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Purification and Properties of Penicillin Amidase from *Bacillus megaterium*

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ABSTRACT

A penicillin amidase, obtained from the exogenous medium of a *Bacillus megaterium* culture, was purified approximately 96-fold by means of two cycles of adsorption on, and elution from, Celite, followed by a further fractionation on carboxymethylcellulose. On the basis of sedimentation centrifugation analysis, the final preparation was deemed to be homogeneous with an apparent molecular weight of approximately 120,000. The enzyme is specific for benzylpenicillin and has a *p*H optimum between 8 and 9. Complete hydrolysis of benzylpenicillin was obtained at low substrate concentrations. At higher substrate concentrations, the hydrolysis of benzylpenicillin was incomplete, apparently due to enzyme inhibition by phenylacetic acid and 6-aminopenicillanic acid, which were formed during the hydrolysis. Under the assay conditions, phenylacetic acid was a competitive inhibitor of penicillin amidase with an inhibitor constant (K_i) of 0.45 M, whereas 6-aminopenicillanic acid was noncompetitive in nature with a K_i of 2.6×10^{-2} M. The Michaelis constant of this enzyme was found to be 4.5×10^{-3} M when benzylpenicillin was used as substrate.

An enzyme which hydrolyzes penicillins to give carboxylic acid and 6-aminopenicillanic acid has been widely demonstrated in bacteria, yeast, and filamentous fungi (3, 8, 10, 14, 16, 17; R. C. Erickson and R. E. Bennett, Bacteriol. Proc., p. 65, 1961), as well as in plants (H. E. Alburn, N. H. Grant, and D. E. Clark, U.S. Patent 3,032,473, 1962) and in animals (G. A. Weitnauer, U.S. Patent 3,070,511, 1962). The enzyme has been given different names by various investigators, depending upon the particular experimental conditions and the results obtained (4, 9, 12). According to the source from which it is isolated, the penicillin amidase may be classified into two types. In one type, the enzyme, obtained from fungi and *Streptomyces* species, hydrolyzes phenoxymethylpenicillin much more readily than it does benzylpenicillin. In the second type, the enzyme, isolated from bacterial species, hydrolyzes benzylpenicillin much more readily than phenoxymethylpenicillin. Most of the published studies are concerned either with a crude cell-free preparation or with intact cells. This paper is a report on the purification and some characteristics of an extracellular penicillin amidase produced by a strain of *Bacillus megaterium* ATCC 14945.

MATERIALS AND METHODS

Substrates. The potassium benzylpenicillin used in these experiments was commerical grade material produced by E. R. Squibb & Sons, New Brunswick, N.J., with a potency of 1,500 units per mg. The ammonium salts of Δ^2-pentenyl- and *n*-heptyl penicillins, phenoxymethylpenicillin (free acid), allylmercaptomethylpenicillin (penicillin O), ethylthiomethylpenicillin, phenoxyethylpenicillin, 2(phenylthio)-ethylpenicillin, dimethoxyphenylpenicillin, and 5-methyl-3-phenyl-4-isoxazolylpenicillin were obtained from the collection of the Squibb Institute.

Determination of enzymatic activity. The enzymatic activity of the penicillin amidase preparations was determined by measuring the amount of 6-aminopenicillanic acid or phenylacetic acid produced from a reaction mixture containing 10 mg of benzylpenicillin in 1 ml of 0.1 M borate buffer, *p*H 8.7, and the enzyme after 20 min of incubation at 37 C. The hydroxylamine method of Batchelor et al. (1) was used for determination of the 6-aminopenicillanic acid, and the amount of phenylacetic acid was determined by the gas chromatographic method of Niedermayer (15). A unit of enzymatic activity is defined as the amount of enzyme required to produce 1 μmole of 6-aminopenicillanic acid (or phenylacetic acid) per hr under these test conditions. The rate of hydrolysis was linear between 0 and 10 units of enzyme per ml during the

first 30 min of incubation. Specific activity is defined as units of enzyme activity per milligram of protein.

