TWO ANTIGENICALLY DIFFERENT STATES OF ACTIVE PENICILLINASE

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The isolation of exopenicillinase of *B. cereus* NRRL 569[1] permitted application of immunological methods to the study of this enzyme[2], while its subsequent purification[3] and crystallization led to a more detailed study of its physico-chemical properties.

In contrast to the exoenzyme, which is almost completely neutralized by the homologous antibodies[2], about 50 to 70% of the cell-bound penicillinase (which forms at least one-tenth of the total penicillinase in the culture) remain active in the presence of anti-exopenicillinase serum. This observation led to the discovery of an immunologically distinct penicillinase (designated as γ-penicillinase) found alongside with the exoenzyme (now called α-penicillinase) in cell preparations of *B. cereus* NRRL 569. γ-Penicillinase has been separated from the α-type by fractional ammonium sulphate precipitation. Most of the other properties studied (including substrate specificity, Michaelis constant, and sedimentation rate) were found to be practically identical, and the only other ways in which γ-penicillinase differed from α-penicillinase were in its rapid inactivation by iodine and lower activity in the alkaline region[4].

The concept of one cell independently producing two distinct kinds of protein having identical biological properties seemed unsatisfactory.

A more plausible alternative—namely that the cell produces one kind of penicillinase which can assume both α- and γ-penicillinase properties—has accordingly been explored.

The evidence obtained suggests that one type of protein molecule—in this case that of a bacterial penicillinase—can exist in two antigenically different, biologically active, states.

MATERIALS

Bacillus cereus NRRL 569, an inducible penicillinase-producing strain.

569/*H Exo-penicillinase*, a purified preparation (from a constitutive mutant of the 569 strain) found not to differ from the induced enzyme of the parent strain by any of the physico-chemical or immunological characters examined[2,3]; subsequently referred to as α-penicillinase.

Anti-α-penicillinase serum, γ-globulin fraction of serum of a rabbit immunized with induced 569 exo-penicillinase; neutralizes about 98% of the activity of 569/H exo-penicillinase in solution[2]; susebquently referred to as anti-α-serum.

METHODS OF ASSAY

Total penicillinase activity, assayed by the manometric method of HENRY AND HOUSEWRIGHT[5]; expressed in units/ml, as previously defined[1].

Unneutralizable penicillinase activity. Samples were incubated for 30 min at room temperature

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with anti-α-serum and subsequently assayed by the manometric method. The amount of serum (50 neutralization units, as defined by POLLOCK[2]), was always somewhat in excess of the concentration required for complete combination with the homologous enzyme.

The iodometric assay method

The method is based on the observation that the product of activity of penicillinase and time required to decolorize a given amount of iodine by the penicilloic acid formed is constant[6].

Reagents:

Iodine solution $M/40$ I_2 in $M/8$ KI aqueous solution.
Penicillin solution 5000 units per ml of $M/10$ phosphate buffer solution, pH 7.0. (freshly prepared, kept on ice).
Gelatin solution 0.5% gelatin in distilled water.

Procedure: Reagents are added to a test tube kept in a 30° C water bath in the following order:

Time before reaction started	*Solution*	*Volume*
5 minutes	Gelatin	3 ml
1 minute	Penicillin	1 ml
½ minute	Iodine	0.5 ml

The reaction is initiated by transferring the contents to another tube where the enzyme sample (0.5 ml) has been prepared. The time taken for complete decolorization of the iodine at 30° C (endpoint determined by adding a drop of starch solution) is recorded. The result is then read from a calibration curve of decolorization time against activity in units/ml.

The inaccuracies in the method are mainly due to iodine uptake by penicilloic acid initially present in the penicillin solution and by crude enzyme preparations. If these are controlled, the results are reproducible well within ± 10%

Determination of iodine sensitivity by delayed iodine addition

γ-Penicillinase is rapidly inactivated by iodine in the concentration used for iodometric assay, so that decolorization of iodine is never obtained even with the most active samples. If, however, instead of having iodine present before the reaction has started, it is added some time afterwards, decolorization eventually takes place. The time of decolorization can be progressively shortened by allowing more time before adding iodine, until a point is reached where the excess of penicilloic acid already formed is sufficient to prevent any appreciable inactivation of the enzyme on adding iodine. This method of assaying iodine-sensitive enzyme will be referred to as the "delayed iodine" technique. Since α-penicillinase is not appreciably inactivated by iodine during the period of test, the proportion of iodine-sensitive enzyme in a mixture can be calculated by subtracting the reading of the ordinary assay from that of the delayed iodine technique. The agreement with manometric assay results is satisfactory.

