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Thiol-Group Binding of Zinc to a β-Lactamase of *Bacillus cereus*: Differential Effects on Enzyme Activity with Penicillin and Cephalosporins as Substrates

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Zinc, which is required for the hydrolysis of cephalosporins by a crude enzyme from *Bacillus cereus* 569, also increased the stability of this activity during storage. A loss in activity of the zinc-activated enzyme which occurred on prolonged hydrolysis of cephalosporin C was not restored by further addition of zinc. The thiol reagents *N*-ethyl maleimide (NEM), iodoacetic acid (IAA), $CdCl_2$, and *p*-chloromercuribenzoate, all at 10^{-3} M, and iodine at 1.6×10^{-3} N prevent zinc activation of the "cephalosporinase" activity. However, NEM and IAA have minimal or no demonstrable inhibitory effect if the enzyme is first treated with zinc. This suggests that zinc is linked to the apoenzyme by a thiol group. Activation by zinc is only partially prevented by NEM if the crude enzyme is pretreated with nickel, which alone causes negligible activation of the apoenzyme. The order of affinities of these metals for the apparent thiol group is thus Hg^{++}, $Cd^{++} > Zn^{++} > Ni^{++}$. The "cephalosporinase" inhibition by Hg^{++} was reversible with dithiothreitol. These metals and thiol reagents do not decrease the ability of the crude enzyme to hydrolyze benzylpenicillin, which is consistent with the report that purified "penicillinase" from *B. cereus* contains no cysteine residue. This suggests that the β-lactamases of *B. cereus* that hydrolyze penicillin and cephalosporins differ from each other by at least one amino acid (cysteine).

Zinc has been shown to be a cofactor required for the demonstration of the "cephalosporinase" activity (hydrolysis of the β-lactam ring of cephalosporin C) of a crude enzyme from *Bacillus cereus* 569 (14), whereas no cofactor requirement has been found for its "penicillinase" activity (hydrolysis of benzylpenicillin; 12). In addition to the zinc content of the assay menstruum, some procedures that selectively reduce either "penicillinase" or "cephalosporinase" activity are: purification (1), heat (4), reaction with antibody (13), repeated hydrolysis of cephalosporin C (15), and reaction with certain metal-binding substances (14).

In relation to these findings, two possibilities have been considered (15). (i) *B. cereus* produces two different β-lactamases, one primarily a "penicillinase" (EC 3.5.2.6.) and the other primarily a "cephalosporinase," each with a different primary structure (14). (ii) Only one primary protein structure with β-lactamase activity is synthesized, but various physicochemical situations alter its conformation and thus the relative rates of hydrolysis of the two substrates, benzylpenicillin and cephalosporin C (14).

This report describes studies of some factors that selectively affect zinc activation of the "cephalosporinase" protein.

MATERIALS AND METHODS

Enzyme induction. Stationary overnight cultures of *Bacillus cereus* 569, grown at 37 C in Brain Heart Infusion (BHI; Difco) broth, were used as inocula (10%, v/v) in BHI broth, which was then incubated for 3 hr or less at 35 C in a Dubnoff metabolic shaker in lots of 10 ml (total fluid) in 50-ml Erlenmeyer flasks, 100 oscillations per min. The inducer, cephalothin, was then added to each flask in a volume of 0.1 ml to give a final concentration of 20 μg/ml; induction was allowed to proceed for 3 hr. A 1-ml amount of 8-hydroxyquinoline (0.01 M) was then added to each flask of induced culture, and, after mixing, the cells were removed by centrifugation at $3,000 \times g$ for

10 min at 6 ± 3 C. The supernatant fraction of this induced culture is referred to as "crude enzyme."

Preliminary enzyme purification. Partial purification was performed at 4 ± 2 C by chromatography on columns of Sephadex G-25 or G-75. After packing the column (30 g of G-25 or 10 g of G-75 powder per 40 × 2 cm column), the system was saturated with 0.067 M sodium-potassium phosphate buffer containing 3×10^{-5} M zinc; this solution was also used for elution. Crude enzyme (10 to 20 ml) containing 10^{-3} M zinc was added to the column. The flow rates were 25 to 30 ml/hr; the effluent was collected in 5- to 6-ml portions.

