# The Interaction between Lysozyme and Penicillin

H. FELSENFELD AND R. E. HANDSCHUMACHER

*Department of Pharmacology, Yale University School of Medicine, New Haven, Connecticut 06510*

(Received October 11, 1966)

SUMMARY

The hydrolytic action of lysozyme (muramidase) was inhibited 50% by benzylpenicillin (24 mM) in 0.1 M phosphate buffer at pH 7.0. 7-(Thiophene-2-acetamido)cephalosporin (cephalothin) exerted a similar inhibitory action.

Modification of some of the functional groups of these molecules, with a resultant decrease in their structural similarity to *N*-acetylmuramic acid, destroyed their inhibitory activity. Integrity of the lactam ring was not essential for activity.

Fluorescence measurements and ultraviolet difference spectroscopy indicated the formation of an enzyme–inhibitor complex. Penicillin and *N*-acetylglucosamine produced a perturbation peak at 293 mμ when mixed with the enzyme. *N*-Bromosuccinimide oxidation of the tryptophan residue in position 62, one of six tryptophan units in the enzyme molecule, destroyed enzymatic activity. This modification reduced the size of the perturbation peak induced by penicillin and eliminated the peak caused by *N*-acetylglucosamine.

The experimental results agree with previous indications that penicillin bears a structural resemblance to a portion of the bacterial cell wall, *N*-acetylmuramic acid.

In these studies, lysozyme has been used as a model for demonstrating the interaction between penicillin and an enzyme protein. The concentrations of penicillin required for inhibition of lysozyme are far greater than the level of the drug required for the inhibition of sensitive microorganisms.

## INTRODUCTION

The development of several new physical techniques for the characterization of molecules has made it possible to construct molecular models of enzymes, substrates, and inhibitors, and to predict to some degree their interactions. This paper describes an attempt to determine whether the structural similarity of penicillin and *N*-acetylmuramic acid, observed by constructing models of the molecules (1), can be demonstrated by using a biological system.

The position of the functional groups and the sites of potential hydrogen or ionic bonding have been stated to be similar in penicillin and *N*-acetylmuramic acid (Fig. 1) (1). If such a structural similarity between the two compounds exists in solution, it would be reasonable to expect that penicillin and *N*-acetylmuramic acid would compete for the same binding site on an enzyme. Lysozyme (muramidase, *N*-acetylmuramide glycanhydrolase, EC 3.2.1.17), was used as the model enzyme since it hydrolyzes the 1,4-glycosidic linkage between *N*-acetylmuramic acid and *N*-acetylglucosamine in substrates of either synthetic or natural origin. This enzyme also catalyzes a transglycosylation reaction between the fragments that are produced (2). Thus, lysozyme has sites that bind both *N*-acetylmuramic acid and *N*-acetylglucosamine, as demonstrated by studies with synthetic substrates (3). X-ray crystallographic data suggested that penicillin is bound at a specific site, believed to be the *N*-acetylmuramic site, when peni-

cillin was allowed to diffuse into preformed crystals of lysozyme (3).

In this study, the interaction of penicillin with lysozyme was investigated by observing the effect of the antibiotic on the enzymatic lysis of bacterial cells and by studying the binding of penicillin to the

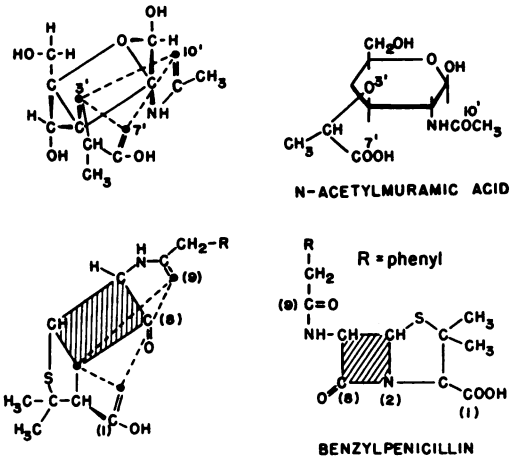


FIG. 1. *Structure of penicillin and N-acetylmuramic acid*

enzyme using fluorimetric measurements and ultraviolet difference spectroscopy. The effect of structural modifications of the penicillin molecule on the inhibitory activity and binding was also determined.

