THE NATURE OF RESISTANCE OF A PENICILLIN TO HYDROLYSIS BY PENICILLINASE

BY N. CITRI AND N. GARBER*

From the Department of Bacteriology, The Hebrew University—Hadassah Medical School, Jerusalem, Israel

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5-Methyl-3-phenyl-4-isoxazolyl penicillin is a competitive inhibitor of penicillinase. Interaction with this compound causes marked conformational changes in the enzyme. The changes are reflected in increased susceptibility to iodination, urea and heat. Present observations are related to previously recorded evidence, and the conclusion is reached that the side-chain of a penicillin may confer resistance by distorting the catalytic orientation of penicillinase.

PENICILLINASE hydrolyses the β-lactam ring in 6-aminopenicillanic acid and in many of its derivatives, including clinically important penicillins such as benzylpenicillin and the phenoxyalkyl penicillins. The widespread occurrence of penicillinase producing bacteria has prompted attempts to synthesise new derivatives of 6-aminopenicillanic acid which would combine high antibiotic potency with resistance to hydrolysis by penicillinase. One such derivative, 6-(2,6-dimethoxybenzamido)penicillanic acid (methicillin) (Rolinson, Batchelor, Stevens, Cameron-Wood, and Chain, 1960) has already proved very effective in clinical practice.

Experiments with methicillin have yielded information which may explain the structural basis of resistance to penicillinase. There is now good evidence that the active site of penicillinase acquires a specific catalytic orientation when in contact with a hydrolysable penicillin (Citri and Garber, 1958; Citri and Garber, 1960; Citri, 1960). In contrast, interaction with methicillin causes loss of the specific conformation which is apparently essential for enzymic activity (Citri and Garber, 1961; Garber and Citri, 1962).

Our experiments with 5-methyl-3-phenyl-4-isoxazolyl penicillin, [P-12] here presented, indicate that such distorting effect may indeed account for the resistance of some penicillins to hydrolysis by penicillinase.

EXPERIMENTAL

Penicillinase. The penicillinase was prepared from the culture supernatant of strain 569/H of *Bacillus cereus*, grown as previously described (Citri and Garber, 1960) and the supernatant enzyme concentrated and purified by the procedure described elsewhere (Citri, Garber and Sela, 1960). The specific enzymic activity of the penicillinase preparations used throughout this work was similar to that reported by Kogut, Pollock and Tridgell (1956) for crystalline penicillinase of *B. cereus*.

Assay of penicillinase. Total penicillinase activity was assayed by the manometric method of Henry and Housewright (1947). The iodine

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resistant (α-type) activity was assayed directly by the iodometric procedure previously described (Citri, Garber and Sela, 1960).

Iodination of penicillinase. The iodinating reagent consisted of 0·003 M I_2 and 0·02 M KI (final concentration). The treatment exposed the enzyme to the iodinating reagent for 5 min. at 0° and pH 7·3.

Heat treatment. The heat treatment was by immersing test tubes in a thermostatic water-bath. The temperature was regulated by Thermonix II Immersion Thermostat within ±0·1°. At the end of the incubation the tubes were immersed in an ice-bath for 1 min. and transferred to a 30° water-bath where the enzyme was assayed.