Adsorption of enzyme on cellulose phosphate powder and subsequent elution. This procedure was carried out as previously described (15), with three exceptions. (i) The elution was performed either with a series of 10-ml portions of buffers in 10% increments up to 0.2 M phosphate (*p*H 7.6), or with 0.2 M phosphate (*p*H 7.6) immediately, rather than with a continuous elution gradient of *p*H and ionic strength. (ii) The ion exchange powder was Whatman P11. (iii) Zinc sulfate was added to the entire system before beginning the purification. The zinc content of the crude enzyme was raised to about 10^{-3} M, and that of the buffers for washing the powder and eluting the protein was raised to 3×10^{-5} M.

Assays of protein and enzyme. Protein content was determined by the method of Lowry et al. (8). "Penicillinase" activity (benzylpenicillin, 5.3 mM, as substrate) was determined quantitatively by the manometric method of Henry and Housewright (5) as described by Pollock (11), but was estimated during purification by the spot test of Perret (10). Cephalosporinase activities were estimated manometrically (as for "penicillinase"), in some instances potentiometrically (20), and spectrophotometrically (16), by using a Beckman DU monochrometer with an automatic cuvette changer and recorder model 2000 (Gilford Instrument Laboratories, Inc., Oberlin, Ohio). (Cephalosporin C or cephalothin was used as substrate; their rates of hydrolysis by zinc-activated *B. cereus* 569 β-lactamase were essentially the same. For manometric assay, the concentration was 4.2 mM, and additional zinc was added to the reaction mixture to make a final concentration of 10^{-3} M, unless otherwise stated.)

Inducers, substrates, and reagents. The cephalothin used as inducer and substrate and the cephalosporin C used as substrate were provided by Eli Lilly & Co., Indianapolis, Ind. The benzylpenicillin potassium salt (Squibb) used as substrate, and the other chemicals, were all of reagent grade and were obtained through commercial sources.

Reaction with metals and thiol reagents, dialysis, and storage. *p*-Chloromercuribenzoate (*p*CMB) was dissolved in 0.9% NaCl and 0.1 N NaOH; when it was added to enzyme preparations, the final *p*H was adjusted to equal the control (near *p*H 7.0). Crude enzyme was incubated in some instances with metals or other thiol reagents [for iodoacetic acid (IAA), the *p*H was adjusted to 7.3 ± 0.1 before incubation] at indicated concentrations for 30 to 180 min either at room temperature (24 ± 2 C) or at 37 ± 0.5 C. The mixture was then dialyzed for 16 to 18 hr against distilled water at 4 ± 2 C in Visking dialysis tubing (8-mm diameter) to remove unbound reagent. Usually, 10 to 30 ml of an enzyme preparation was inside the tubing and 1 to 3 liters of precooled distilled water was in the outer bath, which was changed three times over the dialysis period. After dialysis, the preparations were transferred to glass or polyethylene containers and stored at 4 ± 2 C until assayed (usually the same day), unless a second dialysis followed. In other experiments, enzyme was dialyzed before incubation with thiol reagents. Materials used to study the effect of zinc on stability were not dialyzed; storage was at 4 ± 2 C. The 1.6×10^{-3} N iodine was prepared in 6×10^{-3} M aqueous KI, and this was diluted as required.

Repeated hydrolysis of cephalosporin C. Enzyme inactivation by repeated hydrolysis of cephalosporin C was performed in a Radiometer *p*H-stat as previously described (15).

Handling of glassware. Warburg reaction vessels were boiled in Haemo-sol (Meinecke & Co., Inc., Baltimore, Md.), rinsed in distilled water, and then in 2 N nitric acid to remove adsorbed zinc (9) before a final distilled water rinse.

RESULTS

Effect of zinc on stability of enzyme storage, repeated hydrolysis, and purification. Crude enzyme stored at 4 ± 2 C in the presence of added $ZnSO_4$ (final concentration, 10^{-3} M) for 7 weeks had about 70% of original cephalosporinase activity, whereas enzyme from the same batch stored without additional zinc retained only 20% of the original activity. In both instances, there was little loss of activity after 2 weeks of storage (Fig. 1). Assays were performed manometrically with additional zinc (1 μmole/ml in reaction vessel) added for all "cephalosporinase" determinations. The "penicillinase" activity of these fluids showed no loss of activity during the 7 weeks, irrespective of zinc content during storage (Fig. 1). "Cephalosporinase," but not "penicillinase," activity of crude enzyme (2 ml) was lost after three successive additions of cephalosporin C (5.4 mg each) at 24 ± 2 C, during which hydrolysis was followed in a *p*H-stat. The activity with cephalosporin C as substrate was restored to about its original value by addition of zinc (1 μmole/ml of enzyme), but it did not reach the higher value obtained when zinc was added before hydrolysis. Addition of 1 μmole of zinc sulfate per ml of enzyme before addition of substrate resulted in an initial reaction velocity which was about 10 times the original rate, but, once activity was lost on continued hydrolysis, additional zinc (1 μmole/ml) failed to restore any detectable "cephalosporinase" activity (Table 1).