Determination of protein and of ammonia. Protein was determined on enzyme preparations by use of the method of Lowry et al. (13), or spectrophotometrically by the method of Warburg and Christian (19) with bovine serum albumin as standard. The protein content in crude broth supernatant fluid was determined after the protein had been precipitated with 10% trichloroacetic acid and taken into 0.1 N NaOH solution. In those experiments in which the effect of the penicillin amidase on various amides was studied, the amount of ammonia released from the amides was determined by the method of Conway (6).

Growth of B. megaterium. The culture of *B. megaterium* ATCC 14945, obtained from S. Murao (University of Tottori, Tottori City, Japan), was maintained on agar slants and as lyophilized cell suspensions. For large-scale experiments, the culture was grown in 30-liter fermentors according to the method described in Example 3 by Murao et al. (U.S. Patent 3,144,395, 1964), in which phenylacetic acid is added to induce maximal production of penicillin amidase. After an incubation period of 70 to 72 hr, the fermentation broth was harvested and passed through a Westphalia centrifuge to remove the solids. The cell-free supernatant liquid was retained for recovery of the penicillin amidase.

Purification of enzyme. A batch of 34 liters of supernatant liquid, containing 20 enzyme units per ml, was acidified with dilute acetic acid to *p*H 6.4, and was mixed with acid-washed Celite (no. 545, Johns-Manville Co., New York, N.Y.) at a ratio of 15 g per liter. The suspension was stirred for 2 hr, during which period sufficient acetic acid was added to maintain the *p*H at 6.4. The Celite was collected by filtration and was washed once with 3.4 liters of distilled water. Approximately 95% of the original enzyme activity was recovered on the Celite, and less than 1% was lost by the water wash. The Celite cake was slurried in 24% ammonium sulfate (w/v) in 0.1 M tris(hydroxymethyl)-aminomethane (Tris) buffer (adjusted to *p*H 8.4 with NH_4OH) and was transferred to a glass column of suitable size (Celite column I). Elution of the enzyme from the Celite column I was carried out by further addition of the ammonium sulfate solution, and complete elution was obtained when approximately 2 liters of eluate had been collected. The eluate was concentrated in vacuo by maintaining it at 40 C in a water bath. The protein gradually precipitated from the solution, and the evaporation was halted at the first appearance of ammonium sulfate crystals. The precipitated protein was separated by filtration and was resuspended in a minimal amount of 0.05 M phosphate buffer, *p*H 7.0. The suspension was dialyzed against a 50-fold volume of 0.025 M phosphate buffer (*p*H 6.4), with three changes of buffer in a 24-hr period. The final solution was clarified by centrifugation, with a total yield of 58 ml.

The enzyme was further purified by fractionation on a carboxymethylcellulose column at 4 C. The column was prepared by applying pressure at 15 psi and by extensive washing with 0.025 M phosphate buffer, *p*H 6.4. A 45-ml amount of a solution containing 6 mg of protein per ml, which had been obtained from the previous step, was placed on a cellulose column (4.7 by 47 cm). The column was continuously eluted at a flow rate of 100 ml/hr by the addition of 0.025 M phosphate buffer, *p*H 6.4. About 750 ml of eluate was collected, and most of the impurities and pigmented materials were contained in this eluate. At this point, the eluting solution was changed to 0.1 M phosphate buffer, *p*H 6.5. Then, another 460 ml of eluate was collected before any appreciable activity was eluted. A subsequent 565-ml fraction, which contained most of the enzyme activity, was obtained. The pooled active eluate was mixed with acid-washed Celite at a ratio of 75 g of Celite per liter and was stirred for 1 hr. The Celite, upon which the enzyme was adsorbed, was collected, slurried with 24% ammonium sulfate in 0.1 M Tris buffer (*p*H 8.4), and transferred to a glass column (Celite column II). Additional ammonium sulfate solution was added to the column until 4 ml of eluate per g of Celite had been collected. This latter eluate was concentrated in vacuo, and the precipitated protein was recovered as described above for Celite column I. The protein was suspended in a minimal amount of 0.05 M phosphate buffer, *p*H 7.0, and was dialyzed against a 200-fold volume of the same buffer with three changes over a 16-hr period. The final volume of this solution was 10 ml. This preparation was used in most of the studies.