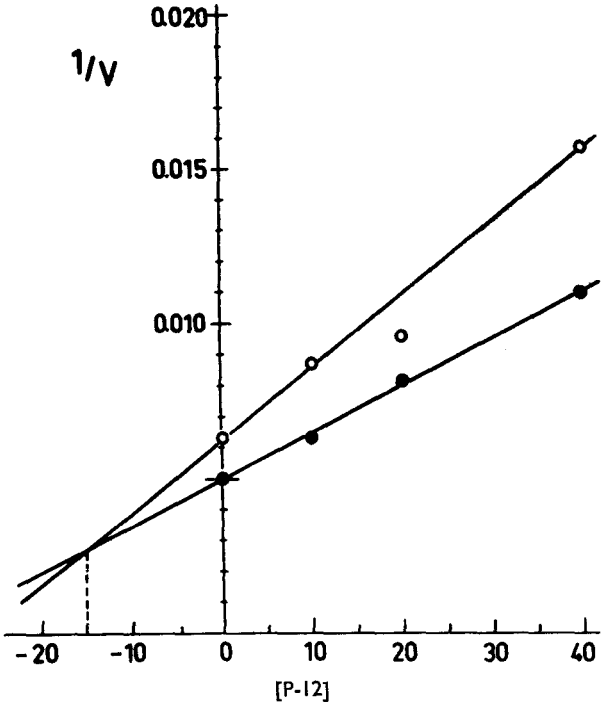


FIG. 1. Competitive inhibition of penicillinase by P-12. Initial velocities for the hydrolysis of benzylpenicillin in the presence of P-12, plotted according to the method of Dixon (1953). The assay system contained 0·05 μg. of penicillinase protein, 3–10 μM benzylpenicillin and 10–40 μM P-12 in a total volume of 3·0 ml. The reactions were run in a Warburg respirometer under conditions used in the manometric assay of penicillinase.

○—○ 3 μM benzylpenicillin. ●—● 10 μM benzylpenicillin.

Assay of residual activity. The assay was based on the iodometric procedure for the determination of iodine resistant (α-type) activity (Citri and Garber, 1958). The reagent mixture (0·5 ml. of I_2, 0·025M in 0·125M KI, 1·0 ml of 0·1M phosphate buffer, 3·0 ml. of 0·5 per cent gelatin and 3 mg. of benzylpenicillin) was kept for 5 min. at 30° before the assay. The assay was started by the transfer of the reagent mixture into the

tube containing the treated preparation. The total volume of the assay was 5 ml.

In the assay of iodinated samples of penicillinase the iodine content of the assay reagents was reduced by the amount used for iodination. Hence the iodine concentration used in the assay was identical in all samples and equal to that used in the original procedure.

Penicillins. Sodium benzylpenicillin B.P. was obtained from Merck and Co., Inc.

Sodium 5-methyl-3-phenyl-4-isoxazolyl penicillin monohydrate [P-12] was supplied by Bristol Laboratories.

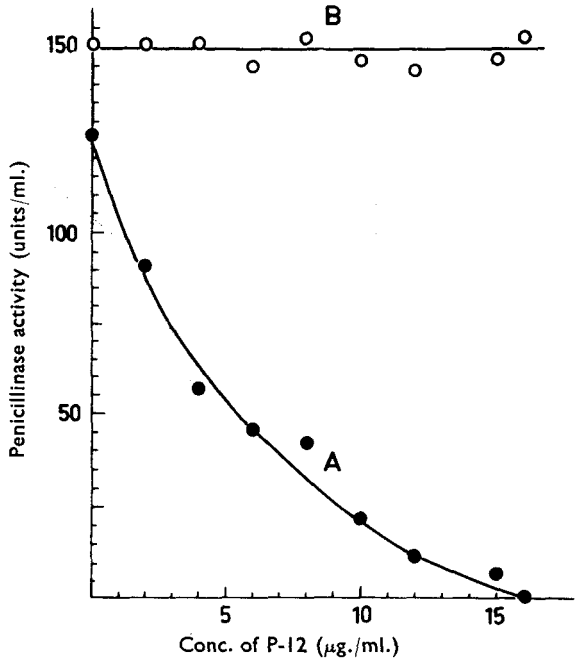


FIG. 2. Sensitisation of penicillinase to iodine as a function of P-12 concentration. Penicillinase (0·5 μg. of enzyme protein) was exposed to varying concentrations of P-12 in the presence of iodine (0·0038M I_2 in 0·02M KI) (Curve A) and in its absence (Curve B). The treatment was at 0° C. in 1·0 ml. of 0·006M phosphate buffer at pH 7·3. At the end of 5 min. the reactants were diluted with an excess of the substrate (3,000 μg. benzylpenicillin in 4 ml. of 0·03M phosphate buffer pH 7·0 containing 0·4 per cent gelatin). The rate of hydrolysis of benzylpenicillin by the residual enzyme was immediately assayed.