More "cephalosporinase" than "penicillinase"

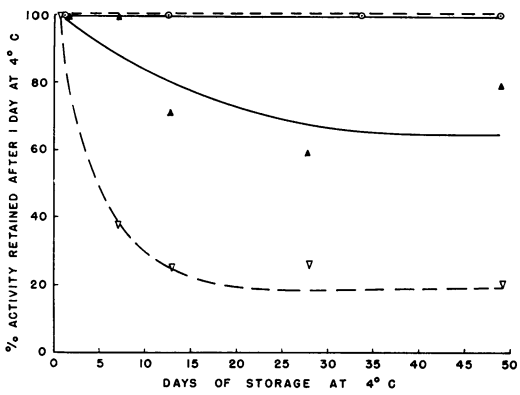

FIG. 1. *Effect of zinc on stability of "cephalosporinase" and "penicillinase" of Bacillus cereus 569. "Cephalosporinase" was measured in the presence of Zn^{++} (10^{-3} M). Symbols: •, activity of "penicillinase," stored without added Zn^{++}; ○, "penicillinase" activity, stored after addition of Zn^{++} (10^{-3} M); ▽, "cephalosporinase" activity, stored without Zn^{++}; ▲, "cephalosporinase" activity, stored after Zn^{++} (10^{-3} M) added.*

TABLE 1. *Effect of zinc on cephalosporinase activity*

State of enzyme	Cephalosporinase activity (μliters of CO_2 per ml per hr ± SD)	
	Before addition of Zn^{++}	After addition of Zn^{++} (1 μmole/ml)
Stored for 2 days without Zn^{++}	21 ± 3[a]	132 ± 0
Same, inactivated by repeated hydrolysis of cephalosporin C	2.5 ± 0.5[a]	22.5 ± 1.5
Stored for 3 days after addition of $ZnSO_4$ (10^{-3} M)	217 ± 7	303 ± 3
Same, inactivated by repeated hydrolysis of cephalosporin C	10 ± 10	11 ± 11

[a] "Penicillinase" activity not appreciably decreased after repeated hydrolysis of cephalosporin C.

activity was recovered in most cases after procedures involving dialysis or chromatography on Sephadex G-25 and G-75 (Table 2), whereas, after elution from cellulose phosphate, more "penicillinase" than "cephalosporinase" was recovered. No significant separation of "penicillinase" and "cephalosporinase" activities could be detected in these systems.

TABLE 2. *Recovery of β-lactamase after partial purification*[a]

Procedure	β-Lactamase activity recovered		Maximal change in specific activity[b]	
	P-ase	C-ase	P-ase	C-ase
	%	%	%	%
Chromatography Sephadex G-25	72	107	6.2	11.7
Sephadex G-25	58	123	5.4	12.8
Sephadex G-25	65	123	6.9	10.3
Sephadex G-25	95	126	8.1	9.0
Sephadex G-25	123	123	13.2	12.0
Chromatography Sephadex G-75	31.0	63.0	2.6	6.0
Cellulose phosphate P11 (adsorption and elution)	>56[c]	32	54.3	26.8
Dialysis	64–73	554–800	8.2	58.9
Dialysis followed by cellulose phosphate P11 (total effect)	>34[c]	101	39.7	80.5

[a] No clear separation of "penicillinase" (P-ase) and "cephalosporinase" (C-ase) activity was obtained in any procedure.

[b] The numbers represent the ratios: actvity per milligram of protein of fraction with highest specific activity/activity per milligram of protein of starting material.

[c] Last fraction still contained "penicillinase." It is assumed that further elution would have increased the percentage recovered.

