Enzymatic Hydrolysis of Organophosphorus Compounds

VI. Effect of Metallic Ions on the Phosphorylphosphatases of Human Serum and Swine Kidney

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The effects of metallic ions on the phosphorylphosphatases of purified preparations of human serum and swine kidney were studied with tabun as substrate.

The serum enzyme is activated by Sr^{2+} and Ba^{2+}, the kidney enzyme by Mn^{2+} and Co^{2+}. Both types of phosphorylphosphatases are strongly inhibited by the following ions: Ni^{+2}, Pd^{+2}, Cu^{+2}, Ag^+, Au^{3+}, Zn^{2+} and Hg^{2+}; the serum enzyme is also inhibited by Mn^{2+} and Co^{2+}. The most potent inhibiting ion of the kidney enzyme is Ag^+ (pI_{50} 5.55).

Some of the metallic ions studied also catalyse the non-enzymic hydrolysis of tabun; the most active ones in this respect are: Cu^{2+}, $Pd^{2+} > Au^{3+} > Ag^+ > Ni^{2+} > Co^{2+} > Zn^{2+}$.

Mn^{2+} has a similar effect on the enzymic reactions when DFP is used as substrate. Similarly, Ag^+ is a strong inhibitor of the DFP hydrolysis by the kidney enzyme. Sr^{2+}, on the other hand, inhibits this enzymic hydrolysis of DFP by the serum enzyme, in sharp contrast to the activating effect of this ion on the tabun hydrolysis.

Activation (by Mn^{2+}) and inhibition (by Ag^+) of kidney phosphorylphosphatase are time reactions. The interaction between the serum enzyme and metallic ions, on the other hand, is independent of time.

Activating and inhibiting ions compete with each other for active groups of the enzyme molecules.

After incubation of the phosphorylphosphatases of serum and kidney with metallic ions and subsequent dialysis the two enzymes behave differently.

The effects of metallic ions on the enzyme activities have been discussed from a more general point of view.

Recent papers by the present authors reported the existence and properties of phosphorylphosphatases [1]. In most of these studies tabun (dimethyl-amido-ethoxy-phosphoryl cyanide) was used as substrate. Employing DFP (di*iso*propoxy-phosphoryl fluoride) as a substrate and kidney as a source for "dialkylfluorophosphatase" (DFPase) in similar studies, Mounter *et al.*[2] recently reported the effects of various cations on the enzymic hydrolysis. In a preliminary comparative study of the phosphorylphosphatases of human
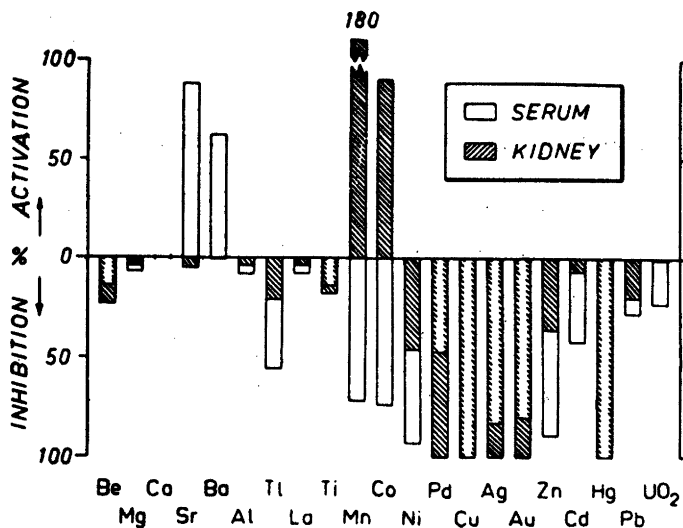
Fig. 1. Effect of various metallic ions on the phosphorylphosphatase activities of human serum and swine kidney (purified preparations). Substrate, 5.3 × 10⁻³ M tabun. Concentration of ions during activity determinations, 1.0 × 10⁻³ M. Time of incubation with ions, 60 minutes. Enzyme activity values corrected for non-enzymic hydrolysis rates, spontaneous substrate hydrolysis as well as non-enzymic hydrolysis catalysed by the metallic ions.

