The Effect of Urea and Guanidine Hydrochloride on Activity and Optical Rotation of Penicillinase*

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The penicillinase of Bacillus cereus exists as an exoenzyme, called α-penicillinase, and as an immunologically distinct cell-bound enzyme, designated as γ-penicillinase (1). Properties such as substrate specificity, Michaelis constant, and sedimentation rate were found to be practically identical, and the only other ways in which the cell-bound γ-penicillinase differed from α-penicillinase were in its rapid inactivation by iodine and in its lower activity in the alkaline region. More recently it has been shown that the exposure of α-penicillinase to dilute alkali, or the adsorption on glass or cell surfaces, results in a reversible change to a form behaving like the cell-bound γ-penicillinase, as followed serologically and by sensitivity to iodine (2). This change was called a shift from the α-state to the γ-state of penicillinase. A more prolonged exposure to alkali caused an irreversible change to the γ-state.

The above findings have suggested that the difference between the various forms of penicillinase of B. cereus is due to configurational changes in the molecule. In order to test this hypothesis, the effect of urea on the iodine sensitivity of the enzymatic activity of α-penicillinase has been investigated (3). Urea is known to break hydrogen bonds in proteins and thus cause changes in their secondary and tertiary structure (4, 5). It was found that although the total penicillinase activity remains constant over a wide range of concentration of urea, a shift from the α-type to the γ-type activity on increasing the concentration of urea occurs, which is reversible upon dilution. At higher concentrations of urea, on the other hand, a loss in penicillinase activity takes place and the shift to the γ-state becomes irreversible (3).

In the following paper, we report the results of an investigation of the effect of urea as well as of another hydrogen bond-breaking reagent, guanidine hydrochloride, on the enzymatic activity and the optical rotation of penicillinase. The effect of the ionic strength on the α- and γ-states of penicillinase was also correlated with conformational changes in the molecule.

EXPERIMENTAL PROCEDURE

The penicillin used was the sodium salt of benzylpenicillin (Merck). Urea, sodium chloride, potassium chloride, sodium nitrate, sodium citrate, potassium iodide, and iodine were of reagent grade, and were used without further purification, whereas reagent grade guanidine hydrochloride (Matheson Company) was recrystallized once from methanol and stored in a desiccator over phosphorus pentoxide. DFP1 was a gift from Dr. W. Dreyer. Celite 535 (diatomaceous earth filter aid) was obtained from Johns-Manville, and Geon (polyvinyl chloride particles) from the Goodrich Company. Gelatin, U.S.P. granular, was from the Fisher Company. Polyethylene glycol (molecular weight 100,000) and polyacrylic acid (molecular weight 200,000) were gifts from Dr. Z. Alexandrowicz. Polyvinylamine was a gift from Dr. P. Spitnik. Polyvinyl alcohol (Elvanol) was obtained from Du Pont.

Penicillinase—The penicillinase used throughout this work was prepared from the culture supernatant of strain 569/H of B. cereus. This mutant strain, which produces penicillinase constitutively, has been derived from the inducible penicillinase-forming B. cereus 569 (6). The culture was started from spores inoculated into a medium consisting of Difco Peptone (1%), Bovril Meat Extract (0.3%), and sodium chloride (0.2%), and prepared according to Pollock (7). After reaching the logarithmic phase, the cells were transferred to a casein hydrolysate-citrate medium, prepared according to Kogut et al. (6), and dispersed in 500 ml lots into 2-liter Erlenmeyer flasks. The casein hydrolysate citrate medium cultures were incubated at 35° in a rotary type shaker for 6 to 8 hours.

The supernatant was then collected by centrifugation (3000 r.p.m., 10 minutes), adjusted to pH 5, and percolated through a column of fine diatomaceous earth (Celite 535). The filtrate was found to be free of penicillinase activity and was discarded. The column was washed with twice-distilled water until no material absorbing in the 260- to 280-mu range of the spectrum could be detected in the eluate. The enzyme was then gradually eluted with a solution consisting of 1 M NaCl and 0.1 M sodium citrate, adjusted to pH 8.5. Most of the activity was found in a small fraction, about 0.1 of the original volume of the eluate. This fraction was subjected to dialysis, at 2°, against 50 volumes of 0.001 M phosphate buffer, pH 7, for 4 hours.