RESULTS

Purification. The data obtained in a typical enzyme purification experiment are presented in Table 1. The first cycle of Celite adsorption was evidently the most efficient step, since 95% of the enzyme was recovered from the fermentation broth in this fraction. During the process of concentrating the ammonium sulfate solution, it was frequently observed that a considerable amount of protein was coagulated on the liquid surface as insoluble material. This denatured protein no doubt accounts for the significant loss in enzyme activity in this step. In subsequent small-scale experiments, the percentage of recovery was usually higher than that obtained on the large column described above.

Ultracentrifuge studies. The final enzyme preparations were found to be homogeneous in molecular species when samples were examined in a Spinco model E ultracentrifuge equipped with a Schlieren diaphragm at an angle of 70°. In a typical experiment, in which the protein concentration was 0.8% in 0.05 M phosphate buffer (*p*H 7.0) after reaching a maximal speed of 44,700 rev/min for a period of 64 min at a temperature of 24 C, a single component was found in the sedimentation pattern. Figure 1 illustrates the sedimentation patterns taken at various time

TABLE 1. *Purification of penicillin amidase*

Fraction	Vol	Total activity[a] (× 10^5)	Total protein	Specific activity (units per mg)	Purification	Recovery
	ml	*units*	*mg*			%
Crude broth supernatant fluid	34,000	4.56	23,000	19.8	1	100
First-cycle Celite adsorption and ammonium sulfate precipitation	58	3.15	340	930	47	69
Carboxymethylcellulose column fractionation	565	2.36	160	1,470	75	52
Second-cycle Celite adsorption and ammonium sulfate precipitation	10	1.53	81	1,900	96	33

[a] Unit of enzyme activity is defined as the amount of enzyme required to produce 1 μmole of 6-aminopenicillanic acid (or phenylacetic acid) per hr in 0.1 M borate buffer (*p*H 8.7) at 37 C.

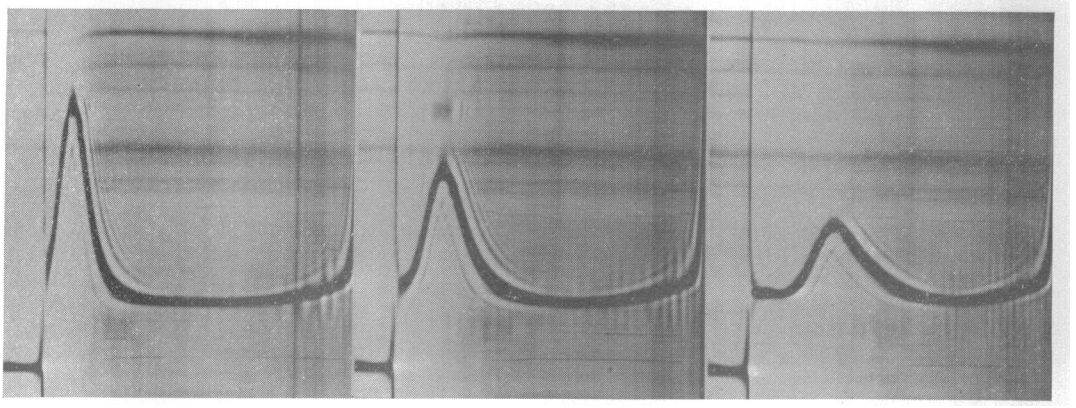

FIG. 1. *Sedimentation pattern of the purified penicillin amidase. Pictures taken at 16, 32, and 64 min after reaching 44,700 rev/min at 24 C.*

periods. The sedimentation constant of this enzyme protein was calculated from the data to be 5.5 Svedberg units. The apparent molecular weight was estimated to be about 120,000 in a separated sedimentation-equilibrium experiment conducted at 9,341 rev/min at 20 C for 47 hr, according to the method employed by Van Holde and Baldwin (18). (Thanks are due to James B. Ifft for his assistance in this experiment.) The partial specific volume of this protein was assumed to be 0.75 ml/g (7).

