photoreduction procedure produces other minor components (Cerutti *et al.* 1966), the major reaction is the conversion of uridylic acid into dihydro-uridylic acid. The hydrated oligonucleotide offers the advantage, which was kindly pointed out to us by Professor D. Shugar, that the 5-hydro-6-hydroxyuridylic acid can be specifically reconverted into uridylic acid on heating (Sinsheimer, 1954). Table 1 shows that, not only is the s-RNA binding capacity of ApUpG destroyed by converting uridylic acid into 5-hydro-6-hydroxyuridylic acid, but the effect is completely reversed on heating, proving that the loss of activity is not due to other reactions or degradation of the oligonucleotide.

5-Hydro-6-hydroxyuridylic acid would be expected to have the same template properties as dihydrouridylic acid. Grossman (1963) has reported that u.v.-irradiated polyU, comprising uracil dimers, uridylic acid and 5-hydro-6-hydroxyuridylic acid, incorporates serine as well as having a decreased phenylalanine incorporation, which is in contrast with poly(U,H₂U), which only has a decreased incorporation of phenylalanine relative to polyU (Rottman & Cerutti, 1966). However, although Grossman (1963) attributed the serine incorporation to the 5-hydro-6-hydroxyuridylic acid, because of the behaviour of the copolymer after irradiation at different wavelengths, it is possible that the serine incorporation was due to chain scission (Logan & Whitmore, 1966), rather than a template difference between uridylic acid and its hydrate.

The experiments reported here show that when ApUpG is converted into either ApH₂UpG or Ap(H₂O)UpG the triplet loses its ability to bind methionyl-s-RNA. These results are compatible

with the idea that the interconversion of uridylic acid and dihydrouridylic acid in a chain-initiating coding triplet could be involved in the regulation of protein synthesis at the translational level.

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Some Properties of Two Extracellular β -Lactamases from *Bacillus cereus* 569/H

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Many bacteria produce β -lactamases that hydrolyse both benzylpenicillin and cephalosporin C, although the relative maximum rates of hydrolysis of these two substrates vary greatly with the source of the enzyme. Cephalosporinase has been used as an operational term to describe β -lactamases that

hydrolyse cephalosporin C at a significant rate (Abraham & Newton, 1956; Crompton, Jago, Crawford, Newton & Abraham, 1962; Fleming, Goldner & Glass, 1963; Sabath, Jago & Abraham, 1965).

The cephalosporinase activity of culture fluid of

Bacillus cereus 569, which was about 5% of its penicillinase activity, was lost selectively during purification of the penicillinase (Abraham & Newton, 1956) and during hydrolysis of cephalosporin C (Sabath & Abraham, 1965), whereas penicillinase activity of this culture fluid was lost selectively on heating at 60° (Crompton *et al.* 1962). Further, the cephalosporinase activity of the culture fluid, unlike its penicillinase activity, was shown to be zinc-dependent (Sabath & Abraham, 1966), and more recent experiments on the effect of thiol reagents on culture fluid have led to the conclusion that a thiol group is probably involved in the binding of zinc to the enzyme (L. D. Sabath & M. Finland, 1967).

As a preliminary to obtaining information about the relationship of the protein structures responsible for these two types of β -lactamase activity further studies have been made of the purification of material from *B. cereus* 569/H. Culture supernatant from this organism (referred to as crude enzyme solution) was obtained as described by Miller, Bach & Markus (1965) except that the casein-hydrolysate medium used for growth contained ZnSO_4 (1 mM). Assays of enzymic activity were carried out at pH 7.0 and at 30° either manometrically (Henry & Housewright, 1947; Pollock, 1952) or by measurement of acid liberated during the reaction in a pH-stat (Sabath *et al.* 1965). The crude enzyme solution contained about 3200 units of penicillinase/ml. and 100 units of cephalosporinase/ml., the units (Pollock & Torriani, 1953) being the amounts of enzyme required to hydrolyse 1 μ mole of benzylpenicillin and cephalosporin C respectively/hr. with substrate concentrations about 10 mM. Protein estimations were made by the method of Lowry, Rosebrough, Farr & Randall (1951) with a crystalline bovine serum albumin standard. Protein N was calculated on the assumption that the protein contained 15.8% of N.