The nondialyzable material was again adsorbed on a Celite column and then washed and eluted as before. At this stage, the enzyme concentration was 50 to 100 times greater than that of the original supernatant and its specific activity was close to that reported by Pollock et al. (8) for a crystalline preparation of the related 569 strain of B. cereus. The second eluate was dialyzed extensively against 20 volumes of 0.001 M phosphate buffer.

1 The abbreviation used is, DFP, diisopropyl phosphorofluoridate.
buffer, pH 7, at 2° for 48 hours, followed by dialysis against distilled water for 6 hours, and was subsequently lyophilized.

**Assay of Penicillinase**—The assays of total penicillinase activity and of the activity of the iodine-resistant fraction, α-type activity, were performed as follows:

**Reagents**—Iodine solution, I<sub>2</sub>, 0.025 M, in 0.125 M KI aqueous solution. Penicillin solution, a freshly prepared solution of 5000 units per ml of 0.1 M phosphate buffer, pH 7.0, which was kept cool. Enzyme solution, penicillinase diluted in the assay medium so as to contain 150 to 200 penicillinase units per ml. One unit is defined as the amount of penicillinase which hydrolyzes 1 μmole of penicillin in 1 hour at 30° and pH 7 (9), based on manometric determination (10). Assay medium, 0.5% gelatin in water or an aqueous solution of the reagent to be investigated for its effect on the enzyme activity.

**Procedure**—(a) **α-Type activity.** Test tubes containing 3 ml of the assay medium are placed in a water bath at 30°. After 5 minutes, 1 ml of penicillin solution and 0.5 ml of iodine solution are added. After equilibration to the temperature for 1 minute, the reaction is started by the addition of 0.2 ml of the enzyme solution to each tube. The time required for a complete decolorization of the iodine by the penicilloic acid formed is recorded. Since the rate of penicilloic acid formation under these conditions is linear (11), and its reaction with iodine is very rapid, the decolorization time can be translated into activity units by means of a simple calibration curve.

(b) **Total activity.** Since the assay described above is performed in the presence of 0.0025 M iodine, it measures only penicillinase activity of the α-type (2). For the measurement of total activity, Procedure α is followed except that the iodine is not present when the assay is started. It is introduced after approximately two-thirds of the amount of penicilloic acid required for complete decolorization has been formed. The validity of this procedure has been demonstrated elsewhere (11). In practice, iodine is added 3 minutes after introducing the enzyme, which is diluted so as to cause complete iodine decolorization in 5 minutes. Whenever the time of decolorization is longer than 5 minutes, the addition of iodine is delayed accordingly. Total activity measured by this procedure is in good agreement with results obtained by the manometric method (10).

(c) **γ-Type activity.** The proportion of the iodine-sensitive (γ-type) fraction in a mixture is calculated by subtracting the values obtained for the α-type fraction from those obtained in the total activity assay.

**Assay of Total Protein**—The protein content was determined either by the Kjeldahl nitrogen method or by the Folin-biuret procedure (12).

**Anti-α-Penicillimase Serum** The antiserum was obtained from rabbits immunized with pure 569/H penicillinase. One neutralization unit (1 NU) will neutralize 1 unit of the enzyme, as defined by Pollock (13).

**Optical rotations** were measured at the sodium D line with a Rudolph high precision ultraviolet polarimeter, model 80, equipped with the Rudolph photoelectric polarimeter attachment and an oscillating polarizer prism.

**Sedimentation** analysis was carried out in a Spinco model E ultracentrifuge, at 21° with the Schlieren optical system.

**Electrophoresis** was carried out in borate buffer of pH 7.6 and 8.7, respectively, of 0.05 ionic strength, at 2°, on a Geon resin block measuring 30 X 2 X 1.5-cm at 25 ma. and 100 volts.