Effect of thiol reagents on β-lactamase activity. Maximal "cephalosporinase" activity of crude enzyme samples was obtained by conducting the manometric assay in the presence of 10^{-3}M zinc sulfate. This maximal activity was usually 5 to 6, but occasionally 10, times that of crude enzyme to which no zinc had been added. When crude enzyme was treated with iodine (1.6×10^{-3} N), IAA (10^{-2} and 10^{-3} M), *N*-ethyl maleimide (NEM; 10^{-2} and 10^{-3} M), $CdCl_2$ (10^{-3} M), $HgCl_2$ (10^{-2} and 10^{-3} M), or *p*CMB (10^{-2} M) before zinc activation, its activity with cephalosporins as substrate was only a fraction (<1 to 35%) of that of the untreated but zinc-activated control, whereas pretreatment with nickel sulfate (10^{-3} M) had no significant effect on "cephalosporinase" activity measured after zinc activation (Table 3). The inhibitory effect of $HgCl_2$ could be reversed by incubating with dithiolthreitol (10^{-3} M) and then dialyzing.

In contrast, when the crude enzyme was first exposed to zinc sulfate (10^{-3} M) and then (after dialysis to remove unbound zinc) to some of

TABLE 3. *Effect of thiol reagents on β-lactamase activity of Bacillus cereus enzyme before and after treatment with $ZnSO_4$, 10^{-3} M*[a]

Reagent	Concn	Exposure		Enzyme activity (as percentage of control ± SD)		
				"Cephalosporinase"		
		Time	Temp	Exposure to thiol reagent before zinc	Exposure to zinc before thiol reagent	"Penicillinase" (no zinc added)
		min	*C*			
None (water control)		30–60	24 or 37	100 ± 5	100 ± 4	100 ± 3
Iodoacetic acid[b]	10^{-2} M	60	37	5 ± 1	86 ± 3	84 ± 2
	10^{-3} M	60	37	11 ± 3	85 ± 1	81 ± 4
	10^{-4} M	60	37	55 ± 13	85 ± 3	79 ± 2
N-ethyl-maleimide[b]	10^{-2} M	70	24	23 ± 2	100 ± 0	95 ± 0
	10^{-3} M	70	24	25 ± 1		
	10^{-4} M	70	24	52 ± 1		
	10^{-5} M	70	24	73 ± 4		
Iodine[b]	1.6×10^{-3} N	30	24	25 ± 5		98 ± 2
	1.6×10^{-4} N	30	24	58 ± 0		93 ± 14
	1.6×10^{-5} N	30	24	76 ± 0		
	1.6×10^{-6} N	30	24	98 ± 2		
$HgCl_2$	10^{-2} M	150	37	<1[c]	1.4 ± 0	230 ± 11
	10^{-3} M	150	37	11 ± 0[c]	10.6 ± 0	193 ± 10
	10^{-4} M	150	37	54 ± 0		109 ± 5
	10^{-5} M	150	37	86 ± 3		100 ± 0
p-Chloromercuribenzoate	10^{-2} M	60	37	2 ± 2		249 ± 13
	10^{-3} M	60	37	57 ± 5		125 ± 3
	10^{-4} M	60	37	97 ± 1		
	10^{-5} M	60	37	99 ± 5		
	10^{-6} M	60	37	101 ± 1		
$CdCl_2$	10^{-3} M	30	24	35 ± 1	22 ± 1	94 ± 2
$NiSO_4$	10^{-3} M	30	24	106 ± 1		

[a] Each enzyme preparation was dialyzed, then exposed to the indicated reagents for 30 to 150 min at 24 ± 2 C or 37 ± 0.5 C, and again dialyzed before zinc activation. In some instances (next to last column), crude enzyme was exposed to $ZnSO_4$ (10^{-3} M) at 24 ± 2 C for 30 to 45 min before dialysis. Zinc was not added to the assays of penicillinase. All assays were done by manometric technique; results are expressed as percentage ± SD of the control sample in water rather than in any of the listed reagents.

[b] Enzyme not dialyzed before exposure to thiol reagent.

[c] Inhibition resulting from treatment with $HgCl_2$ was partially reversible by incubating with dithiothreitol (Cleland's reagent), 10^{-3} M at 37 C for 30 min, followed by dialysis for 18 hr. The enzyme, which was first treated with $HgCl_2$ (10^{-2} or 10^{-3} M) could thus be reactivated to 27 or 108%, respectively, of control activity.

these concentrations of metals or nonmetallic thiol reagents, the inhibition of "cephalosporinase" activity by NEM and IAA was markedly reduced, but not that of $HgCl_2$ nor of $CdCl_2$ (Table 3). None of these reagents significantly decreased "penicillinase" activity under the stated conditions, but $HgCl_2$ and *p*CMB treatment of crude enzyme resulted in some increase in "penicillinase" activity. Heating to 122 C for 15 min, either before or after adding the mercurials, abolished all β-lactamase activity. Uninduced culture supernatant fluid which was treated with $HgCl_2$ or *p*CMB and dialyzed failed to show any "penicillinase" activity. A purified *B. cereus* "penicillinase" (Neutrapen, Riker) showed no significant change in "penicillinase"