It must be recognized that inhibition of the action of lysozyme requires much higher levels of penicillin than are needed for inhibition of sensitive microorganisms.

## MATERIALS AND METHODS

*Assays.* The assays were performed by the method of Shugar (4), using acetone-dried cells of *M. lysodeikticus* and 2× recrystallized lysozyme, purchased from Worthington Biochemical Company. A Beckmann DB spectrophotometer with an automatic recorder was used to measure initial reaction rates at 450 mμ. In some experiments, 3× recrystallized lysozyme from Gallard-Schlesinger Corporation was used. Potassium benzylpenicillin was purchased from Nutritional Biochemicals and *N*-acetylglucosamine from Mann Laboratories. 6-Aminopenicillanic acid and α-phenoxyethylpenicillin (phenethicillin) were supplied by Bristol Laboratories. Potassium benzylpenicilloic acid was prepared from potassium benzylpenicillin by alkaline hydrolysis and also by treatment with penicillinase (Neutrapen, Riker Laboratories) (5). A Radiometer automatic titrimeter was used to assay the progress of the reaction and to test the potency of the penicillinase preparation. 7-(Thiophene-2-acetamido) cephalosporin sodium (cephalothin) and other cephalosporin derivatives were supplied by Eli Lilly and Co.

Fluorescence spectra were obtained with an Aminco-Bowman spectrophotofluorometer using the continuous scan mechanism and a Beckmann recorder attached to the fluorometer. The fluorescence of penicillin required that activation and measurements be made at longer wavelengths than are optimal for activation of tryptophan in proteins. Ultraviolet difference spectroscopy measurements were made with a Cary model 15 automatic recording spectrophotometer, using matched split cells, according to the method of Laskowski (6). *N*-Bromosuccinimide, for enzyme oxidation, was purchased from Aldrich Chemical Company and recrystallized from acetone (7).

## RESULTS

### *Penicillin Inhibition of Lysozyme*

Potassium benzylpenicillin, 24 mM, inhibited the lysis of a suspension of *M. lysodeikticus* by lysozyme, as measured by the decrease in optical density with time (Fig. 2). As a control on the possible effect of inorganic ions, the effect of 24 mM KCl or NaCl was tested; these inorganic ions could account for a maximum of 20% of the observed inhibition. Potassium benzylpenicillin, 48 mM, produced almost complete inhibition of lysis. Preincubation of the drug with the enzyme, or with the cells, for 1 hour did not affect the degree of inhibition.

Initial reaction velocities were measured with a constant amount of enzyme and varying substrate concentrations. The data obtained indicated that penicillin was not so strongly bound to the cells as to eliminate them as a substrate (Fig. 3).

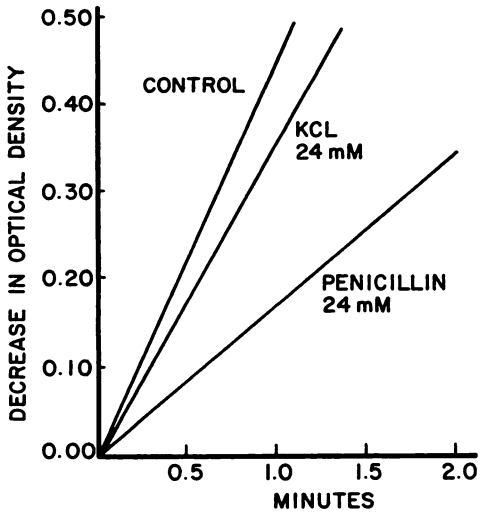


FIG. 2. *Effect of penicillin on lysozyme activity*

The incubation mixture consisted of 0.68 mg of acetone-dried cells of *Micrococcus lysodeikticus* (1.5 mg/ml), in 0.1 M potassium phosphate, pH 7.0; 10 μg of lysozyme (3×-recrystallized); and KCl and penicillin in 0.1 M potassium phosphate, pH 7.0, in a final volume of 3 ml.