### Results

**Characterization of the Exopenicillinase**

The specific enzymatic activities of our penicillinase preparations were similar to that reported by Kogut et al. (6) for their crystalline penicillinase. A penicillinase preparation with an activity of 2 X 10<sup>4</sup> units per mg of protein nitrogen was subjected to electrophoresis on Geon at pH 7.6 and 8.7. After completion of the experiment, the Geon block was cut crosswise into 1-cm segments. The protein was then eluted with 0.5 M aqueous sodium chloride. Enzymatic activity was measured by means of the iodometric assay, and the protein content was determined by the Folin-biuret procedure (12). Only one sharp peak migrating towards the cathode and containing the enzymatic activity was obtained. In a typical experiment carried out at pH 8.7 for 22 hours, the peak moved a distance of 10 cm from the origin.

For the ultracentrifugation the penicillinase sample was dialyzed against a buffer of pH 7.3 and 0.2 ionic strength, composed of 0.1 M NaCl, 0.05 M NaCl, 0.05 M Na<sub>2</sub>HPO<sub>4</sub> and 0.0167 M NaH<sub>2</sub>PO<sub>4</sub> and sedimented at 56,100 r.p.m. At a protein concentration of 0.2%, a single, slightly asymmetrical peak was observed with a sedimentation constant s<sub>20</sub>,<sub>0</sub> = 2.5 S, which is in good agreement with the values of 2.6 to 2.8 S obtained by Hall and Ogston (14) for several preparations of bacterial penicillinase.

**Effect of Urea**

**Iodine Reactivity of Urea-treated Penicillinase**—The effect of the exposure to urea on the shift of penicillinase activity from α-type to γ-type has been demonstrated in a previous communication (3). In the experiments described below, the urea-induced shift was studied under somewhat different conditions. Here, gelatin was excluded from the assay system and preincubation with urea was, therefore, not required. This simplified procedure gave a very consistent picture of α → γ transition as a function of the concentration of urea (Fig. 1).

**Correlation between Iodine Sensitivity and Antigenicity of Urea-treated Penicillinase**—The modified enzyme is of the γ-type as judged by its sensitivity to iodine. It is obviously difficult to consider its antigenic structure in the presence of high concentrations of urea. However, reversion upon dilution results in the recovery of the initial α-type activity as judged both by the resistance to iodine and by the antigenic identity of the enzyme. This was demonstrated by the following experiment. Aliquots of exopenicillinase were exposed to 5 M urea at 30° for 10 minutes. The urea-treated enzyme was then assayed either in 4.5 M urea or in 0.5% gelatin for iodine-resistant and iodine-sensitive activity. The enzyme activity was also tested in 0.5% gelatin in the presence of anti-α-penicillinase serum. The results are summarized in Table 1. It will be seen that penicillinase recovered after exposure to 5 M urea regains its resistance to iodine. Moreover, as in the case of the untreated enzyme, the resistance to iodine may be correlated with the susceptibility to neutralization by anti-α-penicillinase serum, i.e. to the inhibition of the enzymatic activity by the antiserum.

**Effect of Guanidine Hydrochloride**

Since guanidine hydrochloride is a powerful hydrogen bond-breaking agent (4, 5), it was expected to cause an α → γ shift.
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tions its action in breaking hydrogen bonds predominates and the over-all effect is one of a sharp $\alpha \rightarrow \gamma$ shift.

Reversibility of the $\alpha \rightarrow \gamma$ Shift

Reversal by Dilution—The lowering of the concentration of urea by dilution causes a reversal from the $\gamma$-state to the $\alpha$-state, as judged both by loss of sensitivity to iodine (3) and by recovery of the antigenic identity of $\alpha$-penicillinase (Table I).