EXPERIMENTAL

Alkali treatment

We found that a short exposure of purified α-penicillinase to $N/30$ NaOH results in modification of its properties which persists for some time after the enzyme has been neutralized; there is a loss in activity, and the remaining activity is completely abolished by $M/400$ I_2. This modification and the subsequent reversal is demonstrated in Fig. 1. In this experiment, α-penicillinase was diluted in $N/30$ NaOH to contain 134 units/ml. After 10 minutes 0.2 ml of $M/1$ phosphate buffer, pH 7.0, was added to 0.3 ml aliquots of the enzyme, which were then incubated at 30° C for 2, 10, and 20 minutes, respectively, before being assayed. The values for the iodine-insensitive fraction were obtained directly from an ordinary iodometric assay. The iodine-sensitive fraction was measured by subtracting the above values from those obtained by the "delayed iodine" technique (Fig. 1).

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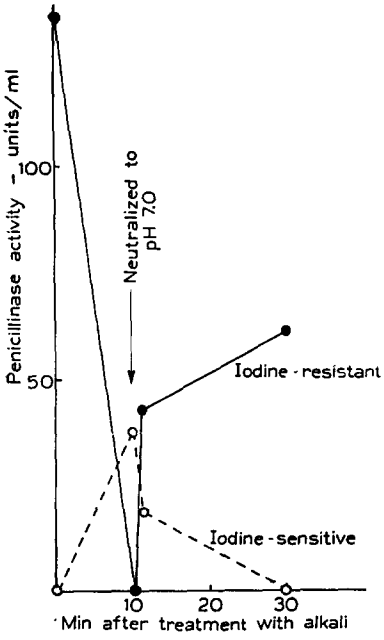

Fig. 1. Effect of treatment of *B. cereus* exo-penicillinase with *N*/30 NaOH for 10 min, followed by neutralization to pH 7.0, on iodine-resistant and iodine-sensitive fractions of penicillinase activity.
●——● = iodine-resistant
○- - -○ = iodine-sensitive.

It will be noted that the enzyme recovered upon reversing the effect of alkali is indistinguishable from α-penicillinase in that it is insensitive to iodine (Fig. 1) and is neutralized by anti-α-serum (Table I). A more prolonged exposure to alkali results, however, in a change which is apparently irreversible; the enzyme is now indistinguishable from γ-penicillinase in that it is not neutralized by anti-α-serum (Table I), is iodine-sensitive, and, like γ-penicillinase, retains these properties irrespective of subsequent treatment.

Adsorption on cells

That α-penicillinase can assume alternative antigenic properties under more physiological conditions is shown in the following experiment.

Uninduced cells of *B. cereus* NRRL 569, harvested from broth culture and washed in distilled water, were suspended at 1.2 mg dry bacterial wt./ml in an aqueous solution containing 780 units purified α-penicillinase per ml, left for 6 h at room temperature, followed by 18 h at 2°. The bacteria were then washed in distilled water to remove unadsorbed enzyme and resuspended at 0.6 mg/ml in (a) 0.5 % gelatin solution and (b) distilled water. The activity of the suspension in gelatin was found to be roughly 10 % of the original enzyme to which the cells were exposed; practically all the enzyme was found to be released to the supernatant and to be fully active in the presence of iodine. However, no enzyme was released to the supernatant from the suspension in distilled water and it was found to be largely iodine-sensitive. The cell-adsorbed enzyme was only partly neutralized by anti-α-serum (Table I).

TABLE I

EFFECT OF ANTI-EXOPENICILLINASE SERUM ON *B. cereus* EXO-PENICILLINASE AFTER (1) TREATMENT WITH ALKALI AND (2) ADSORPTION ON UNINDUCED CELLS OF *B. cereus* 569

	Activity (units/ml)	
	Total	*+ antiserum*
(1) Treated with *N*/30 NaOH		
(a) for 10 minutes	15.3	1.8 (12%)
(b) for 240 minutes	12.8	11.2 (87%)
(2) Adsorbed on cells of strain 569	25.6	16.5 (65%)

Adsorption on glass

The observation that exo-penicillinase is readily and preferentially adsorbed on glass particles has been applied to the task of concentrating and purifying the enzyme[3].

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It was also found that it can be quantitatively and rapidly eluted by 0.3 to 1.0 % gelatin solution. We had thus a simple system where the effect of adsorption could be isolated from any other interactions likely to occur in cell-enzyme systems.

