 mM. The presence of Mg^{2+} ions did not affect the pH optimum. Versenol (*N*-hydroxyethylethylenediaminetriacetic acid) inhibited activity at all concentrations used. Both pyridoxamine phosphate and inorganic orthophosphate inhibited pyridoxal phosphate breakdown competitively, the K_i values being 0·15 and 0·06 mM respectively. Neither pyridoxal nor NH_4^+ ions affected the phosphatase activity.

3. The enzyme preparation also hydrolysed pyridoxamine phosphate at optimum pH 8·9. With the rate of hydrolysis of *p*-nitrophenyl phosphate used as a standard, the relative reaction velocities for pyridoxal and pyridoxamine phosphate were approx. 0·41 and 0·64 respectively. With an alternative preparation of alkaline phosphatase, similar relative reaction rates were observed.

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REFERENCES

Aebi, H. (1949). *Helv. chim. acta*, **32**, 464.

Begum, A. & Bachhawat, B. K. (1960). *Ann. Biochem.* **20**, 143.

Braunstein, A. E. (1960). In *The Enzymes*, vol. 2, p. 113. Ed. by Boyer, P. D., Lardy, H. & Myrbäck, K. New York: Academic Press Inc.

Britton, H. T. S. & Robinson, R. A. (1931). *J. chem. Soc.* p. 1456.

Delory, G. E. & King, E. J. (1945). *Biochem. J.* **39**, 245.
Dixon, M. (1953). *Biochem. J.* **55**, 170.
Dixon, M. & Webb, E. C. (1958). In *Enzymes*. New York: Academic Press Inc.
Gomori, G. (1941–42). *J. Lab. clin. Med.* **27**, 955.
Holmes, W. (1943). *Anat. Rec.* **86**, 163.
Lineweaver, H. & Burk, D. (1934). *J. Amer. chem. Soc.* **56**, 658.
Motzok, I. & Wynne, A. M. (1950). *Biochem. J.* **47**, 187.
Pogell, B. M. (1958). *J. biol. Chem.* **232**, 761.
Roberts, E., Rothstein, M. & Baxter, C. F. (1958). *Proc. Soc. exp. Biol., N.Y.*, **97**, 796.
Roche, J. (1950). In *The Enzymes*, vol. 1, part 1, p. 473. Ed. by Sumner, J. B. & Myrbäck, K. New York: Academic Press Inc.
Schwartz, R. & Kjeldgaard, N. V. (1951). *Biochem. J.* **48**, 333.
Snell, E. E. (1958). *Vitam. & Horm.* **16**, 78.
Turner, J. M. & Happold, F. C. (1961). *Biochem. J.* **78**, 364.
Wada, H., Morisue, T., Nishimura, Y., Morino, Y., Sakamoto, Y. & Ichihara, K. (1959). *Proc. imp. Acad. Japan*, **35**, 229.
Wainwright, S. D. (1959). *Canad. J. Biochem. Physiol.* **37**, 1429.
Wikberg, E. (1960). *Physiol. Plant.* **13**, 616.

Biochem. J. (1961) **80**, 668

Studies on the Interaction of Zinc, Cadmium and Mercuric Ions with Native and Chemically Modified Human Serum Albumin

By D. J. PERKINS
Chemical Pathology Department, St George's Hospital Medical School, Hyde Park Corner, London, S.W. 1

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The ions of zinc, cadmium and mercury vary widely in their effects on living organisms. Zinc ions are well tolerated in mammals, and distribution studies (Gilbert & Taylor, 1956) have shown that they are located in organs rich in enzymes, such as liver, kidney and testes. Zinc has been found essential to the function of alkaline phosphatase (Mathies, 1958), carboxypeptidase B (Folk, Piez, Carroll & Gladner, 1960), liver alcohol dehydrogenase (Vallee & Hoch, 1957), glutamic dehydrogenase (Adelstein & Vallee, 1958), muscle dehydrogenase (Vallee & Wacker, 1956) and leucocyte carbonic anhydrase (Vallee, Hoch & Hughes, 1954). It is also essential in lower organisms to enzymes such as yeast alcohol dehydrogenase (Vallee & Hoch, 1955) and hexokinase in *Neurospora crassa* (Medina & Nicholas, 1957). The requirement of zinc for these enzymes does not necessarily always imply direct zinc–protein linkage. Nevertheless, if such an interaction is involved, a detailed study of the interaction of Zn^{2+} ions with albumin over a range of pH should provide a model with which to compare the effect of pH on these enzymes.

Cadmium ions, although chemically similar to Zn^{2+} ions, are toxic to many organisms. They have been found, however, in the cortex of the normal horse kidney in a protein also containing Zn^{2+} ions (Kägi & Vallee, 1960).

Mercuric ions are almost universally toxic to living matter. This toxicity is associated with the high affinity for protein thiol groups.

The affinity of proteins for these three metal ions has led to their use as precipitants in plasma-fractionation procedures. Zinc ions were used by Ketz & Mahl (1955), Cd^{2+} ions by Tolok (1959) and Hg^{2+} ions by Astrup, Schilling, Birch-Anderson & Olsen (1954).

Though previous workers have studied the interactions of these ions with various soluble proteins and have postulated their sites of binding, there are no studies over a range of pH. In this paper quantitative studies are reported on the interactions of native human albumin and some of its chemical modifications with the ions of zinc, cadmium and mercury over the accessible pH range. The techniques used were equilibrium dialysis and radioactive-isotope dilution.

METHODS

Zinc. $^{65}ZnCl_2$ of approximate specific activity 100 mc/g. of Zn was obtained from The Radiochemical Centre, Amersham, Bucks. Carrier inactive $ZnSO_4$ was added to bring the specific activity to the required value.

Cadmium and mercury. Both these materials were obtained by neutron irradiation of inactive material in BEPO at A.E.R.E., Harwell. Cadmium metal (0·5 g.) was irradiated for 4 weeks at a neutron flux of 10^{12}/cm.^{2}/sec. Mercuric oxide (2 g.) was irradiated for 4 weeks at a neutron flux of $1{\cdot}2 \times 10^{12}$/cm.^{2}/sec. No significant quantities of isotopes of other elements are produced by these irradiations. Both samples were dissolved in warm 2N-HNO_3. Portions were diluted to the required concentration and activity before use.

Measurement of radioactivity. The γ-radiation of each isotope was used for isotope-dilution analysis. Samples (10 ml.) of each solution to be analysed were placed in an annular NaI crystal attached to a photomultiplier and an Ekco automatic scaler. Each sample was counted for a minimum of 160 000 counts.