Further studies of the shift induced by urea or by guanidine hydrochloride have shown that the extent of the reversal depends on the initial concentration of the reagent, as well as on the extent of dilution. Typical results are presented in Table II. Of particular interest is the observation that exposure to 3.3 M guanidine hydrochloride causes a reversible inactivation of the enzyme and that all the activity recovered on dilution is of the

At low concentrations. The effect of guanidine hydrochloride on the activity of penicillinase was, therefore, tested in the same way as that of urea. The $\alpha \rightarrow \gamma$ shift as a function of the concentration of guanidine hydrochloride is presented in Fig. 2. The expected correlation between hydrogen bond-breaking capacity and occurrence of an $\alpha \rightarrow \gamma$ shift is borne out by the sharp drop in, and disappearance of, $\alpha$-type activity at concentrations up to 1.5 M guanidine hydrochloride. Higher concentrations of guanidine cause a gradual inactivation of the enzyme.

An inversion of the $\alpha \rightarrow \gamma$ shift has been observed at concentrations lower than 0.6 M guanidine hydrochloride. This apparent anomalous behavior is due to an interaction of the dilute enzyme solution with the glass surface of the vessel (see legend to Fig. 1) which, in the absence of electrolytes, causes an $\alpha \rightarrow \gamma$ shift. An increase in the ionic strength of the solution suffices to prevent transition to the $\gamma$-state. Thus, guanidine hydrochloride has a dual effect; at low concentration it acts as an electrolyte and favors an $\alpha \rightarrow \gamma$ shift, whereas at higher concen-
Effect of Electrolytes—The reversal of the $\alpha \to \gamma$ shift at low concentrations of guanidine hydrochloride has been ascribed to its electrolytic nature. No such effect has been observed in experiments with urea. On the other hand, a similar result has been obtained in dilute aqueous solutions of $\alpha$-penicillins assayed in the presence of other electrolytes, e.g. NaCl, NaNO$_3$, KCl. The effect of the concentration of the electrolyte on the state of penicillinase is illustrated in Fig. 3. It will be noted that a full reversal of the $\alpha \to \gamma$ shift caused by the adsorption on glass occurs at concentrations of 1.0 to 1.5 M NaCl. On further increase of the ionic strength, the enzyme is converted to the $\gamma$-state and the total activity decreases. As shown in Table III A, this shift is reversible. Moderate concentrations of electrolytes, e.g. 0.6 M NaCl, cause a marked reversal of the $\alpha \to \gamma$ shift induced by exposure to alkali or to urea. The shift can be prevented by increasing the ionic strength of the enzyme preparation (Table III).

The influence of polyelectrolytes on penicillinase in aqueous urea was also investigated. The addition of 1 mg of polycrylic acid or polyacrylic acid per ml of penicillinase solution in 3.5 M urea decreased the proportion of the enzyme in the $\gamma$-state from 53 to 20% of the activity, without any loss in total activity. The basic polyelectrolyte, polyvinylamine, and the nonionizable polyvinyl alcohol did not have any effect on the proportion of the enzyme present in the $\gamma$-state.

**Optical Rotation of Penicillinase**

Proteins possess in their molecules areas of regularity, due to their secondary and tertiary structure, and measurements of the optical rotation (15, 16) reflect the extent of the macromolecular asymmetry. In view of the changes in the conformation of proteins brought about in many cases by urea or guanidine hydrochloride and reflected in optical rotatory properties (5, 17, 18), it seemed of interest to examine the optical rotation of penicillinase under conditions causing changes in the type of enzymatic activity. Table IV summarizes the results obtained.

Penicillinase in the $\alpha$-state has a low levorotation, which increases slightly upon conversion to the $\gamma$-state. A high concentration of guanidine hydrochloride brings about an increase in the levorotation of penicillinase to a value similar to those of other denatured, unfolded, proteins. Under these conditions penicillinase is enzymatically inactive.

A significant increase in the levorotation was also obtained when the optical rotation of penicillinase was measured in dilute alkali. Under these conditions the enzyme changes to a form which is, after neutralization, active and sensitive to iodine (2).