RESULTS

Inhibition of penicillinase by P-12. P-12 is highly resistant to hydrolysis by penicillinase of *B. cereus.* The low rate of hydrolysis of P-12 compared with benzylpenicillin has been previously reported (Gourevitch and others, 1961). As with methicillin (Garber and Citri, 1962), P-12 was found to inhibit competitively the hydrolysis of benzylpenicillin by *B. cereus* penicillinase. The kinetics of the inhibition have been investigated with two concentrations of benzylpenicillin and a series of concentration of

P-12. The results, plotted according to the method of Dixon (1953) are presented in Fig. 1. The K_I value obtained from the Dixon plot is 5×10^{-3}M. This value is quite close to that obtained for methicillin ($K_I = 1{\cdot}8 \times 10^{-3}$M) (Garber and Citri, 1962).

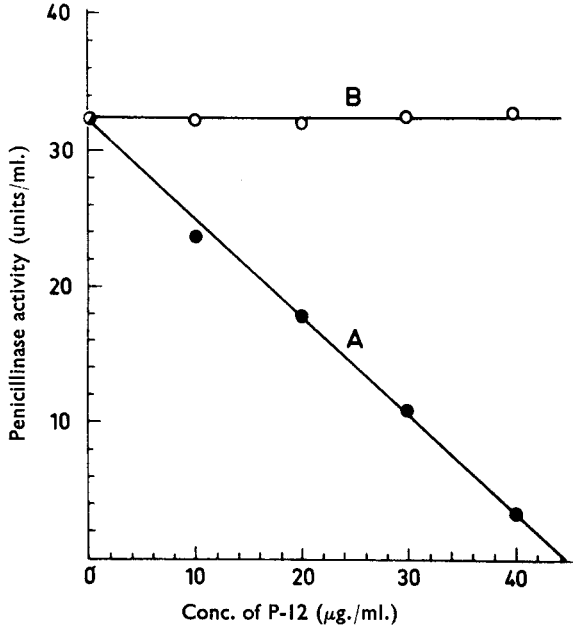


FIG. 3. Sensitisation of penicillinase to urea as a function of P-12 concentration. Penicillinase (0·14 μg. of enzyme protein) was exposed for 5 min. at 0° C to increasing concentrations of P-12. The treatment was in 3·0 ml. phosphate buffer (0·25M, pH 7·0) containing 0·5 per cent gelatin, in the presence of 4M urea (A) and without urea (B). Assay was started by adding benzylpenicillin (3,000 μg. in 1·0 ml. of 0·1M phosphate buffer pH 7·0) and iodine (0·5 ml. of I_2, 0·0025M in 0·125M KI) to the treated samples.

Sensitisation of penicillinase to iodination. Changes in the susceptibility to iodination have been found to provide a sensitive indicator of structural changes in the molecule of penicillinase (Citri, 1958; Citri, Garber and Sela, 1960). Using this criterion the effect of methicillin was demonstrated on the conformation of penicillinase (Citri and Garber, 1961; Garber and Citri, 1962).

P-12 affects the sensitivity of penicillinase in the same way as methicillin as shown in the following experiment.

The enzyme was exposed to varying concentrations of P-12 in the presence of iodine and in its absence. The residual activity of the treated enzyme was assayed, and the results are summarised in Fig. 2. It will be noted that whereas exposure of penicillinase to P-12 alone had no effect (Fig. 2,B), exposure to P-12 in the presence of iodine caused a loss in activity which was proportional to the concentration of P-12 (Fig. 2,A).

