Interconversion of \( \alpha \)- and \( \gamma \)-Penicillinase from Bacillus cereus 569*

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SUMMARY

Extracellular penicillinase from Bacillus cereus 569 was found to exist in two states, an iodine-sensitive state and an iodine-insensitive state. Iodine-sensitive penicillinase is inactivated by \( 2.5 \times 10^{-8} \) m iodine within 1 min, while the iodine-insensitive enzyme is inactivated at a much slower rate. Approximately 20% of the extracellular penicillinase activity found in crude or partially purified preparations of penicillinase is iodine-sensitive. The two states of penicillinase were shown to be interconvertible. In the presence of saturated ammonium sulfate, penicillinase is converted quantitatively into the iodine-sensitive state, and, upon removal of the ammonium sulfate, it spontaneously reverts to the iodine-insensitive state.

It is concluded that \( \alpha \)- and \( \gamma \)-penicillinase correspond to the iodine-insensitive and iodine-sensitive penicillinases, respectively, and that the two penicillinases are different states of the same enzyme in dynamic equilibrium. The relative proportion of the two states depends upon the conditions imposed upon the enzyme preparation.

Bacillus cereus 569 is reported to produce two types of penicillinase: \( \alpha \), which represents approximately 95% of the penicillinase produced by the cell and is defined as totally extracellular and insensitive to iodine; and \( \gamma \), which represents approximately 5% of the total penicillinase produced and is defined as cell-bound, sensitive to iodine, and not neutralized by antiserum prepared against extracellular penicillinase (1, 2). A third form, \( \beta \), was also described; however, \( \beta \) is neutralized by antiserum prepared against extracellular penicillinase and appears to be a small fraction of the \( \alpha \)-penicillinase that is superficially adsorbed onto the cell. Partial purification of \( \gamma \)-penicillinase by ammonium sulfate precipitation was reported by Pollock (1). He compared various properties of \( \alpha \)- and \( \gamma \)-penicillinase and found that, although the two enzymes differed in the properties indicated above, their substrate specificities, Michaelis constants, and sedimentation rates were very similar.

Studies by Citri (2) showed that when \( \alpha \)-penicillinase is treated with base or adsorbed onto glass it acquires many of the characteristics of \( \gamma \)-penicillinase (i.e. it becomes iodine-sensitive and cannot be neutralized by antiserum prepared against extracellular penicillinase). Therefore, we undertook studies to determine whether \( \alpha \)- and \( \gamma \)-penicillinase are the same protein or two similar proteins.

Data presented in this communication show that the iodine-sensitive and the iodine-insensitive penicillinases are readily interconvertible. It is concluded that \( \alpha \)- and \( \gamma \)-penicillinase correspond to the iodine-insensitive and iodine-sensitive penicillinases, respectively, and that the two penicillinases are different forms of the same protein.

MATERIALS AND METHODS

Organism—B. cereus 569 was obtained from Dr. Martin Pollock. A variant strain of B. cereus 569, which is used in the commercial production of penicillinase, was obtained from Riker Laboratories, Northridge, California, and is referred to here as the Riker strain. The doubling time and amino acid requirements of the two strains are the same; however, they produce a slightly different penicillinase as shown by disc gel electrophoresis.

Preparation of Penicillinase—For these studies both a commercial preparation of penicillinase and enzyme partially purified in our laboratory were used. The commercial preparation (Neutrapen) was obtained from Riker Laboratories, Northridge, California, and taken up in distilled water and used directly (3).

Partial purification of penicillinase was accomplished as follows: Two 1-liter cultures of B. cereus 569 were grown overnight in media which contained per liter: trisodium citrate \( 2H_2O \), 5.9 g; (NH\(_4\)\(_2\))\(_2\)SO\(_4 \), 2 g; KH\(_2\)PO\(_4 \), 3 g; MgSO\(_4 \cdot 7H_2O \), 0.52 g; casamino acids, 10 g; glucose, 2 g. These cultures, in log phase, were used to inoculate 13.5 liters of previously warmed medium in a carboy. The culture was aerated by passage of air and in early log phase penicillinase synthesis was induced with 2 units per ml of benzylpenicillin. When exponential growth ceased the cells were harvested with a Szent-Györgyi and Blum continuous flow apparatus. To the spent medium was added 2 volumes of acetone and, after stirring, the solution was allowed

† This investigation was supported in part by National Institutes of Health Grants HD 02168 and HD 04831 and is Journal Paper J-6480 of the Iowa Agriculture and Home Economics Experiment Station, Ames.