Stability. Samples of purified penicillin amidase in 0.05 M phosphate buffer, *p*H 7.0, were stable when stored at 4 C for a 3-month period. No change in activity was noted when enzyme samples were diluted with 0.1 M borate buffer, *p*H 8.4, to a protein concentration of 1 mg/ml and were stored for 8 hr at −20, 5, 24, and 37 C. With the same protein concentration, 70% of the enzyme activity was lost at 45 C, and the enzyme was completely denatured at 60 C. A study of penicillin amidase stability in solutions of different *p*H is summarized in Table 2. Maximal enzyme stability was observed at 24 C for 9 hr over the *p*H range of 6.5 to 10.05.

Optimal pH and temperature. The enzyme has an optimal *p*H range between 8 and 9. A rapid decrease in activity was observed when the *p*H was raised above 9.5 or lowered below 7.5 (see Fig. 2). The decrease in activity was not the result of chemical inactivation of substrate (benzylpenicillin) or of product (6-aminopenicillanic acid), since both compounds were found to be unchanged after incubation at 37 C for 20 min in solutions more alkaline than *p*H 10 and more acidic than *p*H 5.

The effect of temperature on the initial rate of enzyme reaction is summarized in Table 3. Although a temperature of 45 C was optimal for enzymatic activity, it was not suitable for routine use, because the enzyme protein was considerably inactivated at this temperature.

Substrate specificity. The penicillin amidase of *B. megaterium* was found to be rather specific for benzylpenicillin. Under the same assay conditions, it was much less active on other penicillins con-

TABLE 2. *Stability of enzyme solution at different pH levels*[a]

pH	Per cent maximal activity
4.15	0
5.15	74
6.5	100
7.2	100
9.0	100
10.05	100
10.8	2

[a] Enzyme solutions were diluted to 1 mg/ml with appropriate buffer, and were kept at 25 C for 9 hr. The treated enzyme solutions were further diluted before assay.

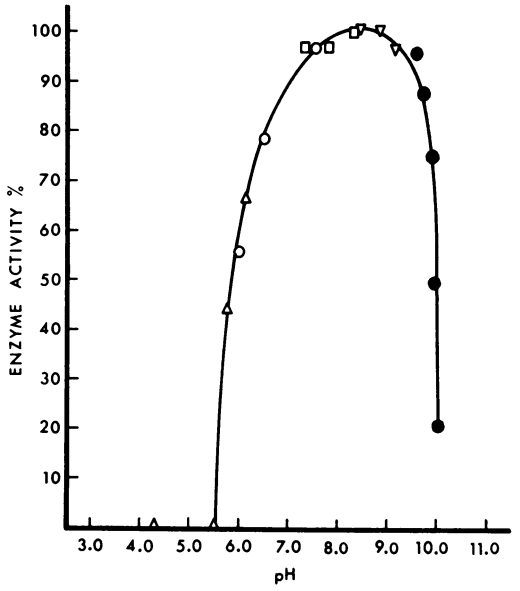

FIG. 2. *Effect of pH on reaction rate of penicillin amidase. The assay conditions were the same as those used in routine tests, except that the buffer system was changed as noted. The pH values were measured by use of a glass electrode 5 min after incubation. Symbols: △, acetate buffer; ○, phosphate buffer; □, Tris chloride; ▽, sodium borate; ●, carbonate hydrochloride.*

taining a variety of "side chains" instead of the phenylacetyl group. On the other hand, the 6-aminopenicillanic acid moiety was also necessary for the maximal rate of enzyme action. For instance, the enzyme hydrolyzed nonpenicillin derivatives of phenylacetic acid, such as phenylacetamide, at a much slower rate, as measured by the liberation of ammonia, than it hydrolyzed the 6-aminopenicillanic acid derivative of phenylacetic acid (i.e., benzylpenicillin). The ideal comparison would have been the measurement of the rate of hydrolysis of a derivative which is called cephalothin, which has, as its nucleus, 7-aminocephalosporanic acid. Unfortunately, this substance was not available to us at the time this investigation was being carried out. The enzyme had no activity on a number of other amides, dipeptides, polypeptides, and proteins. These data are summarized in Table 4.