The effect of changing the concentration of the enzyme is shown in Fig. 4. Initial reaction velocities, measured at varying concentrations of potassium benzylpenicillin, indicated that the enzyme was not titrated in an irreversible manner by the drug.

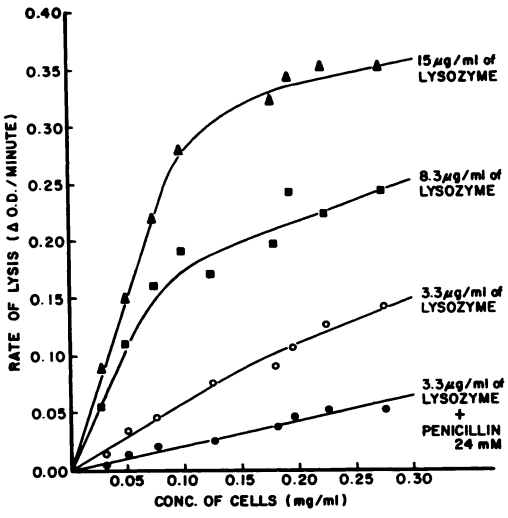


FIG. 3. *Effect of cell concentration on lysozyme activity and penicillin inhibition*

The incubation mixture was the same as for Fig. 2, except that 2×-recrystallized lysozyme was used.

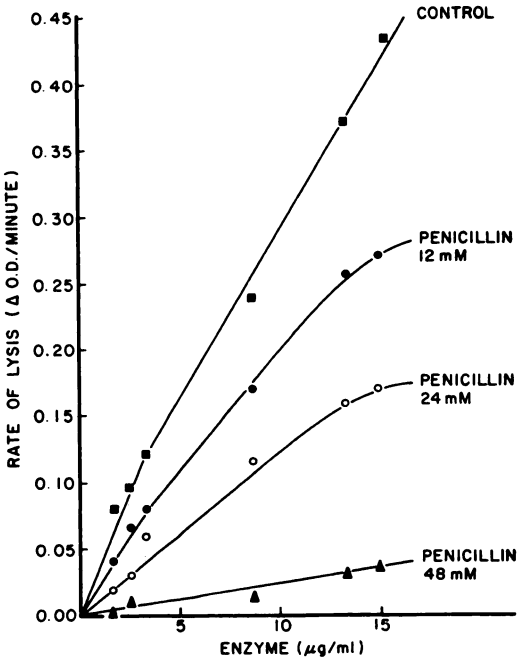


FIG. 4. *Effect of enzyme concentration on penicillin inhibition*

The incubation mixture was the same as for Fig. 2, except that 2×-recrystallized lysozyme was used.

*N*-Acetylglucosamine has been shown to be a competitive inhibitor of lysozyme (3). The kinetics of enzyme inhibition by penicillin also were those of a typical competitive inhibitor (Fig. 5); since the substrate concentration cannot be defined in molar units, a $K_i$ has not been calculated.

The inhibition of the reaction caused by 24 mM potassium benzylpenicillin was equivalent to that observed with 24 mM *N*-acetylglucosamine. When both compounds were present in the reaction mixture at this concentration their inhibitory effects were approximately additive (Fig. 6). Although the presence of 0.1 M Tris buffer, pH 7.0, prevented the inhibitory effect of potassium benzylpenicillin, the inhibitory effect of *N*-acetylglucosamine on lysozyme was unaffected; a similar lack of inhibitory action was observed with penicillin in 0.1 M imidazole buffer at pH 7.0, 0.1 M glycyl-

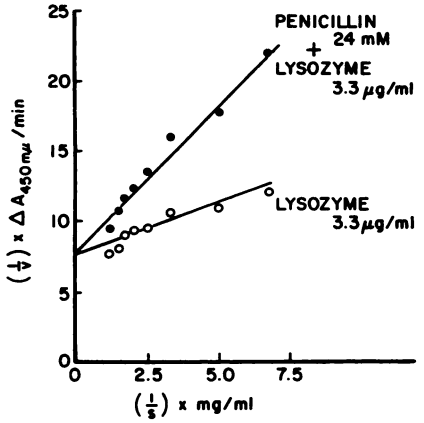


FIG. 5. *Kinetics of inhibition of lysozyme by penicillin*

The incubation mixture was the same as for Fig. 2, except that 2×-recrystallized lysozyme was used, and an optical wedge was inserted into the sample cuvette to reduce the light path to 2 mm.

glycine buffer at pH 8.0 and phosphate buffers of lower ionic strength.

