The effect of zinc on 5-nucleotidase of cobra venom and the interference of other nucleotides

Cobra venom contains much zinc (see[1]). The dried venom used in the present work is that described in an earlier publication[2] and contains 2.2 mg/g Zn. This corresponds to $1.2 \cdot 10^{-4}$ *M* Zn in the natural venom (assuming a sp. gr. of 1.058 and that the venom dries to one third of its fresh weight[3]). The experiments to be reported were carried out in 0.02 *M* barbitone buffer (pH 8.4) at 30° with 0.1% dried cobra venom (giving about $4.6 \cdot 10^{-5}$ *M* Zn) using as substrate adenosine-5'-phosphate (AMP) (about $7 \cdot 10^{-3}$ *M*).

Partial removal of the naturally occurring Zn from the system effected a twofold increase in the 5-nucleotidase activity and more complete removal with excess ethylenediaminetetraacetic acid followed by addition of Mg, increased the activity almost by four. Addition of Zn to this mixture caused inhibition again. It was found that Zn and AMP mutually precipitate as a gelatinous mass and titration curves suggest that a compound is formed between equal moles of Zn and AMP. However, since in the above incubations the AMP concentration is 140 times that of Zn, it seems unlikely that the Zn exerts its influence by precipitating the substrate. Addition to the crude venom of large amounts of Zn up to $2.1 \cdot 10^{-2}$ *M* does cause further inhibition of the enzyme (probably by removal of AMP) but smaller additions, up to 10^{-3} *M*, have no effect. It is likely, therefore, that the naturally occurring Zn inhibits the 5-nucleotidase activity by combining with the enzyme rather than with the substrate. The venom contains Mg (0.14 mg/g dried venom)[3] and the enzyme seems to require this, or Mn, for its activity[4]. The similarity between Mg and Zn both in atomic radius and ionic charge, and the greater tendency of Zn to form complexes support the hypothesis that Zn inhibits the enzymic activity by replacing Mg on the enzyme. EPPERSON[5] also found Zn to inhibit AMP breakdown but he used 4 m*M* Zn and does not report the substrate concentration. EPPERSON's more recent publication[6] has not been available to the author.

During preliminary experiments to investigate possible inhibition of 5-nucleotidase by other nucleotides an enhancement of the enzyme by ATP and diphosphopyridine nucleotide (DPN) was discovered. Several other nucleotides and purine derivatives behaved similarly (Table I col. 2). Only ADP had any significant inhibitory effect. Inorganic phosphate and pyrophosphate have insufficient influence to account for these observations.

TABLE I

EFFECT OF SOME SUBSTANCES UPON 5-NUCLEOTIDASE ACTIVITY OF COBRA VENOM

Substance added with equimolar AMP	*Initial activity (arbitrary units) of 5-nucleotidase in:*	
	Crude venom ($4.6 \cdot 10^{-5}$ M Zn)	*Dialysed venom* (minus Zn)*
None	100	382 166 (dialysed only)
ATP** (Na salt)	165	
ATP** (Ba salt regenerated)	125	267 (30%)
ADP	60 (40%)	198 (48%)
ITP	118	
Inosine	110	
Hypoxanthine	118	
Adenine	100 (0%)	
Adenosine	100 (0%)	
Ribose	91 (9%)	
Ribose-5-phosphate	91 (9%)	
Thiaminpyrophosphate	180	
DPN	210	382 (0%)
NMN	140	382 (0%)
FMN	124	

(Figures in brackets are % inhibition with respect to first figure of column)

* Venom dialysed against barbitone buffer with subsequent addition of ethylenediaminetetraacetic acid and Mg ion.

** ATP only one half molar concentration of AMP.

The only simple explanation seems to be that the added substances remove an inhibitor from the scene of activity. If such were the case these substances should effect no enhancement of the enzyme in the absence of the inhibitor. After removal of Zn from the venom this is the case. After dialysis of the venom overnight against barbitone buffer, with subsequent addition of ethylenediaminetetraacetic acid to remove remaining metals, followed by Mg addition, no enhancement was obtained with those nucleotides tried. (Table I, col. 3). Inhibition occurred with ATP and ADP (probably competitively). If competitive inhibition is superimposed upon the actual enhancement then the recorded enhancement for ATP in Table I, col. 2 will be low. In the presence of extra Zn ($4 \cdot 10^{-3}$ *M*), ATP ($3.5 \cdot 10^{-3}$ *M*) inhibited the activity 60%. Under these conditions it is possible that the effective concentration of AMP was reduced and that ATP was able thereby to compete more strongly for the enzyme. (The optimum concentration of AMP in this system is about $3 \cdot 10^{-3}$ *M*.) However this supports the idea that the nucleotides enhance by combining with Zn, and when they are previously "saturated" with the metal there is no enhancement. There was no competitive inhibition by DPN or nicotinamidemononucleotide (NMN) confirming the similar result of HEPPEL AND HILMOE[7].

As there is a great excess of AMP capable of precipitating the Zn, simple salt formation and precipitation of the metal by the added substances cannot explain these results. The formation of a less dissociated compound of Zn, such as a complex or chelate, must be considered. Furthermore, ADP has no enhancing effect on the enzyme system: it inhibits to a similar extent irrespective of the presence of Zn, so that an explanation of the effect of ATP must differentiate it from ADP.

Titration curves confirm that both ATP and ADP form complexes with Zn. Fig. 1 shows a paper ionophoretic strip of some of the nucleotides with and without Zn (0.5 mole). The extent of trail of Zn towards the positive electrode presumably gives a measure of the strength of a Zn-nucleotide complex. The more such a complex is dissociated the sooner will it break down as the products move away from one another. There is some Zn-ATP complex left after 1.5 h but the Zn-ADP compound is broken down sooner, suggesting a weaker binding of Zn. The Zn-AMP precipitate is slowly dissolved as the products move in opposite directions and disturb the equilibrium.