Attempts to devise a purification procedure in which the enzyme was adsorbed on glass powder (Kogut, Pollock & Tridgell, 1956), Celite (Miller *et al.* 1965) or calcium orthophosphate were unsuccessful. In each case penicillinase activity was recovered, but most of the cephalosporinase activity was lost. Both activities were recovered together after passage of the crude culture fluid through a column of Sephadex G-25, but much of the cephalosporinase activity was lost during attempts to separate it from penicillinase activity on a column of Sephadex G-100. However, a separation was achieved by precipitation with $(\text{NH}_4)_2\text{SO}_4$ at pH 7.0 and 4° in tris-acetate buffer (made by addition of 50 mM-sodium acetate-acetic acid buffer, pH 6.0, to 0.1 M-tris-HCl buffer, pH 8.0, to give pH 7.0) containing ZnSO_4 (1 mM). The first enzyme to be precipitated by increasing

concentrations of $(\text{NH}_4)_2\text{SO}_4$ showed penicillinase activity but no significant cephalosporinase activity. Further fractionation by precipitation with ethanol and with $(\text{NH}_4)_2\text{SO}_4$ yielded a crystalline penicillinase, presumably identical with that obtained by Kogut *et al.* (1956), and a crystalline enzyme with cephalosporinase activity. These two enzymes have been called β -lactamase I and β -lactamase II respectively. Isolation of β -lactamase II was facilitated by the inclusion of a step in which a solution of partially purified material was heated at 65°, when selective inactivation of β -lactamase I was accompanied by the precipitation of denatured protein and about a 1.5-fold increase in cephalosporinase activity. The estimated specific activity of β -lactamase I was 3.27×10^6 units/mg. of protein N and that of β -lactamase II was 3.54×10^6 units/mg. of protein N.

In the present communication we report on the behaviour of the enzymes to various penicillin and cephalosporin substrates.

The activity of β -lactamase I was unaffected by Zn^{2+} when the latter was added in concentrations up to 1 mM, and was inhibited by Zn^{2+} in higher concentration. β -Lactamase II was zinc-dependent. Dialysis at 4° for 6 hr. against 5 mM-sodium citrate buffer, pH 4.0, containing NaCl (1 M), followed by dialysis overnight against tris-acetate buffer, pH 7.0, resulted in virtually complete loss of activity. More than 80% of the activity was restored by the addition of Zn^{2+} . Under the conditions used the optimum concentration of Zn^{2+} was between 0.1 and 0.4 mM. After dialysis of the enzyme and treatment with 0.5 mM-N-ethylmaleimide or 50 mM-iodoacetate (10 min. at 30° in 5 mM-tris-sodium acetate buffer, pH 7.0) its activity was no longer restored by the addition of Zn^{2+} . In its zinc-dependence and inactivation by N-ethylmaleimide, or iodoacetate, after dialysis, β -lactamase II showed properties similar to those of the cephalosporinase component of the original culture fluid (Sabath & Abraham, 1966; L. D. Sabath & M. Finland, personal communication. But studies with crude enzyme solutions left open the questions whether the β -lactamase responsible for the cephalosporinase activity also showed high penicillinase activity and, if so, whether the latter activity was dependent on the presence of zinc. The properties of purified β -lactamase II indicate that both these questions can be answered in the affirmative.

Table 1 shows that β -lactamase II, unlike β -lactamase I, hydrolyses cephalosporin C, cephalothin, cephaloridine, methicillin and cloxacillin at a maximum rate (V_{max}) comparable with that of the hydrolysis of benzylpenicillin. The Michaelis constants found with different substrates were of the same order, ranging from 3.3 to 0.5 mM. But most of them were more than one order higher than

Table 1. *Maximum rates of hydrolysis ($V_{\max}$) and Michaelis constants (K_m) for different penicillins and cephalosporins with β -lactamase I and β -lactamase II*Values of $V_{\max}$ and K_m were obtained from Lineweaver-Burk plots.

Enzyme	Substrate	$V_{\max}$ (relative)	K_m (mM)
β -Lactamase I	Benzylpenicillin	100	0.037*
	Phenoxymethylpenicillin	102	0.061
	Ampicillin	100	1.3
	Penicillin N	38	0.053
	Methicillin	<0.1	
	Oxacillin	<0.1	
	Cephalosporin C	<0.1	
	Cephalothin	<0.1	
	Cephaloridine	<0.1	
	Cephaloglycin	<0.1	
	Deacetylcephalosporin C	<0.1	
	Deacetylcephalosporin C lactone	30	2.0
β -Lactamase II	Benzylpenicillin	100	3.3
	Ampicillin	64	3.3
	Methicillin	89	1.6
	Oxacillin	89	1.6
	Cloxacillin	89	2.3
	Quinacillin	10†	
	Cephalosporin C	80	1.1
	Cephalothin	89	1.0
	Cephaloridine	41	3.3
	Cephaloglycin	50	0.5
	Deacetylcephalosporin C	0.02‡	
	Deacetylcephalosporin C lactone	<0.1	

* Manson, Pollock & Tridgell (1954) reported K_m 0.065 mM for crude penicillinase from *B. cereus* 569 with benzylpenicillin as substrate.