A number of compounds derived from the penicillin molecule have been tested for activity. The results are shown in Table 1. The replacement of the benzyl group by the α-phenoxyethyl group (phenethicillin) did not decrease the inhibitory effect of penicillin, but cleavage of the benzylpenicillin molecule produced inactive compounds, as seen with 6-aminopenicillanic acid and phenylactic acid. On the other hand, benzylpenicilloic acid, prepared by either alkaline hydrolysis or penicillinase cleavage of the β-lactam ring, was as inhibitory as an equimolar concentration of penicillin. Cephalothin sodium, 24 mM, produced an inhibitory effect that was approximately equivalent to that observed with potassium benzylpenicillin (Table 1). Several other cephalosporin derivatives were tested for inhibitory activity (Fig. 7); thus, 7-amino-cephalosporanic acid (*a*) was inactive, as was the pyridinium derivative (*b*). A derivative of cephalosporin with an aliphatic side chain (*c*) and one with a substituted carboxyl group (*d*) produced much less inhibition than cephalothin (Fig. 7).

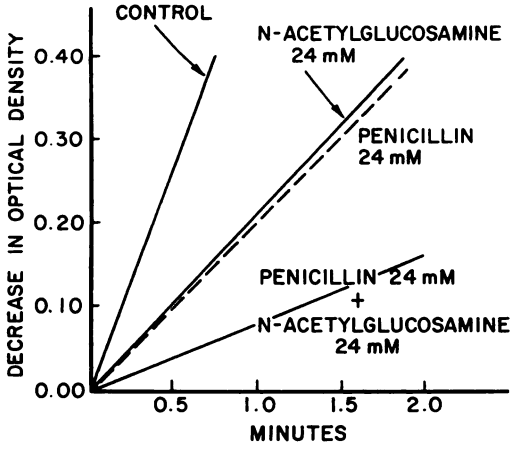


FIG. 6. *Effect of penicillin and N-acetylglucosamine on lysozyme activity in phosphate buffer, 0.1 M, pH 7.0*

The incubation mixture was the same as for Fig. 2, except that 30 μg of 2×-recrystallized lysozyme was used.

TABLE 1

*Effect of benzylpenicillin and related compounds on lysozyme activity*

| Compound | Concentration (mM) | Per cent inhibition of lysis[a] |
|---|---|---|
| Potassium benzylpenicillin | 24 | 42.5 |
| Potassium benzylpenicillin | 48 | 87.5 |
| α-Phenoxyethylpenicillin | 17 | 30.0 |
| 6-Aminopenicillanic acid | 24 | 1.5 |
| 6-Aminopenicillanic acid | 48 | 1.5 |
| Phenylacetic acid | 24 | 3.0 |
| Cephalothin sodium | 24 | 48.5 |
| Benzylpenicilloic acid[b] | 24 | 50.4 |
| Benzylpenicilloic acid[c] | 24 | 48.2 |

[a] Incubation mixture was the same as for Fig. 2 except that 2×-recrystallized lysozyme was used. Percentage of inhibition was measured by comparing the decrease in optical density with the control for the first minute of the reaction. The experimental values are the average of two determinations.

[b] Prepared by alkaline hydrolysis of potassium benzylpenicillin.

[c] Prepared by treatment of potassium benzylpenicillin with penicillinase.