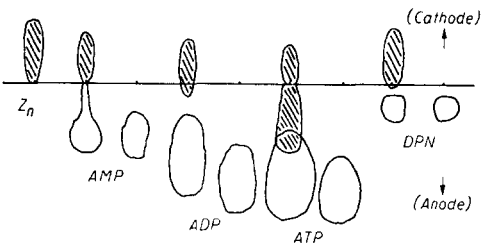

Fig. 1. Paper ionophoresis of adenine nucleotides with and without approximately half mole of zinc. Tris buffer pH 8.2. 1.5 h at 25° C. 10 V/cm. 0.2 mA/cm width. Nucleotides (open areas) detected by ultra-violet light. Zinc (shaded areas) detected by dithizone-in-chloroform spray.

It is supposed by CALVIN[8] and MELCHIOR[9] that in certain enzymic phosphate transferring reactions involving ATP, ADP, DPN, coenzyme A, *etc.*, the metal ion required, usually Mg or Mn, is joined to two phosphate oxygens forming a six-membered ring, the metal then being able to chelate with suitable groups on the enzyme. In the absence of protein such metal compounds may be less stable and more dissociated. Studies by MONK[10] on metal complexes with the inorganic polyphosphates have shown that a slight increase in stability is obtained with increase in phosphate chain length. Similarly, one would expect a greater stability for Zn-ATP than for Zn-ADP. If the stability of the 5-nucleotidase-Zn complex happens to fall between these two, then the enhancement of the crude venom activity by ATP but not by ADP is explained. However, it can be shown with atomic models that by folding ATP about the ribose moiety a tetrahedral arrangement (preferred by co-ordinated Zn) can be provided to co-ordinate a Zn atom by means of the amino-N, the N at position 7 and the β- and γ-phosphate oxygens. This would presumably afford an extra stability to the complex which is not possible with ADP owing to the shorter chain length. Recently RAAFLAUB[11,12] has suggested that metalcomplex formation may be a function of ATP and the results of NEUBERG's studies[13] on the solubilising properties of ATP may be explained similarly.

ALBERT[14] has shown that inosine, hypoxanthine and riboflavine have an avidity for metals, chelating them through a tertiary heterocyclic N and an acidic OH in the peri position. Likewise the former may remove Zn ion from the systems under consideration. Inosinetriphosphate (ITP) and flavinemononucleotide (FMN) may do so by a similar mechanism.

The titration curves for DPN with and without Zn do not differ markedly until pH 6 is reached, when there is evidence of ionisation of the DPN molecule in the presence of Zn. The groups responsible for this are not known but cannot be the phosphate hydrogens as these are ionised at this pH (*cf.*[15,16]). Above about pH 8 the Zn-DPN curve approximately coincides with the sum of the DPN and Zn curves separately. From Fig. 1 it would seem that DPN does not form a complex with Zn at pH 8 or above, at least in the absence of protein. It may be that

protein present in the cobra venom incubations stabilises a Zn-DPN complex in the manner described by CALVIN[8]. However, it is of interest that the DPN molecule can theoretically accommodate a tetrahedrally-co-ordinated Zn atom using two phosphate oxygens, adenine amino-N and nicotinamide amide-N.

Evidence is now available for the functional role of Zn in DPN-alcohol dehydrogenase systems[17,18,19]. Other instances have been reported of the effect of Zn and other metals on DPN and dehydrogenases[20,21] and upon general phosphate metabolism[22].

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Bacterial protoplasts: growth and division of protoplasts of *Bacillus megaterium*

The sub-cellular organelles produced by digesting away the cell walls and cross septa of *Bacillus megaterium* are capable of synthesising protein, nucleic acids and adaptive enzymes (MCQUILLEN[1,2]), of supporting the growth of bacteriophages (SALTON AND MCQUILLEN[3]; BRENNER AND STENT[4]) and of allowing the development of endospores (SALTON[5]). The digestion with lysozyme, if carried out in suitable media of high osmotic pressure, converts the rod-shaped bacilli into from one to four spherical protoplasts (WEIBULL[6,7]; MCQUILLEN[8]). These have now been shown to be able to increase in size and dry weight, and to divide.

Strain KM of *B. megaterium* was grown in a glucose/NH_3/salts medium; the cells were harvested, washed and converted to protoplasts as described earlier (MCQUILLEN[1]) except that in some experiments phosphate buffer (0.5 *M*, pH 7.0) was used as stabiliser in place of sucrose (7.5% w/v). Samples of intact cells and protoplasts were centrifuged, rinsed twice with the stabilising medium and resuspended in the same medium to which had been added glucose (0.5% w/v) and either Bacto peptone (0.1% w/v) or a mixture of 18 amino acids each at a final concentration of 50 μg/ml (MCQUILLEN AND SALTON[9]). 250 ml lots of these suspensions (*ca.* 100 μg dry weight per ml) were incubated at 28° C in rocked Roux bottles fitted with an air vent through a rubber bung. At intervals, 50 ml samples were removed, treated with formaldehyde, centrifuged, washed twice with distilled water and dried to constant weight. Table I records some typical results.

Both the protoplasts and the intact cells more than doubled in dry weight during the first two hours of incubation but whereas the latter continued to grow exponentially, the protoplasts increased more or less linearly. Whether this is significant of a difference in behaviour or whether it is due to lysis of some of the very fragile protoplasts, is not known but similar results have been obtained on many occasions. During the course of such an experiment which was prolonged,