TABLE 4. *Effect of zinc, cadmium, nickel, and mercury on β-lactamase activity*[a]

Metal treatment of enzyme	Enzyme activity (as percentage of zinc-treated sample)			
	"Cephalosporinase"			"Penicillinase"
	No zinc before assay	Treated with zinc (10^{-3} M) before assay	Exposure to *N*-ethyl maleimide (10^{-3} M)[b]	
None (water control)	10 ± 0	62 ± 3	23 ± 2	87 ± 6
$CdCl_2$, 10^{-3} M	17 ± 4	35 ± 1	—	94 ± 2
$NiSO_4$, 10^{-3} M	5 ± 2	106 ± 1	38 ± 1	—
$HgCl_2$, 10^{-3} M	1 ± 1	11 ± 0	—	193 ± 10
$ZnSO_4$, 10^{-3} M	100 ± 8	100 ± 10	100 ± 0	100 ± 4

[a] Crude enzyme from *Bacillus cereus* 569 was treated for 30 to 45 min at 24 or 37 C with indicated metals and then dialyzed.

[b] Exposure for 70 min at 24 C after treatment with metal (first column) but before second dialysis and before addition of zinc (10^{-3} M) for assay.

activity on identical treatment with $HgCl_2$; "cephalosporinase" activity was not detected either before or after the exposure to $HgCl_2$.

Cadmium, mercury, and nickel were themselves relatively ineffective activators of the "cephalosporinase" when compared with zinc; however, pre-exposure to nickel could partially prevent the NEM effect, namely, prevention of zinc activation of "cephalosporinase" (Table 4). About 70% of the "cephalosporinase" inactivation by IAA occurred in 20 min at 37 C (Table 5).

TABLE 5. *Effect of time of exposure to iodoacetic acid (IAA) on inhibition of Bacillus cereus "cephalosporinase" activity*[a]

Time of exposure to IAA (min)	"Cephalosporinase" activity (as percentage of control)[b]	
	No prior treatment with zinc	Pretreatment for 20 min with zinc (10^{-3} M)
0 (control)	100	110 ± 1
20	28 ± 5	111 ± 2
60	3 ± 2	86 ± 3
180	<1	

[a] The crude enzyme preparation was exposed to IAA 10^{-2} M (final concentration) at 37C (pH 7.2) for various periods, then dialyzed for 18 hr. $ZnSO_4$, final concentration 10^{-3} M, was added just before manometric assay

[b] "Penicillinase activity after exposure for 60 min to IAA was about 84% of its untreated control.

DISCUSSION

Although it was previously shown that zinc is clearly required for the hydrolysis of cephalosporins by a β-lactamase produced by *B. cereus* 569 (14), the present studies indicate that either storage (in the absence of zinc) or prolonged activity of the enzyme (with cephalosporin C as substrate) causes it to decrease or lose its ability to be activated by zinc. Enzyme lability in the absence of zinc may have accounted for the selective loss of "cephalosporinase" activity during the earlier purification procedures previously conducted without supplementary zinc (15) and the preservation of such activity when zinc was added.

The highly purified *B. cereus* 569 "penicillinase" of Kogut, Pollock, and Tridgell (6) did not show any increase of "cephalosporinase" activity when zinc was added (14, 15). This could be attributed to a selective removal of the "cephalosporinase" protein during purification of the "penicillinase" (on the assumption that the two enzyme activities are associated with different proteins), as well as to inactivation (denaturation) of "cephalosporinase" in the absence of zinc during purification. Kuwabara and Abraham (7), by adding zinc to the system, recently succeeded in separating from *B. cereus* 569/H supernatant fluid, a crystalline β-lactamase with primarily penicillinase activity (β-lactamase I) and another crystalline enzyme (β-lactamase II) which, in the presence of zinc, hydrolyze cephalosporins and penicillins at relatively similar rates.