**Effect of DFP**

Penicillinase cleaves an amide bond in penicillin. Several hydrolytic enzymes acting on peptide (amide) bonds are inactivated by DFP (19), whereas others, e.g. carboxypeptidase (20), maintain their activity in the presence of this reagent. It seemed, therefore, of interest to see if penicillinase, either in $\alpha$ or $\gamma$ state, is inactivated by DFP.

DFP, dissolved in isopropanol, was preincubated with penicillinase in water, 0.5% gelatin or 2 M guanidine hydrochloride, at 25° for 3 hours, and the enzymatic activity of penicillinase was subsequently measured iodometrically. It was found that 0.001 M DFP failed to inhibit either the $\alpha$ or the $\gamma$ state of penicillinase, even when tested at an excess of 10,000 moles DFP per mole of penicillinase. This suggests that no reaction with the active site has occurred.

**DISCUSSION**

The interconversion of the iodine-resistant and the iodine-sensitive forms of exopenicillinase may be accomplished with no appreciable changes in the total enzymatic activity. It follows, therefore, that the specific activity of the two forms of the enzyme is practically identical.

The optical rotation of penicillinase changes in the presence of urea and guanidine hydrochloride and parallels the iodine sensitivity of the enzyme. Up to a certain concentration these
Reagents cause almost no loss in total activity but bring about a complete change from iodine-resistant to iodine-sensitive type of activity. From the optical rotatory measurements it may be concluded that a small but definite change occurs in the configuration of the molecule. A drastic change in the configuration of penicillinase, at high molarities of the hydrogen bond-breaking agent, brings about the complete inactivation of the enzyme.

The configurational changes of penicillinase induced by moderate concentrations of urea or guanidine hydrochloride are easily reversed upon dilution as shown both by the antigenic identity of the recovered enzyme and by its resistance to iodine.

It is quite possible that the small conformational changes in the architecture of the enzyme have no influence on the configuration of the catalytic site of penicillinase, and resulted only in the exposure of a grouping sensitive to iodine. On the other hand it is conceivable that the identity of the kinetics of the two types of the enzyme is due to the fact that the enzyme in the γ-form reverts in the presence of the substrate to the α-form, a situation not unlike that observed in the case of ribonuclease (5), where the modified molecule reverts to the enzymically active conformation through contact with the substrate. The results of an investigation of the effect of penicillin on urea-modified penicillinase (11) are consistent with such an interpretation.

Penicillinase is a basic protein in which lysine and arginine account for one-fourth of its total nitrogen. Basic polypeptides such as polylysine are known to be strongly adsorbed on glass (21) and it is thus not surprising that glass surface adsorbs penicillinase. Under these conditions penicillinase is converted into the iodine-sensitive form of activity. Higher molarities cause a complete loss of the enzymic activity.

The observations on the reversible and irreversible transition to the iodine-sensitive form of exopenicilllnase may prove relevant to the mechanism of formation of the cell-bound enzyme, γ-penicillinase, which, according to the view presently maintained (22) is an irreversibly iodine-sensitive derivative of exopenicilllnase.

**SUMMARY**

1. A method of purification of the exopenicilllnase of Bacillus cereus is described. The substance was characterized by electrophoresis on polyvinyl particles and by sedimentation.

2. Penicillinase changes from an iodine resistant to an iodine-sensitive state in urea or guanidine hydrochloride. This change is reversible up to a certain concentration of the hydrogen bond-breaking agent, and becomes irreversible above this concentration. Higher molarities cause a complete loss of the enzymic activity.

3. As judged from optical rotatory measurements, a small but definite change in the configuration of the penicillinase molecule occurs upon the conversion from the iodine-resistant to the iodine-sensitive type of activity. The inactivation in high molarities of the hydrogen bond-breaking agent is accompanied by a drastic change in configuration.

4. The iodine-resistant form of penicillinase is stabilized by an increase in ionic strength, or by addition of acidic polyelectrolytes.

5. Neither form of penicillinase is inactivated by diisopropyl phosphorofluoridate.

**REFERENCES**


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