## *Spectroscopy*

Fluorescence measurements (Table 2) showed a decrease in fluorescence emission when penicillin and lysozyme were mixed, relative to the values obtained with com-

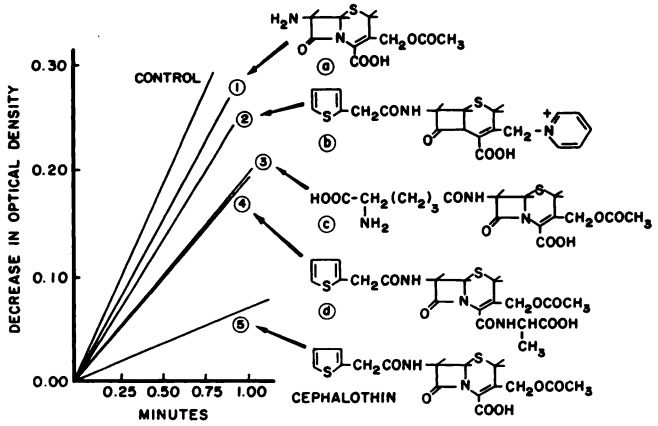


FIG. 7. *Effect of cephalothin and its derivatives on lysozyme activity*

The incubation mixture was the same as for Fig. 2, except that 30 μg of 2×-recrystallized lysozyme was used. The concentration of the inhibitors was 24 mM.

parable solutions of penicillin or lysozyme examined independently. These data were obtained by activation at 320 mμ and 365 mμ, while emission at 350 mμ and 400 mμ, respectively, were recorded. Since the absorbance of these solutions at 350μ and 400 mμ was less than 0.010, this was not responsible for the decreased emission.

The interaction between penicillin and lysozyme also was demonstrated by ultraviolet difference spectroscopy (Fig. 8). A peak at 293 mμ was observed, characteristic of perturbation of the absorbance of tryptophan residues in proteins (8).

Mixtures of *N*-acetylglucosamine and lysozyme showed a similar peak that was additive with that produced by penicillin, but 6-aminopenicillanic acid, which did not inhibit the enzyme, did not cause the appearance of a peak at 293 mμ when mixed with the enzyme. Penicillin, but not *N*-acetylglucosamine, gave the characteristic perturbation peak with tryptophan monomer at 291 mμ (8). Tryptophan monomer, at a concentration (0.84 mM) equivalent to the tryptophan content of the enzyme solution gave a peak of equal size to that observed with lysozyme in the presence of 50 mM penicillin.

To attempt a localization of the intramolecular site of binding of the inhibitors, three times-recrystallized lysozyme was oxidized according to the method of Hayashi *et al.* (7, 8), with 3 moles of *N*-bromosuccinimide per mole of enzyme in 0.1 M potassium acetate pH 4.0, at an

TABLE 2

*Quenching of lysozyme fluorescence by penicillin*

| Sample | Fluorescence emission | |
|---|---|---|
| | Activating wavelength 320 mμ Fluorescence wavelength 365 mμ | Activating wavelength 350 mμ Fluorescence wavelength 400 mμ |
| | (intensity units) | (intensity units) |
| A = Enzyme[a] | 69 | 88 |
| B = Penicillin (25 mM) | 32 | 78 |
| C = Enzyme[a] + penicillin (25 mM) | 59 (A + B = 101)[b] | 71 (A + B = 166)[b] |
| D = Penicillin (50 mM) | 56 | 59 |
| E = Enzyme[a] + penicillin (50 mM) | 60 (A + D = 125)[b] | 64 (A + D = 147)[b] |

[a] Lysozyme 33 μg/ml in 0.1 M $KPO_4$, pH 7.0, at 25°.

[b] Figures within parentheses represent the calculated emission for the mixture.

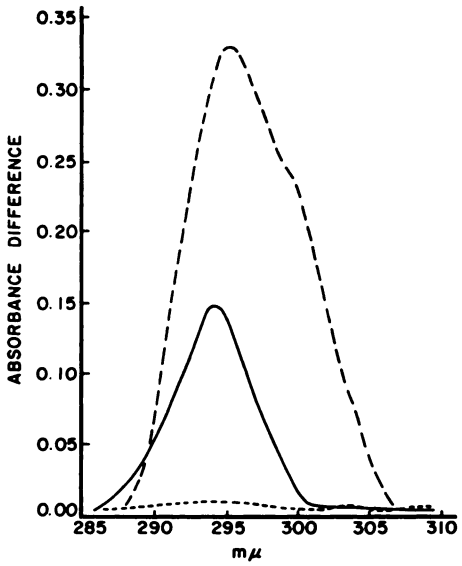


FIG. 8. *Ultraviolet difference spectra*

*N*-Acetylglucosamine ———, benzylpenicillin ———, and 6-aminopenicillanic acid --- were present at a concentration of 100 mM in 1 ml of 0.1 M potassium phosphate, pH 7.0, in one compartment of the cell. The second compartment contained 2 mg of lysozyme (2×-recrystallized) dissolved in 1 ml of the same buffer.