Ten mg of fine-mesh glass powder were added to 535 units of purified penicillinase in 6 ml distilled water. The suspension was left for 2 h at room temperature, centrifuged, the supernatant fluid discarded, and the glass powder pellet washed once in distilled water and resuspended in 5 ml of distilled water. This "coated glass" suspension was found not to release the enzyme into solution unless gelatin was added to the sample or present in the assay mixture. Since it was known from previous experiments that gelatin by itself had no effect on the interaction between iodine or antiserum and the enzyme *in solution*, it could be used as a neutral eluting agent, and it would be presumed that the effect of adding gelatin would be that of recovering much of the original α-penicillinase by desorbing it from glass surfaces. This has been borne out by experiment (Table II). The experiment also shows that the enzyme, while on glass, is largely in the unneutralizable, iodine-sensitive state.

TABLE II

EFFECT OF IODINE AND OF ANTI-EXOPENICILLINASE SERUM ON *B. cereus* EXOPENICILLINASE AFTER (1) ADSORPTION ON GLASS AND (2) ADSORPTION ON, AND SUBSEQUENT ELUTION FROM, GLASS

	Activity (units/ml)	
	(1) On glass	*(2) After elution from glass with gelatin*
A. Effect of iodine		
"Delayed iodine technique"	30.8	42.5
Ordinary iodometric assay	not measurable	30.8
% inactivation by iodine	100	27
B. Effect of anti-exopenicillinase serum:		
No antiserum	38.9	43.5
+ 50 neutralization units of antiserum	33.5	7.1
% neutralization	14	84

DISCUSSION

The results have shown that purified penicillinase of *B. cereus* can, under certain specified conditions, change some of its properties in a reversible manner. Since we have no direct evidence as to the chemical nature of this change, it is referred to as a change in the state of the enzyme. Exopenicillinase in solution would then be in the α-state, whereas when adsorbed or pretreated with alkali, so as to become iodine-sensitive and unneutralizable by anti-α-serum, it would be in the γ-state. The cell-bound γ-penicillinase, both *in situ* and as isolated by the POLLOCK procedure, could perhaps be regarded as representing that proportion of penicillinase which, within the cell's economy, had been diverted from the enzyme-secreting pathway and irreversibly fixed in the γ-state. This concept would be compatible with the mounting evidence, notably that obtained by KAPLAN[7], that the release of an enzyme from a cell involves alteration of its properties.

Judged by the two independent criteria used, penicillinase, regardless of prepara-

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tion, was found to be either in the α- or in the γ-state. In fact, in no case was *B. cereus* penicillinase found to be unneutralizable by anti-α-serum, without at the same time being iodine-sensitive, and *vice-versa*. This suggests that both criteria originally used by POLLOCK[4] to characterize γ-penicillinase pertain to a fundamental property of *B. cereus* penicillinase. This property could be described as freedom to exist in one state or another without change in function. Possible mechanisms of transition from one state to another remain to be investigated, but it seems likely that some unfolding of the molecule takes place in the transition from α- to γ-state, and that this results in exposure of iodine-sensitive groups and in loss of the antigenic identity. The iodine-sensitive groups exposed may in fact be tyrosyl residues which might have been involved in hydrogen bonds before the treatment[8]. Un-hydrogen-bonding of tyrosyl residues takes place in the ribonuclease molecule when exposed to similar concentrations of alkali, but the treatment there results also in an irreversible loss of enzymic activity[9]. On the other hand, studies on ribonuclease demonstrate in a striking way that the active centre can survive changes in the secondary structure of the molecule[10]. The rather unexpected sensitivity of the serological procedure, which served to detect changes in a protein molecule not reflected in its biological activity, may be found to have wider application. Thus, in some cases at least, the enzyme-neutralizing antibody could be considered a tool subtle enough to explore the range within which the enzyme can vary its structure while retaining its function.

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SUMMARY

1. *B. cereus* penicillinase can exist in two serologically distinguishable states. In the γ-state its activity is completely abolished by $M/400$ I_2 whereas in the α-state the enzymic activity is not affected by that concentration of iodine.

2. A reversible transition from α- to γ-state can be demonstrated by having penicillinase exposed to alkali, or by letting it adsorb to a glass or cell surface. A prolonged exposure to alkali results in an irreversible transition to γ-state.

3. A possible mechanism for the transition and its relation to naturally occurring γ-penicillinase, are discussed.

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