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1 J. Imsande, unpublished observation.
to stand overnight at 4°. The liquid was decanted and the white precipitate was taken up in as little water as possible (usually 500 ml for a 13-liter culture). To remove any acetone that was carried over the solution was placed in an aspirator flask and maintained under reduced pressure. The crude preparation was flash-evaporated to about 70 ml. After dialysis for 3 hours against 2 volumes of \(2 \times 10^{-4} \text{ M EDTA, pH 7.0}\), the preparation was centrifuged and passed over a phosphocellulose column. Purified phosphocellulose was equilibrated with 0.005 M Tris-HCl, pH 8.1, at 4°. Enzyme was applied to the column (1.5 \times 15 \text{ cm}) at a flow rate of approximately 12 ml per hour. The column was then washed with 15 ml of 0.005 M Tris, pH 8.1, followed by 15 ml of 0.18 M NaCl in 0.005 M Tris, pH 8.1. Penicillinase was eluted from the column with 150 ml of 0.4 M NaCl in 0.005 M Tris, pH 8.1. The fractions with the highest specific activity were dialyzed as above, flash-evaporated to about 10 ml, and again dialyzed (see Imsande et al. (3)). All steps were carried out in the cold. The specific activity of crystalline penicillinase is approximately \(2 \times 10^4\) units per mg of protein (4).

**Enzyme Assay**—Enzyme preparations were diluted with 0.1 M phosphate buffer, pH 7.0, to a concentration of 10 to 100 units of enzyme per ml. At zero time 0-ml fractions of the diluted enzyme preparations were incubated at 30°. At 60 sec, 1 ml of iodine solution (2.5 \times \times 10^{-2} \text{ M iodine and 1.25 M KI}) was added to the enzyme solution (2). Enzyme samples were removed at 1-min intervals for 6 min and penicillinase activity was assayed (5). A control was always run simultaneously in which water instead of iodine was added to the previously incubated enzyme preparation.

A unit of penicillinase activity is defined as the amount of enzyme required to hydrolyze 1 \(\mu\text{ mole of penicillin in 0.05 M phosphate (pH 7.0) per hour at 30° (4).}

**Preparation of Washed Cells**—Cultures of \(B. \text{ cereus 569}\) or the Riker strain were grown to log phase, induced with 2 units per ml of penicillin, and harvested 1 hour later while still in log phase. Cell growth was stopped by adding \(1.6 \times 10^{-4}\) M hydroxyquinolin. An aliquot was kept on ice and the remainder of the culture was centrifuged. The supernatant was kept on ice and the cells were washed with cold 0.3 M phosphate buffer, pH 7.0. The 0.3 M phosphate wash was kept on ice and the cells were resuspended in 0.1 volume of 0.3 M phosphate. Whole culture, spent medium, wash, and washed cells were assayed for both iodine-sensitive and iodine-insensitive penicillinase activity.

**Treatment of Penicillin with Ammonium Sulfate**—Partially purified extracellular penicillinase was diluted 1:1000 with 0.1 M cold phosphate, pH 7.0, or with cold 0.1 M phosphate saturated with ammonium sulfate. These diluted enzyme preparations were kept on ice for 2 hours after which an aliquot of each was assayed for both iodine-insensitive and iodine-sensitive penicillinase. The remainder of the enzyme preparation was dialyzed against 2 changes of 200 volumes of the \(2 \times 10^{-4}\) M EDTA, pH 7.0. After 3 hours of dialysis the volumes of the dialyzed enzyme samples were measured to correct for any volume increase during dialysis. Each sample was again assayed for both iodine-insensitive and iodine-sensitive penicillinase.

**RESULTS**

**Iodine-sensitive Extracellular Penicillinase**—Under normal growth conditions approximately 90% of the penicillinase activity produced by \(B. \text{ cereus 569}\) is secreted by the cells into the culture medium. In an attempt to determine whether the extracellular penicillinase is composed only of \(\alpha\)-penicillinase activity (i.e. is insensitive to \(2.5 \times 10^{-4}\) M iodine) or whether it also contains \(\gamma\)-penicillinase activity (i.e. is partially sensitive to \(2.5 \times 10^{-4}\) M iodine), the penicillinase activity present in spent medium of either \(B. \text{ cereus 569}\) or the variant "Riker strain" of \(B. \text{ cereus 569}\) was assayed as described under "Materials and Methods." Enzyme preparations were previously incubated in the absence of iodine (○) or in the presence of iodine (△) prior to assay.

![Figure 1](http://www.jbc.org/)

**Fig. 1.** A and B, Iodine-sensitive penicillinase activity in spent culture medium. Penicillinase activity present in spent culture medium from \(B. \text{ cereus 569}\) (A) or from the variant Riker strain of \(B. \text{ cereus 569}\) (B) was assayed as described under "Materials and Methods." Enzyme preparations were previously incubated in the absence of iodine (○) or in the presence of iodine (△) prior to assay.
Commercial extracellular penicillinase (A) or penicillinase partially purified in the laboratory (B) was assayed as described under "Materials and Methods." Enzyme preparations were previously incubated in the absence of iodine (0) or in the presence of iodine (•) prior to assay.