Potentiation of the effect of urea on penicillinase. The effect of P-12 on the reactivity of penicillinase is similar to that observed with moderate concentrations of hydrogen bond breaking agents such as urea or guanidine hydrochloride (Citri and Garber, 1958; Citri, Garber and Sela, 1960). The susceptibility to iodine induced by urea is reversed by the substrate, but the reversal is precluded if iodine is present before or during the assay. Hence pre-treatment with adequate concentrations of urea causes a loss of enzymic activity under the present conditions of assay. No such loss is noticeable after a short exposure to 4M urea in the cold. If, however, P-12 is present during this treatment the enzyme becomes inactivated. The loss of activity is directly proportional to the concentration of P-12, as will be apparent from the results presented in Fig. 3.

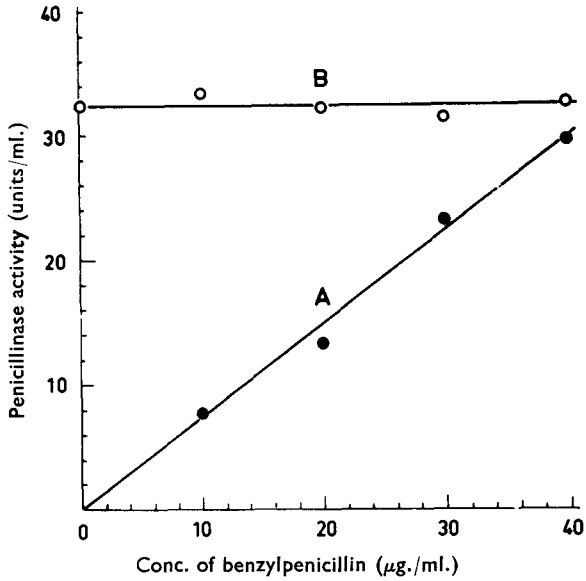


FIG. 4. Reversal of the effect of P-12 by benzylpenicillin. Penicillinase (0·14 μg. of enzyme protein) was exposed for 5 min. at 0° C. to P-12 (50 μg./ml.) and increasing concentrations of benzylpenicillin. Treatment was carried out in the presence of 4M urea (A) and without urea (B). For other experimental conditions see Legend to Fig. 3.

Reversal of the effect of P-12 by benzylpenicillin. As shown previously (Fig. 1) P-12 inhibits competitively the enzymic hydrolysis of benzylpenicillin. It follows that P-12 and benzylpenicillin combine with the same site of the enzyme. It would be expected, therefore, that the interaction of P-12 with penicillinase which lowers resistance to urea would be affected by benzylpenicillin. Since benzylpenicillin is known to reverse the effect of urea, presumably by favouring the active conformation of penicillinase (Citri and Garber, 1960), it was actually expected to reverse competitively the effect of P-12 under the present conditions. Results demonstrating reversal by the substrate are shown

in Fig. 4, where the activity of penicillinase pre-treated with urea and P-12 is plotted against the concentration of benzylpenicillin present during the pre-treatment. It will be noted that the survival of penicillinase activity is proportional to the ratio of substrate to analogue during the pre-treatment with urea.

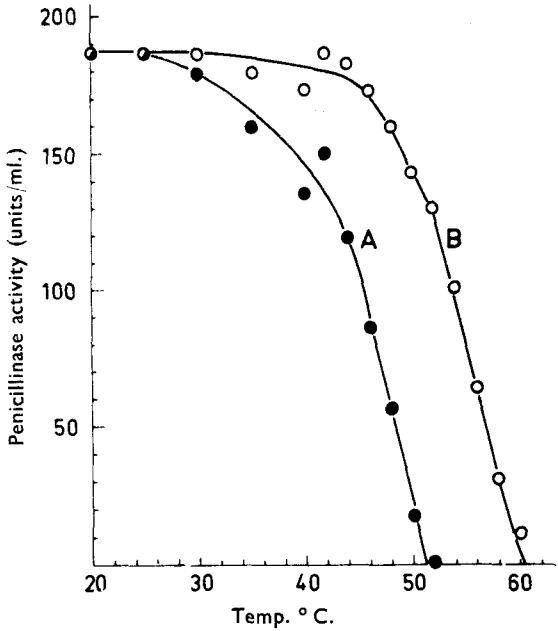


FIG. 5. Effect of P-12 on the thermostability of penicillinase. Reaction mixture (final volume — 0·4 ml.) consisted of penicillinase — 0·5 μg.; phosphate buffer pH 7·3 — 0·05M; gelatin — 0·25 per cent and P-12 — 0·18 μg. (Curve A). Controls contained no analogue (Curve B). At the end of 2 min. exposure to the indicated temperatures the residual activity was determined as described in the Experimental Section.