serum and rabbit kidney we found, among other things, that the two enzymes behave differently, the serum not being activated by Mn^{2+}, but inhibited instead [3]. The activation of the "DFPase" of swine kidney by Mn^{2+} was reported earlier by Mounter et al.[2], who later [4] confirmed our observation that the two enzymes show differences in respect to Mn^{2+} and other cations. The effect of a variety of ions has been investigated in this laboratory, and the present report deals with the results obtained with metallic ions.

METHODS AND MATERIAL

The Warburg manometric technique was used in determining enzyme activity [1]. All measurements were made at 25° C in 0.040 M sodium bicarbonate. The enzyme activity was expressed in b_{30} values. Tabun was used as substrate in most experiments; its concentration in the reaction mixture (total volume, 2.00 ml) was 5.3 × 10⁻³ M. DFP of the same molar concentration was also employed in some comparative studies.

The enzyme preparations used were Fraction IV-1 of human postpartum serum and a purified preparation of swine kidney. The latter was prepared according to the method described by Mounter et al.[2] for "DFPase". Both these phosphorylphosphatase preparations were dialysed free of ions against distilled water and then diluted to a proper concentration for enzyme activity determinations with bicarbonate solution.

The chlorides of the metals were used, except $AgNO_3$, $Pb(NO_3)_2$, $TlNO_3$, and UO_2SO_4. In preliminary testing of the effects of the metallic ions, the salt concentration in the reaction mixture during activity determination was 1.0 × 10⁻³ M. The enzymes were incubated 60 minutes with the ions, unless otherwise stated, before the substrate was added.

RESULTS

Effects of various metallic ions on the enzymic hydrolysis of tabun. The effects of metallic ions, the concentration of which was 1.0×10^{-3} M during enzyme activity determination, were studied using purified preparations of phosphorylphosphatases of human serum and swine kidney. The results are shown in Fig. 1. The serum enzyme was activated by Sr^{2+}, and Ba^{2+}; these ions had practically no effect on the kidney phosphorylphosphatase. Mn^{2+} and Co^{2+} were strong activators of the kidney enzyme, the former being particularly effective; the serum enzyme, on the other hand, was inhibited by these cations. Strong inhibitors of both types of phosphorylphosphatases were Pd^{2+}, Cu^{2+}, Ag^+, and Hg^{2+}. In addition, Ni^{2+} and Zn^{2+} were strong inhibitors of the serum phosphatase and moderate inhibitors of the kidney enzyme. Fe^{2+} (not inserted in Fig. 1) is a weak inhibitor of the serum phosphatase; this ion, however, is not suitable to study in bicarbonate systems, and no further work has therefore been carried out with Fe^{2+}.

Activation and inhibition by certain metallic ions as function of ion concentration. Those ions, which were found to be strong activators or inhibitors of the phosphorylphosphatases, were used in more detailed studies of the effects as a function of ion concentration. The results are shown in Fig. 2 for the serum enzyme and in Fig. 3 for the kidney enzyme. The *activating* ions gave optimum effect when present in a concentration of 10^{-3} M. In the presence of still higher concentrations of these ions the enzyme activity was slightly decreased or remained constant. It was especially noticeable that Mg^{2+} and Ca^{2+} had practi-
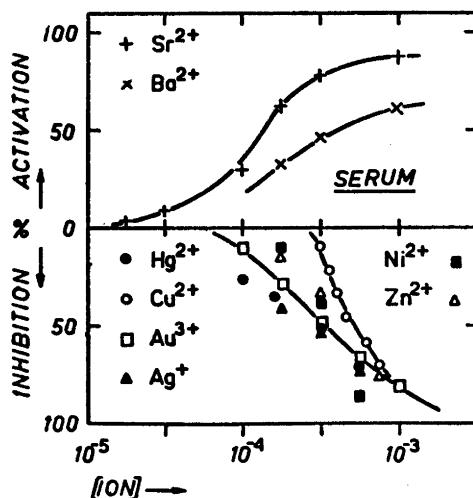

