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Zinc as a Cofactor for Cephalosporinase from *Bacillus cereus* 569

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Crude preparations of β-lactamase from *Bacillus cereus* 569 show a selective loss of cephalosporinase activity on purification (Abraham & Newton, 1956) and during prolonged action on substrate (Sabath & Abraham, 1965). They also show a much greater loss of penicillinase (EC 3.5.2.6) activity than cephalosporinase activity on keeping at 60° (Crompton, Jago, Crawford, Newton & Abraham, 1962). During attempts to purify the crude preparation cephalosporinase activity was repeatedly lost although penicillinase activity was retained. It was therefore decided to investigate the possibility that a cofactor required only for cephalosporinase activity was removed during the purification procedures used.

Solutions of the crude enzyme from *B. cereus* 569 were prepared as described by Crompton *et al.* (1962) with cephalosporin C_A (pyridine) (10 μg./ml.) as inducer and an induction time of 3hr. β-Lactamase activities were measured manometrically (Henry & Housewright, 1947; Pollock, 1952) and related to μl. of CO_2/ml. of enzyme/hr., normally with substrate (cephalosporin C or benzylpenicillin) at a concentration of 2mg./ml.

Table 1 shows that some loss of cephalosporinase activity occurred when the crude enzyme was dialysed against running tap water, but that when EDTA (disodium salt) was added before dialysis (final concn. 0·27mM) the selective loss of cephalosporinase was much more striking. A similar extensive loss of cephalosporinase activity was observed when measurements were made with enzyme that had been treated with EDTA (at concentrations down to 2·7 μM) but not dialysed.

Cephalosporinase activity was restored to solutions of crude enzyme that had been subjected to treatment with EDTA and dialysis by the addition of $ZnSO_4$. When the latter was added in optimum amount (final concn. 1mM) the cephalosporinase activity rose to 4–5 times that of the original crude enzyme (Table 1). Addition of Zn^{2+} to the original crude enzyme, or to the dialysed enzyme, also produced a similar increase in cephalosporinase activity.

Several other metal-binding substances decreased the cephalosporinase activity of the crude enzyme to a relatively low level. After the addition of 1,10-phenanthroline (300 μM) the cephalosporinase activity was less than 10% of its original value and the penicillinase activity 95%. With 240 μM-quinalizarin (tetrahydroxyanthraquinone) the corresponding value for cephalosporinase activity was also less than 10%. After H_2S had been bubbled gently through the solution for about 7min. the values for cephalosporinase and penicillinase activities were less than 10% and 90% respectively. No significant loss of cephalosporinase activity was observed after treatment of the crude enzyme with ammonium aurin tricarboxylate (140 μM), titan yellow (160 μM), sodium azide (10mM) or 8-hydroxyquinoline (1mM). The use of 8-hydroxyquinoline (1mM) to terminate enzyme induction (Crompton *et al.* 1962) did not significantly affect the cephalosporinase activity of the crude enzyme obtained.

The logarithms of the equilibrium formation

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Table 1. *Effect of EDTA and* Zn^{2+} *on the cephalosporinase and penicillinase activities of crude β-lactamase from* B. cereus 569

The cephalosporinase activity of the original crude enzyme (μl. of CO_2/ml.of enzyme/hr.) was about 4% of the penicillinase activity. Dialysis or measurement of activity was begun between 3 and 15 min. after the addition of EDTA. — Signifies that the treatment referred to was omitted.

Treatment of crude enzyme			Activity (% of that of original crude enzyme)	
(1) EDTA (0·27 mM) added	(2) Dialysis (15–18 hr.) after (1)	(3) Final concn. of added $ZnSO_4$ (mM) after (1) and/or (2)	Cephalosporinase	Penicillinase
—	—	—	100	100
—	+	—	75	100
—	+	1·0	530	135†
+	—	—	9	89
+	+	—	< 12*	93
+	+	10·0	191	—
+	+	1·0	443	120†
+	+	0·1	151	—
—	—	1·0	525	150†
—	—	0·01	157	—

* A series of four experiments gave three values of < 8% and one of 11%; but in two other experiments the values were 25 and 30% respectively.

† Some, at least, of the apparent increase in activity in these cases may be attributed to the fact that benzylpenicillin was hydrolysed at a significant rate in the absence of enzyme when Zn^{2+} was present.

constants, K, for 1:1 chelates of EDTA and 1,10-phenanthroline with zinc are 16·1 and 6·43 respectively (Martell & Calvin, 1952). It is therefore not surprising that the former ligand was effective in lower concentrations than the latter in removing cephalosporinase activity. However, the failure of 8-hydroxyquinoline ($\log K$ 10·91) to act as an inhibitor suggests that the effect of a ligand also depends on its ability to approach a zinc-binding site in the enzyme.