![Graph](image)

**Table 1**

**Iodine-sensitive penicillinase associated with log phase culture**

Experiments were performed with *Bacillus cereus* 569 as described under "Materials and Methods."

| Culture fraction      | Units of penicillinase at zero time | Iodine-sensitive penicillinase |
|-----------------------|-------------------------------------|-------------------------------|
|                       | Total  | Iodine-insensitive | Units | Percentage of total |
| Whole culture         | 106    | 87                | 10    | 18                 |
| Spent medium (A)      | 84     | 47                | 37    | 44                 |
| Washed cells (B)      | 9      | 2.5               | 6.5   | 72                 |
| Cell wash (C)         | 2      | 1                 | 1     | 50                 |
| A + B + C             | 95     | 51                | 44    | 46                 |
| Recovery (%)          | 90     | 60                | 225   |                   |

iodine. These results show that iodine-sensitive penicillinase is either secreted by the cells or that penicillinase sensitivity to iodine can develop in the culture medium.

**Iodine-sensitive Penicillinase Associated with Log Phase Cultures** —Measurements similar to those described above were made on the various components of a log phase culture of *B. cereus* 569. Data presented in Table I show that approximately 18% of the penicillinase activity associated with a "whole" log phase culture is iodine-sensitive while approximately 46% of the penicillinase activity associated with the various components of the culture is iodine-sensitive. These data also show that while 90% of the total penicillinase activity was recovered only 60% of the iodine-insensitive penicillinase activity was recovered. On the other hand, 225% of the iodine-sensitive penicillinase activity was recovered. These data suggest that the iodine-insensitive penicillinase activity can be converted into an iodine-sensitive penicillinase activity.

**Conversion of Iodine-insensitive Penicillinase to Iodine-sensitive Penicillinase** —Since iodine-sensitive penicillinase was found to be a normal component of extracellular penicillinase and since the relative amount of iodine-sensitive penicillinase appeared to increase when the cells were collected and washed, an attempt was made to show directly the conversion of iodine-insensitive penicillinase to an iodine-sensitive penicillinase. It should be noted that the first iodine-sensitive penicillinase to be characterized had been obtained by precipitation with 90% saturated ammonium sulfate (1). Therefore, it was decided to examine the effect of saturated ammonium sulfate on extracellular penicillinase. Enzyme preparations were treated as described under "Materials and Methods." As shown in Fig. 3, preliminary incubation of penicillinase in saturated ammonium sulfate causes some inac-
sensitive penicillinase activities can be easily shown.

and Table II). Similar results were obtained with commercially

ated with the bacterial cell while all of the extracellular penicil-

interconversion between the iodine-insensitive and the iodine-

reversible. Data presented in Fig. 3 and Table II show that the

newly formed iodine-sensitive penicillinase activity is spontane-

ously converted back into an iodine-insensitive state when the

amount of iodine-insensitive penicilliiase activity in the same

nium sulfate-treated preparation increased by 62 units while the

amount of iodine-sensitive penicillinase activity in the ammo-

perimentation on the distribution of γ-penicillinase and on

(3)) experimentation on the distribution of γ-penicillinase and on

other data suggested that the iodine-insensitive and iodine-insensitive forms of penicillinase activity are approximately equal. Hence, the ammonium sulfate treatment appears to produce a quantitative interconversion between the two forms of penicillinase activity. From these data it is concluded that α- and γ-penicillinase correspond to the iodine-insensitive and iodine-insensitive forms of penicillinase activity. The ammonium sulfate treatment appears to produce a quantitative interconversion between the two forms of penicillinase activity. From these data it is concluded that α- and γ-penicillinase correspond to the iodine-insensitive and iodine-insensitive states, respectively, and that the two penicillinases are different conformational states of the same enzyme in dynamic equilibrium. This conclusion is supported by the observation that penicillinase can readily undergo reversible denaturation readily (3) and that attempts to separate the iodine-insensitive and the iodine-sensitive forms of penicillinase by acrylamide gel electrophoresis and phosphocellulose chromatography were unsuccessful.2

It is interesting to note that the γ-penicillinase activity associated with the bacterial cell is reported to be attached largely to the cell membrane (9). Since approximately 90% of the penicillinase activity is secreted by the cell (Table I), it is possible that the "membrane-bound" γ, which represents approximately 5% of the total penicillinase activity, is newly synthesized penicillinase that is undergoing secretion from the cell by some, as yet, unknown process. If this prediction is correct it implies that the secretion process requires approximately 5% of a cell doubling time, or about 2 min. Although it has been reported that the "membrane-bound" γ does not serve as a direct precursor to extracellular penicillinase, the data from which that claim was derived were obtained with nonexponentially growing cells and hence do not permit the cited conclusion (10).

2 Unpublished data.
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J. Biol. Chem. 1970, 245:3556-3560.

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