Fig. 2. Effect of metallic ions on the enzymic hydrolysis of tabun by human serum phosphorylphosphatase as function of molar ion concentration. Values corrected for non-enzymic hydrolysis (cf. text to Fig. 1). The values obtained for Mn^{2+} are found in Fig. 4. The pI_{50} values (— log molar ion concentration giving 50 % inhibition of enzyme activity) of the inhibiting ions are summarized in Table 1.
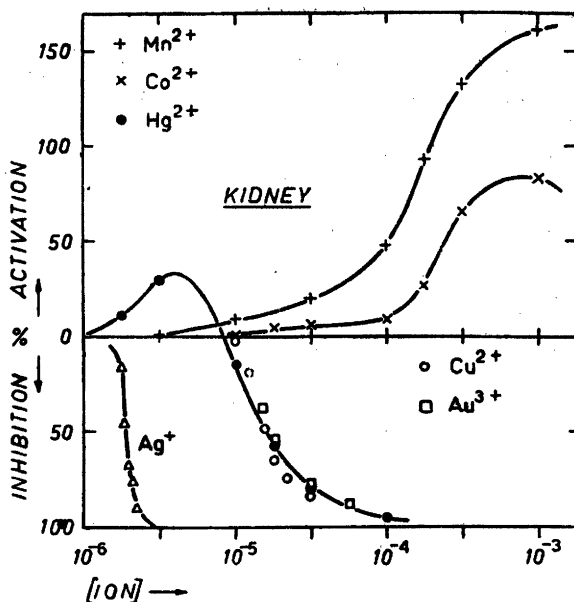
*Fig. 3. Effect of metallic ions on the enzymic hydrolysis of tabun by swine kidney phos-
phorylphosphatase as function of molar ion concentration. Values corrected for non-enzymic
hydrolysis (cf. text to Fig. 1). The pI_{50} values of the inhibiting ions are summarized
in Table 1.*

cally no effect on either of the two enzymes studied. This is in contrast to the
observation made by Mazur [5] who recommended Mg^{2+} or Ca^{2+} as activators
in the enzymic hydrolysis of DFP by liver extracts.

Those ions which are strong *inhibitors* of the phosphorylphosphatases were
found to be equally active for each of the enzymes studied. That ion concentra-
tion which gives 50 % inhibition of the enzymic hydrolysis of tabun, expressed
in pI_{50} values (— log molar ion concentration), is approximately the same for
various ions: 3.2—3.4 for the serum enzyme and 4.7 for the kidney enzyme.

The effects of Hg^{2+} and Ag^+ on the kidney phosphorylphosphatase are
especially interesting. Mercuric ions, being equally active as inhibitors as, for
instance, Cu^{2+} and Au^{3+}, increase the enzyme activity when present in low
concentrations. Such a Hg^{2+}-activating effect was observed by Mazur [5] for
the hydrolysis of DFP by liver, but could not be confirmed by Mounter et al.[2]
with a purified preparation similar to that used in the present investigations.
The inhibiting effect of Ag^+ on the kidney phosphorylphosphatase is very
striking; 50 % inhibition was obtained by 2.81×10^{-6} M Ag^+ (pI_{50} 5.55),
which is a concentration approximately ten times lower than those of Cu^{2+},
Au^{3+}, and Hg^{2+} giving the same degree (50 %) of inhibition. The pI_{50} values
are summarized in Table 1.