enzyme concentration of 5 mg/ml; a control preparation was treated in a similar fashion except that *N*-bromosuccinimide was omitted. Previous workers (7) have indicated that the oxidative procedure converts only the tryptophan residue at position 62 (Fig. 9) to a derivative, without major changes in the secondary or tertiary structure of the protein. The oxidized enzyme was used for ultraviolet difference spectroscopy experiments (Table 3). Although enzymatic activity and the perturbation caused by *N*-acetylglucosamine was completely eliminated by this treatment, the perturbation caused by penicillin was reduced by only 16%.

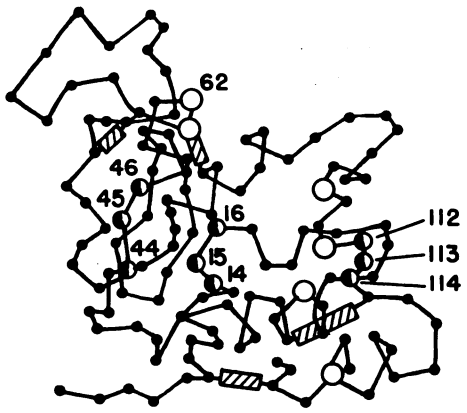


FIG. 9. *Lysozyme*

○, Tryptophan; crosshatched area, disulfide bridge; ◐-◐-◐-◐, basic amino acid cluster. Redrawn from a figure by Sir Lawrence Bragg for a paper by Blake *et al.* (9).

TABLE 3
*Ultraviolet difference spectroscopy with oxidized lysozyme*[a]

| Mixture components[b] | Absorbance difference, 293 mμ |
|---|---|
| Native enzyme + penicillin | 0.027 |
| Control enzyme[c] + penicillin | 0.025 |
| Oxidized enzyme + penicillin | 0.021 |
| Native enzyme + *N*-acetylglucosamine | 0.043 |
| Control enzyme[c] + *N*-acetylglucosamine | 0.038 |
| Oxidized enzyme + *N*-acetylglucosamine | 0.000 |

[a] 3×-recrystallized enzyme. Activity loss after oxidation = 99%.

[b] Enzyme concentration after mixing = 1 mg/ml. Inhibitor concentration after mixing = 50 mM.

[c] 3×-recrystallized enzyme subjected to the same treatment as the oxidized enzyme with the omission of *N*-bromosuccinimide.

## DISCUSSION

These experiments have shown that benzylpenicillin inhibits the lysis by the enzyme lysozyme (muramidase) of the cells of *M. lysodeikticus* (previously dried with acetone). This inhibition was observed in potassium phosphate buffer under the conditions of the standard assay procedure. Unlike the results with *N*-acetylglucosamine, the inhibitory effect of penicillin was not observed in certain buffers, a finding which suggests that important differences in the binding of these inhibitors exists.

The ultraviolet and fluorescence spectroscopic evidence for complex formation between lysozyme and either *N*-acetylglucos-

amine or penicillin corresponds closely with the data obtained by Hayashi (8, 10) and Laskowski (6) and their associates for substrate-lysozyme interaction. A characteristic peak was seen at 293 mμ when either penicillin or *N*-acetylglucosamine was mixed with lysozyme, and at 291 mμ when penicillin and tryptophan monomer interact. These findings support the concept that the inhibitors associate with one or more of the tryptophan residues of the enzyme. The perturbation produced by *N*-acetylglucosamine was completely abolished by oxidation of the tryptophan residue in position 62 of the lysozyme molecule, and the enzyme activity was completely destroyed. The perturbation induced by penicillin, however, was reduced by only one-sixth, a finding which reinforces the idea that important differences exist in the binding of these two compounds. It suggests that penicillin may bind to all (six) tryptophan residues of the enzyme; this result is also consistent with the additive effect of penicillin and *N*-acetylglucosamine on the size of the perturbation peak. The X-ray crystallographic evidence, reported by Johnson and Phillips (3), however, indicated that penicillin was bound at only one site in the crystalline state. This may reflect the special nature of this binding site on the protein. Its location adjacent to the *N*-acetylglucosamine-binding site, as reported in the crystallographic studies, is consistent with such a hypothesis. This specific binding of penicillin may involve the formation of a complex between the ring system of tryptophan and the phenacyl group of benzylpenicillin, plus bonding of the strongly acidic carboxyl group with basic amino acid sequences such as lysine-14, arginine-15, histidine-16; asparagine-44, arginine-45, asparagine-46; or arginine-112, asparagine-113, arginine-114 (Fig. 9). This association, involving the tryptophan residue at position 62, may induce a conformational change in the enzyme that results in the retention of one molecule of penicillin in the crystalline state.