In concentrations from 100 to 1 mM a variety of metal salts other than $ZnSO_4$ did not restore more than a small fraction of the cephalosporinase activity of crude enzyme that had been subjected to treatment with EDTA and dialysis. The metal ions used were Ca^{2+}, Ba^{2+} and Cd^{2+} (added as their chlorides), Mg^{2+} and Mn^{2+} (added as their sulphates) and Co^{2+} (added as its acetate). The addition of $CuSO_4$ or $FeSO_4$,$(NH_4)_2SO_4$ (1 to 0·01 mM) to the original crude enzyme produced no significant change in cephalosporinase activity. At higher concentrations the effect of these metal ions was inhibitory.

Cephalosporin C acted as a competitive inhibitor of the penicillinase activity of the crude enzyme preparation from *B. cereus* 569 and also of the preparation whose cephalosporinase activity had been largely removed by treatment with EDTA and dialysis. Values of K_i/K_m with benzylpenicillin as substrate and cephalosporin C as inhibitor were obtained from data plotted in the manner of Hunter & Downs (1945). The value of K_i/K_m, i.e. 4, with the former preparation did not differ significantly from that with the latter.

The present results suggest that without zinc as a cofactor the crude enzyme from *B. cereus* 569 shows little, if any, of its potential cephalosporinase activity. In contrast, it has been concluded that there is no indication of a cofactor requirement for the penicillinase from this organism (Pollock, 1960). Whether activation by zinc is peculiar to cephalosporinase from *B. cereus* is uncertain, but it does not appear to be a general characteristic of cephalosporinases, since the crude enzyme from *Pseudomonas pyocyanea* (Sabath, Jago & Abraham, 1965) was not inhibited by EDTA or by H_2S. Moreover, changes other than, or additional to, loss of zinc may result in a selective loss of cephalosporinase activity of crude enzyme from *B. cereus*. A preliminary attempt to increase the relatively minute amount of cephalosporinase activity in a highly purified preparation of penicillinase from *B. cereus* 569/H (kindly provided by Professor M. R. Pollock) by the addition of Zn^{2+} (100 to 0·01 mM) was unsuccessful.

The finding that cephalosporin C was a competitive inhibitor of penicillinase in the crude enzyme from *B. cereus* 569 both before and after the cephalosporinase activity of the latter had been lost on treatment with EDTA, as well as an inhibitor of

a purified penicillinase from *B. cereus* 569 that had retained very little cephalosporinase activity (Abraham & Newton, 1956), indicates that cephalosporin C can form a complex with a β-lactamase from this organism in the absence of Zn^{2+}, although Zn^{2+} is necessary for enzymic hydrolysis of cephalosporin C to occur at a significant rate. This situation could be analogous to that with carboxypeptidase, whose zinc-free apoenzyme can form complexes with certain substrates but is unable to catalyse their hydrolysis (Coleman & Vallee, 1962).

Whether Zn^{2+} is incorporated into the active centre of the cephalosporinase or whether a change in conformation at the active centre results from a reaction with Zn^{2+} at another part of the molecule is not revealed by the present data. The question arises whether the cephalosporinase and penicillinase activities are associated with a single protein whose conformation is changed by complex-formation with zinc. Purification of the crude enzyme from *B. cereus* 569 under conditions such that its cephalosporinase activity is retained may be necessary before this question can receive a satisfactory answer.

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Studies on the Biosynthesis of the Corrin Ring of Vitamin B_{12}

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The similarity in structure between the corrin ring of vitamin B_{12} and the porphyrin nucleus of haem has stimulated numerous workers to examine the similarities in the biosynthetic pathways (Bukin & Pronyakova, 1960; Shaposhnikov & Finogenova, 1963; Bardi *et al.* 1958). In the present communication we discuss the identity of the next stable intermediate after porphobilinogen. Our evidence suggests that uroporphyrinogen is an intermediate, and that porphobilinogen does not form a corrole ring directly. Previous evidence for the contrary may have resulted from the choice of experimental material by past workers.

When choosing a micro-organism for the investigation of the biosynthesis of something that is formed in minute amounts, as with vitamin B_{12}, one naturally favours organisms that have an extraordinary ability to synthesize the desired compound. Thus propionibacteria, nocardia and actinomycetes have gained popularity as experimental material. However, these micro-organisms have a drawback for studies involving the question of the nature of the first tetrapyrrolic vitamin B_{12} intermediate, namely they all have the capacity to make haem enzymes. The question is raised whether a porphyrin formed by such organisms is a primordial haem molecule, or a primordial cobamide coenzyme.

Structural considerations make it apparent that no porphyrin occurring in the biosynthetic sequence after uroporphyrinogen can be considered as possible intermediates of vitamin B_{12}. The previous observation that vitamin B_{12}-producing organisms also produce coproporphyrin III tells us little beyond the fact that the organism has the capacity