*Effects of metallic ions on the non-enzymic hydrolysis of tabun in the absence
of phosphorylphosphatase.* It was recently demonstrated by Wagner-Jauregg
et al.[6] that certain metallic ions themselves catalyse the hydrolysis of DFP

Table 1. Effect of metallic ions (10^{-3} M) on the non-enzymic hydrolysis of tabun (expressed in b_{30} values), and molar ion concentration (in pI_{50} values) giving 50 % inhibition of the phosphorylphosphatase activities of purified preparations of human serum and swine kidney. a: activation. Cf. Fig. 1.

Metal ion	No enzyme present	pI_{50}	
		Kidney	Serum
Be^{2+}	8	<2	<2
Mg^{2+}	0	—	—
Ca^{2+}	4	—	—
Sr^{2+}	3.5	a	—
Ba^{2+}	2	a	—
Al^{3+}	4	—	—
Tl^+	5.5	3	<2
La^{3+}	5	—	—
Ti^{3+}	3.5	<2	<2
Mn^{2+}	0	3.33	a
Co^{2+}	17	3.20	a
Ni^{2+}	18	3.26	<3
Pd^{2+}	23 *	3.3	4.3
Cu^{2+}	30	3.18	4.70
Ag^+	21.5	3.43	5.55
Au^{3+}	23	3.28	4.66
Zn^{2+}	13	3.22	<3
Cd^{2+}	6.5	<3	—
Hg^{2+}	4	3.30	4.66
Pb^{2+}	4	<2	<2
UO_2^{2+}	6	<2	—

* $5 \times 10^{-4} M$ Pd^{2+}.

and other organophosphorus compounds. Cu^{2+} is particularly active in such reactions, especially when present as chelates with dipyridyl, histidine, etc. The metallic ions used in the present studies, were also investigated for their effects on the non-enzymic hydrolysis of tabun. Amongst the ions studied the following are the most active catalysts: Cu^{2+}, $Pd^{2+} > Au^{3+} > Ag^+ > Ni^{2+}$ $> Co^{2+} > Zn^{2+}$ (Table 1). It is interesting to note that Mn^{2+} is without any effect on the non-enzymic hydrolysis.

Comparison between the effects of ions on the enzymic hydrolysis of DFP and tabun. In the investigations reported by Mounter et al.[2] the enzymic hydrolysis of DFP by swine kidney was found to be activated by Mn^{2+}. In Fig. 4 the effects of Mn^{2+} on the DFP hydrolysis catalysed by the phosphorylphosphatases of serum and kidney are compared with the results obtained with tabun as substrate. As far as the kidney enzyme is concerned this ion increases the activity for both substrates. The percentage activation of the DFP hydrolysis, however, is much more striking than that of the tabun hydrolysis. For serum phosphorylphosphatase, Mn^{2+} is an inhibitor in both the DFP and tabun reactions. The strongest inhibitor of the cations, Ag^+, studied with tabun as substrate is also a very potent inhibitor of the DFP hydrolysis by the kidney enzyme.
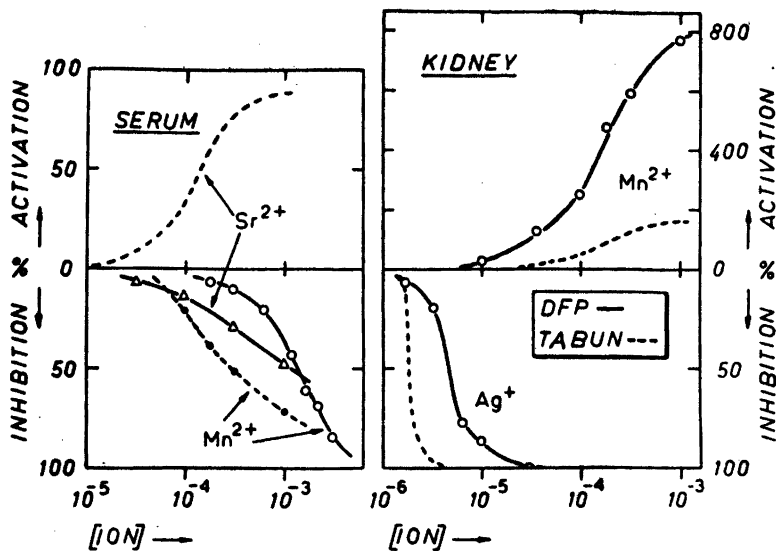
Fig. 4. *Comparison between the effects of ions on the enzymic hydrolysis of tabun and DFP. Data referring to tabun (dotted lines) are taken from Figs. 2 and 3.*