Effect of substrate concentration and the in-

TABLE 3. *Effect of temperature on the rate of enzymatic reaction*[a]

Temp	Per cent of the maximal rate of hydrolysis
C	
15	27.3
22	52.5
30	72.5
36	85
40	95.5
45	100
50	91

[a] The reaction mixtures were the same as those described under routine assay procedure. The temperature conditions were as follows. The mixtures were incubated for 30 min at 22 C and below; 20 min, between 30 and 40 C; and 10 min, at 45 to 50 C. The rates of enzyme reactions at different temperatures were calculated by comparison with the values obtained during the first 10 min of incubation.

TABLE 4. *Substrate specificity of penicillin amidase*

Substrate	Rate of hydrolysis	Relative activity
	μmoles/hr	
Natural penicillins		
Benzylpenicillin	38.0	100
Δ^2-Pentenylpenicillin	6.2	16.3
Allylmercaptomethylpenicillin	4.0	10.5
Phenoxymethylpenicillin	1.3	3.4
n-Heptylpenicillin	0	0
Synthetic penicillins		
Ethylthiomethylpenicillin	4.0	10.5
Phenoxyethylpenicillin	1.5	4.0
2(Phenylthio)ethylpenicillin	0	0
Dimethoxyphenylpenicillin	0	0
5-Methyl-3-phenyl-4-isoxazolylpenicillin	0	0
Amide derivatives		
Phenylacetamide	8.7	23.0
N-methylphenylacetamide	3.8	10.0
DL-*N*-phenylacetylalanine	5.8	15.2
β-(α-Toluylamindo) propionic acid	5.4	14.2

hibition by hydrolysis products. Preliminary experiments showed that the initial rate of hydrolysis was stimulated by the increase in substrate concentration. This result indicates that the relation between the initial rate and the substrate concentration obeys the classical Michaelis-Menton equation. At a substrate concentration equal to or less than 2.7×10^{-2} M, complete hydrolysis was obtained with 5 units of enzyme in the reaction mixture after 4 hr of incubation at 37 C. However, with substrate concentrations greater than 2.7×10^{-2} M, the hydrolysis was not complete even after prolonged incubation. Furthermore, the enzymatic reaction was halted after the same maximal concentration of products was obtained, regardless of the substrate concentration employed. It was presumed that the enzymatic action was inhibited when a sufficient amount of products accumulated in the reaction mixture. Figure 3 is a graphic representation of the Lineweaver-Burk plots of the initial enzymatic reaction rates against different substrate concentrations with and without the addition of hydrolysis products. The data indicate that both products were inhibitors. Phenylacetic acid (Fig. 3, upper half) acted as a competitive inhibitor with an inhibitor constant of 0.45 M, whereas the 6-aminopenicilanic acid acted as a noncompetitive inhibitor with an inhibitor constant equal to 2.6×10^{-2} M (Fig. 3, lower half). The Michaelis constant of the enzyme, with benzylpenicillin as substrate, was estimated from both graphs to be 4.5×10^{-3} M.

Inhibitors. No stimulation of enzymatic activity was observed when divalent metal ions were added under the modified assay conditions. In contrast, some of the metal ions, such as Co^{+2}, Fe^{+2}, Ni^{+2}, and Mn^{+2}, at levels between 10^{-5} and 10^{-3} M, produced an inhibitory effect on the enzyme activity to the extent of 10 to 30% inhibition. Several sulfhydryl reagents, such as *p*-chloromercuribenzoate at 0.001 M, or *o*-iodosobenzoate and iodoacetate at 0.01 M, had no effect on penicillin amidase.