An examination of the activity of the penicillin and cephalosporin series of derivatives suggests that an aromatic side chain and a free carboxyl group are necessary for inhibitory activity. Modifications of the functional groups or either penicillin or related compounds, with a resultant decrease in their structural similarity to *N*-acetylmuramic acid, either eliminated or reduced their inhibitory activity. One unique feature of these experiments was the inhibitory effect of benzylpenicilloic acid, a compound that lacks antibacterial activity. Prior cleavage of the β-lactam ring may prevent acylation of a site of action associated with the bactericidal effect of penicillin, the transpeptidase or cross-linking enzyme (11, 12), but it does not interfere with the association of the molecule and lysozyme. The opening of the ring does not change the position of the nitrogen atom and does not eliminate the binding potential of the C-8 carbonyl group (Fig. 1).

Lysozyme is not the locus, described by Strominger and Park and their associates (13–15), at which penicillin acts as a bactericidal agent since a much greater concentration of penicillin is required for inhibition of lysozyme. The antibiotic action of penicillin, however, may require an association with an *N*-acetylmuramic-like receptor site that possesses some of the structural features of the site on lysozyme to which the drug is bound.

ACKNOWLEDGMENTS

The authors wish to thank Dr. J. E. Coleman for his constructive suggestions and Dr. Harvey Higgins for supplying the cephalosporin derivatives. This study was supported by a grant from the American Cancer Society (T112 E), and an American Cancer Society Professorship (R.E.H.).

REFERENCES

1. J. F. Collins and M. H. Richmond, *Nature* **195**, 142 (1962).
2. N. Sharon and S. Seifter, *J. Biol. Chem.* **239**, PC 2398 (1964).
3. L. N. Johnson and D. C. Phillips, *Nature* **206**, 761 (1965).
4. D. Shugar, *Biochim. Biophys. Acta* **8**, 302 (1952).
5. W. S. Wise and G. H. Twigg, *Analyst* **75**, 106 (1950).
6. M. Laskowski, Jr., *Federation Proc.* **25**, 20 (1966).

7. K. Hayashi, T. Imoto, G. Funatsu and M. Funatsu, *J. Biochem.* (*Tokyo*) **58**, 227 (1965).
8. K. Hayashi, T. Imoto and M. Funatsu, *J. Biochem.* (*Tokyo*) **54**, 381 (1963).
9. C. C. F. Blake, D. F. Koenig, G. A. Mair, A. C. T. North, D. C. Phillips and V. R. Sarma, *Nature* **206**, 757 (1965).
10. K. Hayashi, T. Imoto and M. Funatsu, *J. Biochem.* (*Tokyo*) **55**, 516 (1964).
11. E. M. Wise, Jr., and J. T. Park, *Proc. Natl. Acad. Sci. U.S.* **54**, 75 (1965).
12. D. J. Tipper and J. L. Strominger, *Proc. Natl. Acad. Sci. U.S.* **54**, 1133 (1965).
13. K. Izaki, M. Matsuhashi and J. L. Strominger, *Proc. Natl. Acad. Sci. U.S.* **55**, 656 (1966).
14. E. M. Wise, Jr., and J. T. Park, *Federation Proc.* **25**, 344 (1966).
15. D. J. Tipper, *Federation Proc.* **25**, 344 (1966).