As mentioned above, Sr^{2+} is an activator of the enzymic hydrolysis of tabun by serum phosphorylphosphatase. However, when DFP is used as a substrate for this enzyme, Sr^{2+} acts as an inhibitor. In view of this observation one could surmise that two different enzymes are responsible for the hydrolysis of the two substrates. It was, however, demonstrated in a previous paper by the present authors [1] that DFP and tabun are hydrolysed by the same enzyme of the preparation of human serum used (Fraction IV—1) (see further "Discussion" below). As far as the kidney phosphorylphosphatase is concerned the results reported in the present paper are consistent with the view that DFP and tabun are hydrolysed by the same enzyme.

Activation and inhibition by metallic ions as time reactions. The enzyme activation by a metallic ion cannot be entirely electrostatic (there is no requirement for activation energy in such reactions) if the interaction between the metal and the protein is a time-reaction. Examples of such time-reactions, in which active enzymes are formed, have been described elsewhere [7]. In the present series of studies on the effects of ions on phosphorylphosphatases, the enzymes were incubated with certain metallic ions for various periods of time before the substrate (tabun) was added to the incubation mixture. The enzyme activity values obtained are shown in Fig. 5. It is clear from these results that there is a fundamental difference between the mechanism of the action of metallic ions on the two phosphorylphosphatases. Both the activation (by Mn^{2+}) and the inhibition (by Ag^+) of the kidney enzyme are time-reactions, in sharp contrast to the activation (by Sr^{2+}) and the inhibition (by Mn^{2+}) of the serum enzyme, which are independent of time. It will be noted that the
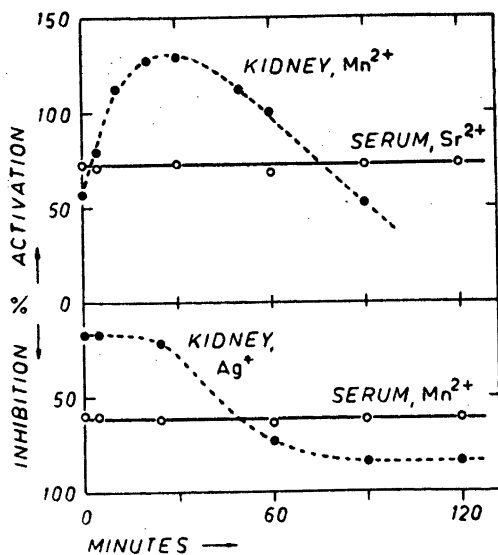
Fig. 5. Activation and inhibition of phosphorylphosphatases incubated for various periods of time with metallic ions. Molar concentrations of the metallic ions during incubation: 1.25×10^{-3} M Mn^{2+}, 1.25×10^{-3} M Sr^{2+}, and 3.75×10^{-3} M Ag^+; N.B. the ion concentration during activity determinations are 1.25 times less than those during incubation.
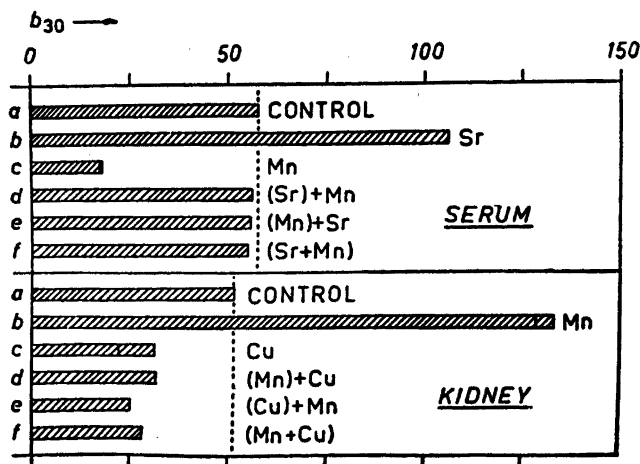