DISCUSSION

Penicillin amidase, an extracellular bacterial enzyme, when purified as described above, appeared to be homogeneous in molecular species based upon the criterion of sedimentation analysis. The best substrate for enzyme activity, among the various compounds tested, was found to be benzylpenicillin. For maximal enzyme activity, 6-aminopenicillanic acid and phenylacetic acid,

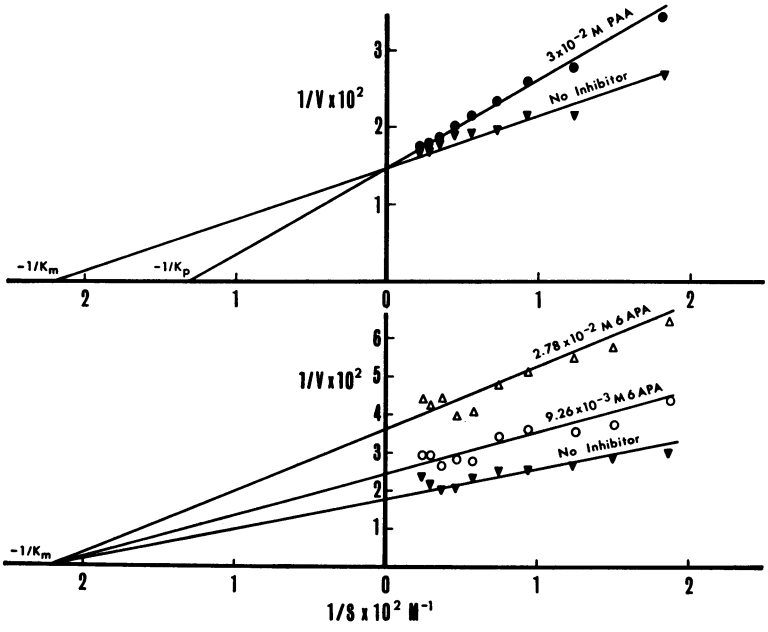

FIG. 3. *Lineweaver-Burk Plots of initial rate at different substrate levels with or without the preaddition of products. In the upper half, curves were obtained in the presence of phenylacetic acid (PAA) by the measurement of production of 6-aminopenicillanic acid (6APA); in the lower half, curves were obtained in the presence of 6 APA by the measurement of PAA.*

which are moieties of benzylpenicillin, were required. Although this penicillin amidase could utilize as substrate various penicillins other than benzylpenicillin, or certain derivatives of phenylacetic acid, the reaction rates in these experiments were much slower. Therefore, in view of the substrate specificity required for penicillin amidase activity, it appears unlikely that this enzyme can be classified as either a deacylase, as suggested by Cole (5), or an acyl transferase, as suggested by Kaufmann and Bauer (11). For the same reasons, the more correct nomenclature for this enzyme would appear to be penicillin amidase, according to Sakaguchi and Murao (17), rather than benzylpenicillin acylase, as suggested by Huang et al. (9).

Although no attempt was made to measure the residual enzyme bound on the cells, most of the enzyme appears to be excreted into the exogenous medium. The maximal growth of the bacterial cells occurred after an incubation period of 20 to 24 hr. At this time, the extracellular enzyme concentration began gradually to increase, and continued during the observation period of 96 hr. There was no evidence of lysis in the bacteria throughout the incubation period.

The role played by penicillin amidase in the metabolism of *B. megaterium* is still obscure. Although the cell releases this penicillin-inactivating enzyme into the medium, the growth of the organism remains sensitive to benzylpenicillin. The inhibition of bacterial growth by benzylpenicillin was observed by use of several media containing antibiotic concentrations as low as 0.1 μg/ml (Basch, *personal communication*).

Batchelor et al. (2) observed that, although 6-aminopenicillanic acid inhibited the enzymatic hydrolysis of phenoxymethylpenicillin by *Streptomyces lavendulae* BRL. 198, phenoxyacetic acid was without effect. However, as noted above, both 6-aminopenicillanic acid and phenylacetic acid were shown to inhibit penicillin amidase obtained from *B. megaterium*. Since penicillin amidase of *B. megaterium* and the enzyme obtained from *S. lavendulae* are both penicillin-hydrolyzing enzymes, these results appear to be inconsistent. This seeming inconsistency may be resolved if one considers that the inhibition of the enzyme obtained from *S. lavendulae* by 6-aminopenicillanic acid could be easily demonstrated because of the noncompetitive character of the compound, with an inhibitor constant close to the value of the Michaelis constant of the enzyme. Conversely, the inhibitory effect of phenylacetic acid may not have been detected, because it would appear to be a competitive inhibitor of the enzyme with a much greater inhibitor constant.