No free thiol group appears to be required for "cephalosporinase" activity after zinc activation, because NEM and IAA (at about 10^{-3} M) failed significantly to inhibit crude enzyme that was first treated with an adequate amount of Zn^{++}. However, one or more thiol groups appear to be required for the binding of zinc essential for activity to the apoenzyme, because several thiol reagents (NEM and IAA at 10^{-2} and 10^{-3} M and iodine at 1.6×10^{-3} N) markedly inhibit zinc activation of the "cephalosporinase." Several of

these reagents may react with non-thiol groups. For example, iodine is known to react with tyrosine residues in protein, and IAA has been reported to inhibit by reacting with non-thiol groups, e.g., a histidine residue in ribonuclease, and to react with methionine residues (19). Some slow non-thiol reactions of NEM have also been reported (17, 18). Thus, although diverse reactions with non-thiol groups are known, these reagents have in common the property of reacting rapidly with thiol groups, and the ability to prevent zinc-activation of the *B. cereus* cephalosporinase. The ability of Hg^{++} and Cd^{++} to inhibit cephalosporinase activity, when added either before or after Zn^{++}, suggests that they have a greater affinity for the zinc-binding site, presumably an SH group, than does zinc itself.

Pretreatment with $NiSO_4$ (10^{-3} M) only partially protected the zinc-binding site, the activity on subsequent addition of zinc being 38% of the activity of the crude enzyme protected from NEM by zinc. These experiments suggest that zinc is bound to the apoenzyme by a thiol group in the active "cephalosporinase"; other metals tested, though relatively ineffective as activators of the crude, induced enzyme, are capable of reacting with the thiol group that binds Zn^{++}. The relative affinities of these metals for that thiol group appear to be Hg^{++}, $Cd^{++} > Zn^{++} > Ni^{++}$.

The present finding that thiol reagents do not decrease "penicillinase" activity is consistent with previous evidence from inhibition studies that *B. cereus* "penicillinase" does not have an essential thiol group, and with the failure to find any cysteine at all on amino acid analysis of crystalline *B. cereus* "penicillinase" (3). On the other hand, *p*CMB has been reported to inactivate *B. cereus* "penicillinase" that had first been exposed to oxacillin or methicillin (2). Thus, the evidence that a thiol group is required for the binding of zinc to the "cephalosporinase" apoenzyme would suggest that the primary protein structure of *B. cereus* 569 "cephalosporinase," β-lactamase II of Kuwabara and Abraham (7), differs from that of the crystalline "penicillinase" of this strain studied by Pollock and co-workers (3, 6), although the missing of a single amino acid residue in the penicillinase analysis cannot be excluded.

"Cephalosporinase" activity appeared to increase during several experiments in which the crude enzyme was dialyzed or passed through a column of Sephadex G-25. This could reflect removal of inhibiting substances from cephalosporinase, but the possibility of conversion of some "penicillinase protein" (with a decrease in "penicillinase" activity) to a "cephalosporinase" form has not been excluded. Heating to 65 C also resulted in a net loss in "penicillinase" activity and doubling of "cephalosporinase" activity (7). Thus, the possible interrelation between these two β-lactamases requires clarification.

Since the synthesis of both *B. cereus* 569 "penicillinase" and "cephalosporinase" can be induced by the same small molecules, viz., penicillins or cephalosporins (15), the structural genes for both enzymes (if there are two, as it appears) would presumably be part of the same operon because the mutant 569/H(6, 7) is constitutive for both β-lactamases. However, the involvement of two structural genes is neither necessary nor established. An alternate possibility is that there is one structural gene, but that ambiguity in translation of messenger ribonucleic acid sometimes results in incorporation of a thiol-containing amino acid (and potential "cephalosporinase" activity), while at other times it does not.

The increased "penicillinase" activity noted after treating the dialyzed enzyme with either *p*CMB or $HgCl_2$ was probably not due to metal-catalyzed hydrolysis of benzylpenicillin, because the activity was heat-labile and because identical treatment of either uninduced *B. cereus* 569 supernatant fluid (no β-lactamase activity) or a highly purified "penicillinase" failed to change pre-existing "penicillinase" activity. The mechanism by which mercury increased "penicillinase" activity requires elucidation. Two possibilities are that (1) the mercury complexed with a penicillinase-inhibitor, thus unveiling more "penicillinase" activity, or (ii) the mercury acted as a cofactor for the "penicillinase" activity mediated by β-lactamase II. Kuwabara and Abraham (7) reported that β-lactamase II requires zinc for hydrolysis of benzylpenicillin, and several of its analogues, as well as for the hydrolysis of cephalosporins. Of the metals tested, zinc was the only one capable of markedly activating the *B. cereus* cephalosporinase (14). The effect of metals other than zinc on the β-lactamase II-mediated "penicillinase" activity has not been reported.

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