Fig. 6. Competition between activating and inhibiting metallic ions. The enzymes (activities: a) were incubated 30 and 60 minutes with either ion (b, activator; c, inhibitor), 30 minutes with the activator and then a further 30 minutes with the inhibitor (d), 30 minutes with the inhibitor and then a further 30 minutes with the activator (e), and 60 minutes with a mixture of activator and inhibitor.

"zero" values were obtained by adding the substrate a few seconds *before* the addition of the metallic ion.

Competition between activating and inhibiting metallic ions. Activating and inhibiting ions were found to compete with each other for active groups of the phosphorylphosphatase molecules. The results showing this phenomenon are demonstrated in Fig. 6 by a preliminary experiment. The activating effect of an ion is depressed by an inhibiting ion even if the enzyme was incubated with the activator 30 minutes before the inhibitor was added. This competition phenomenon will be studied in more detail before any discussion of its meaning is given.

Table 2. *Effect of dialysis of enzyme preparations incubated with metallic ions. Enzyme activity was determined "before" and "after" dialysis and expressed in b_{30} values.*

			Serum		Kidney	
			Metal	b_{30}	Metal	b_{30}
Control			—	85	—	48
Activator	{	Before	Sr^{2+}	145.5	Mn^{2+}	118
		After		85		32
Control			—	116.5	—	41
Inhibitor	{	Before	Mn^{2+}	52	$Ag+$	4.5
		After		101		5

Effect of dialysis of enzyme preparations incubated with metallic ions. The enzyme preparations were incubated with activating and inhibiting metallic ions respectively and the mixtures dialysed against distilled water. The phosphorylphosphatase activities of the dialysed solutions were then determined and compared with those of the non-dialysed ones. The results obtained are recorded in Table 2. The effect of the activator (Sr^{2+}) as well as of the inhibitor (Mn^{2+}) of the serum enzyme is reversed by dialysis. The inhibition of the kidney enzyme, on the other hand, cannot be reversed by this procedure. The effect of dialysis on the Mn^{2+} activated is most interesting and unexpected. The activity of the activated enzyme is depressed markedly after dialysis and is even lower than the activity of the original non-treated enzyme. In one experiment (not demonstrated in Table 2) the enzyme, activated with Mn^{2+} to about 150 %, had lost after dialysis 67 % of its original activity. Before more experiments have been carried out, no explanation of this phenomenon will be attempted.

DISCUSSION

There are a number of ways in which a metal can be visualized as influencing the activity of an enzyme. Apart from the role of metals in the iron and copper oxidation-reduction enzymes, in which the usual function of the protein is to keep the metal in solution and to stabilize the state of lower valence, there may be a primary effect of the metal on the enzyme protein or co-operative effect

of metal and protein. These mechanisms of the action of metals can be exemplified by several well-known enzyme systems [7-10].