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LITERATURE CITED

1. BATCHELOR, F. R., E. B. CHAIN, T. L. HARDY, K. R. L. MANSFORD, AND G. N. ROLINSON. 1961. 6-Aminopenicillanic acid. III. Isolation and purification. Proc. Roy. Soc. (London) Ser. B **154**:498–508.
2. BATCHELOR, F. R., E. B. CHAIN, M. RICHARDS, AND G. N. ROLINSON. 1961. 6-Aminopenicillanic acid. VI. Formation of 6-aminopenicillanic acid from penicillin by enzymic hydrolysis. Proc. Roy. Soc. (London) Ser. B **154**:522–531.
3. CLARIDGE, C. A., A. GOUREVITCH, AND J. LEIN. 1960. Bacterial penicillin amidase. Nature **187**: 237–238.
4. CLARIDGE, C. A., J. R. LUTTINGER, AND J. LEIN. 1963. Specificity of penicillin amidases. Proc. Soc. Exptl. Biol. Med. **113**:1008–1012.
5. COLE, M. 1964. Properties of penicillin deacylase enzyme of *Escherichia coli*. Nature **203**:519–520.
6. CONWAY, E. J. 1947. Microdiffusion analysis and volumetric error. Cosby Lockwood and Sons, Ltd., London, England.
7. EDSALL, J. T. 1953. The size, shape and hydration of protein molecules, p. 549. *In* H. Neurath and K. Bailey [ed.], The proteins, vol. I, part B. Academic Press, Inc., New York.
8. HUANG, H. T., A. R. ENGLISH, T. A. SETO, G. M. SHULL, AND B. A. SOBIN. 1960. Enzymatic hydrolysis of the side chain of penicillins. J. Am. Chem. Soc. **82**:3790–3791.
9. HUANG, H. T., T. A. SETO, AND G. M. SHULL. 1963. Distribution and substrate specificity of benzylpenicillin acylase. Appl. Microbiol. **11**: 1–6.
10. KAMEDA, Y., Y. KIMURA, E. TOYOURA, AND T. OMARI. 1961. A method for isolating bacteria capable of producing 6-aminopenicillanic acid from benzylpenicillin. Nature **191**:1122–1123.
11. KAUFMANN, W., AND K. BAUER. 1964. Variety of substrates for a bacterial benzylpenicillin-splitting enzyme. Nature **203**:520.
12. KAUFMANN, W., K. BAUER, AND H. OFFE. 1960. Enzymatic cleavage and resynthesis of penicillins. Antimicrobial Agents Ann., p. 1–5.
13. LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR, AND R. J. RANDALL. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
14. MURAO, S., AND Y. KISHIDA. 1961. Studies on penicillin amidase. Part V. Studies on the penicillin-amidase producing microorganisms. J. Agr. Chem. Soc. Japan **35**:610–615.
15. NIEDERMAYER, A. O. 1964. Determination of

phenylacetic acid in penicillin fermentation media by means of gas chromatography. Anal. Chem. **193**:938–939.

16. ROLINSON, G. N., F. R. BATCHELOR, D. BUTTERWORTH, J. CAMERON-WOOD, M. COLE, G. C. EUSTACE, M. V. HART, M. RICHARDS, AND E. B. CHAIN. 1960. Formation of 6-aminopenicillanic acid from penicillin by enzymatic hydrolysis. Nature **187**:236–237.
17. SAKAGUCHI, K., AND S. MURAO. 1950. A preliminary report on a new enzyme, "penicillinamidase." J. Agr. Chem. Soc. Japan **23**:411.
18. VAN HOLDE, K. E., AND R. L. BALDWIN. 1958. Rapid attainment of sedimentation equilibrium. J. Phys. Chem. **62**:734.
19. WARBURG, O., AND W. CHRISTIAN. 1941. Isolierung und Kristallisation des gärungsferments enolase. Biochem. Z. **310**:384–421.