† Relative rate of hydrolysis with substrate concentration 3.0 mM.

‡ Relative rate of hydrolysis with substrate concentration 1.0 mM.

those found for several substrates with β -lactamase I (0.037–0.06 mM).

β -Lactamase II shows a wider range of activity than most of the β -lactamases previously studied. Only one enzyme produced by *Escherichia coli* K 12 carrying the Resistance (R) factor 1818 has so far been shown to have a comparable substrate profile (Datta & Kontomichalou, 1965). An area in the region of the active centre of β -lactamase II thus appears to be more open in conformation than is usual with enzymes of the β -lactamase group.

Selective inactivation of β -lactamase I by heat in a partly purified preparation containing both enzymes was accompanied by a small increase in the β -lactamase II activity, but no evidence was obtained that this was due to the formation of β -lactamase II from β -lactamase I. Attempts to convert crystalline β -lactamase I into an enzyme with the activity of β -lactamase II were unsuccessful. The relationship of β -lactamase II to the cell-bound γ -penicillinase from *B. cereus* 569 and 569/H, which has a higher solubility in $(\text{NH}_4)_2\text{SO}_4$ solution than the exopenicillinase described by Kogut *et al.*

(1956) and a higher relative activity against methicillin (Ron-Zenziper & Citri, 1963), remains to be determined.

The term cephalosporinase does not provide an adequate indication of the nature of the hydrolysable substrates of the enzyme from *B. cereus* 569/H that shows activity against cephalosporin C. It has thus been replaced by the term β -lactamase II in this communication.

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Acid Phospholipase A in Lysosomes of the Bovine Adrenal Medulla

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In recent studies of the phospholipid content of different particulate fractions from the bovine adrenal medulla it was found that the chromaffin granules, i.e. the particles that contain the hormones, were characterized by a high content of lysolecithin (Blaschko, Firemark, Smith & Winkler, 1966, 1967; Winkler, Strieder & Ziegler, 1966). These observations have led us to investigate the presence and distribution of particulate phospholipase A activity in the bovine adrenal medulla. The large-granule fraction obtained from homogenates of bovine adrenal medulla contains three different types of cell particle, i.e. mitochondria, lysosomes and chromaffin granules. These three types of cell particle can be separated by sucrose-density-gradient centrifugation. The present communication deals with the first outcome of this study which leads us to conclude that lysosomes contain acid-phospholipase A activity.

Methods. Homogenates of bovine adrenal medulla were fractionated as described by Smith & Winkler (1966). By this method a large-granule fraction is sedimented at $2.42 \times 10^5 g \cdot min.$; this is further resolved into its constituent particles by centrifugation over a sucrose density gradient. The gradient tubes contained 0.5 ml. each of 1.3M-, 1.4M-, 1.5M-, 1.6M-, 1.7M-, 1.8M-, 1.9M- and 2.0M-sucrose, and 0.25 ml. of 2.5M-sucrose. The tubes were centrifuged at $87.4 \times 10^5 g \cdot min.$ in the Spinco swing-out rotor SW39L and seven fractions were collected. Each fraction was dialysed against 25 mM-glycylglycine-NaOH buffer, pH 6.5. The

dialysed fractions were analysed for fumarase activity (Racker, 1950), acid-deoxyribonuclease activity (Gianetto & de Duve, 1955) and catecholamines (Euler & Hamberg, 1949).

Phospholipase activity was determined by incubating 22 $\mu g.$ of [^{32}P]lecithin or of [^{32}P]phosphatidylethanolamine (prepared as described by van den Bosch & van Deenen, 1965) with up to 0.3 ml. of each fraction in sodium acetate buffer, pH 4.16 (37°) and I 0-0125 (final volume 1.0 ml.). The phospholipids were dispersed in water by brief ultrasonic treatment. After incubation for 1 hr. at 37° the reaction was stopped by the addition of 4 vol. of a mixture of chloroform (1 vol.) and methanol (2 vol.). The solvents were removed by evaporation *in vacuo*, and methanol was added to the residue. The [^{32}P]phospholipids and their breakdown products were separated by thin-layer chromatography according to the method of Skipski, Peterson & Barclay (1964). Phospholipids extracted from rat liver were co-chromatographed with each sample to act as markers. The radioactivity of the spots corresponding to phosphatidylethanolamine, lecithin, their respective lyso compounds, and glycerophosphorylethanolamine and glycerophosphorylcholine were measured with a scintillation counter. It was established, for each fraction, that the breakdown of substrate was linear both with time of incubation and with enzyme concentration under the conditions of assay.

Results and discussion. The pH optimum of the acid phospholipase in the large-granule fraction was