Let us first consider the mode of action of a metallic ion on the enzyme. By combining with an enzyme molecule, the metallic ion gives the complex a net charge which is obviously different from the original charge of the protein. At the same time the affinity of the enzyme for the substrate may be changed. This effect can be entirely electrostatic; there is no requirement of activation energy in such reactions and the effect is independent of the time of interaction between metal and protein. In addition to modifying the charge, the metal can change the configuration of the enzyme protein, e.g., by reacting with —SH groups when these groups are necessary for enzyme activity. It may be pointed out that in these instances of metal-enzyme interaction the change in enzyme activity (it may be activation or inhibition) does not necessarily imply that the metal is involved directly in the formation of an enzyme-substrate complex. In the present study on the effects of metallic ions on the human serum phosphorylphosphatase, the activation by Sr^{2+} (tabun as substrate) and the inhibition by Mn^{2+} (and other ions as well) might be explained by this latter idea. The interaction between metallic ion and enzyme in these cases is independent of time (Fig. 5) and the metal most probably does not act as a mediator (Sr^{2+}) nor does it prevent the formation of an intermediate complex (Mn^{2+}, etc.). In fact, the observation made that the DFP hydrolysis by this enzyme is inhibited instead of being activated by Sr^{2+} makes it likely that the metal does not act as a mediator, but reacts at a site of the enzyme molecule which is responsible for the splitting of the P—CN (in tabun) and P—F linkages in the substrate molecules. The Sr^{2+}-protein complex favours this reaction for tabun but inhibits it for DFP.

For many metal-activated enzyme reactions the mechanism of activation postulated is that the metal acts as a mediator (bridge) between enzyme and substrate. It has been suggested that in this type of metal activation a chelate is formed between the metallic ion and the substrate, this chelate-formation being necessary for the enzyme activity. One of the arguments against this hypothesis is that those metals (e.g., Mn^{2+} and Mg^{2+}) which possess weak chelating activity are commonly occurring activators of hydrolytic enzyme reactions. An alternative role for the metallic ion in such reactions was recently postulated by Klotz and LohMing [11]. According to this idea, the metallic ion favours the formation of the activated enzyme-substrate complex by co-ordinating with both enzyme and substrate and by increasing the local concentration of OH^- thus speeding up the hydrolysis. This idea seems to be applicable to Mn^{2+} and Co^{2+} activation of the hydrolysis of tabun by the kidney phosphorylphosphatase. The substituents [$(CH_3)_2N—$, $C_2H_5O—$, and $CN—$] of tabun, determining the specificity of the enzyme, are assumed to be bound directly to the enzyme (not to the metal in form of a chelate). The inhibiting ions (Cu^{2+}, Au^{3+}, Hg^{2+}, etc.) also form complexes with the enzyme protein, but these complexes prevent the substrate from reacting with the enzyme. In this respect, Ag^+ is particularly active.

Various opinions have been formed to explain the fact that metals have different (activating as well as inhibiting) effects on one particular enzyme activity and also that one metal can activate one enzyme and inhibit another.

As demonstrated in the present paper Mn^{2+} is an example of a metallic ion which can activate one enzyme and inhibit another and the explanation might be that there are two different mechanisms for enzyme activity which are influenced in different ways by the metallic ion. As far as the Mn^{2+} activation of the kidney phosphorylphosphatase and other enzymes is concerned and taking into account the fact that Mg^{2+} is without any effect on the enzymes studied by us, in contrast to the similar effect of Mg^{2+} and Mn^{2+} on other enzym systems, it may be useful to compare the electron configuration of Mn with that of Mg. Magnesium has a filled outer shell (3 s) and manganese a half-filled 3 d shell (5 electrons instead of 10). Manganese is the only divalent ion with this electron structure which may explain the different effects of Mn^{2+} and Mg^{2+}. It may also explain the instances when Mn^{2+} behaves like Co^{2+} which has a partly filled 3d shell (7 electrons).

Such an explanation does not seem to be valid for the effect of metallic ions on the serum phosphorylphosphatase. This is particularly true for the Sr^{2+} activation of this enzyme which to the authors' knowledge is the only known enzyme activation by this ion. Whatever the explanations of the effects studied may be, it is obvious that the activating effect of Sr^{2+} on the serum phosphorylphosphatase depends on a different mechanism to that for the Mn^{2+} activation of the kidney enzyme. From this we can conclude that the mechanisms of enzyme action of the serum phosphorylphosphatase and the kidney phosphorylphosphatase differ fundamentally.

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