Potentiation of the effect of heat on penicillinase. The results presented so far indicate that the interaction between penicillinase and P-12 involves breaking of some specific secondary links, such as hydrogen bonds, in the vicinity of the active site. According to this interpretation, P-12 would be expected to lower the thermostability of penicillinase.

The effect of substrate analogues on the stability of the enzyme to heat has been the subject of more experiments (Citri and Garber, 1962). Substrate analogues do indeed cause increased sensitivity to heat, as shown in Fig. 5, where the effect of P-12 is presented as a function of temperature of incubation. The P-12 induced thermolability is proportional to the concentration of the analogue. This is shown in Fig. 6 where the survival of enzyme activity at 45 and 48° is plotted against the concentration of P-12 present during the treatment.

Effect of pH on the thermostability of penicillinase in the presence of P-12. The pattern of thermostability of penicillinase over the range of pH 4–7, shown in Fig. 7 (Curve B) is completely changed in the presence of P-12 (Fig. 7, A). There is practically no loss of activity after 2 min. exposure to 50° at acid pH (pH 4·0–6·0) unless P-12 is present. With P-12 added complete inactivation takes place at pH 4·0–4·5. The thermostability of penicillinase in the presence of P-12 increases sharply in the range of pH 4·5–5·0, and decreases soon after. Again P-12 causes complete inactivation at pH 7·0 when the control enzyme, although not completely stable, retains most of its activity. The curve obtained with P-12 at 50° is similar to that obtained with the enzyme alone at 58°, and in particular to one obtained with methicillin at 48° (Citri and Garber, 1962).

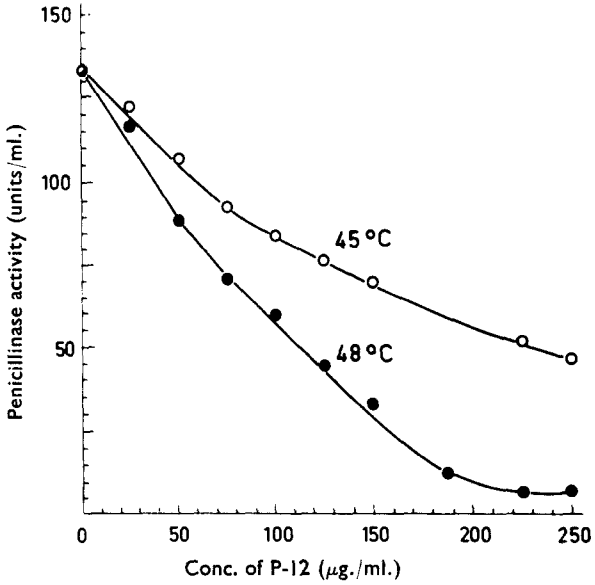


FIG. 6. Thermal inactivation of penicillinase as a function of P-12 concentration. Reaction mixtures (final volume—0·4 ml.) consisted of: penicillinase—0·5 μg.; phosphate buffer pH 7·3—0·05M; gelatin—0·25 per cent, and varying amounts of P-12. At the end of 2 min. exposure to 45° C. (○) and 48° C. (●) respectively, the residual activity was measured as described in the Experimental Section.

DISCUSSION

The penicillin chosen for this study represents a new class of derivatives of 6-aminopenicillanic acid, the 4-isoxazolylpenicillins. When suitably substituted in both *ortho* positions, members of this class are highly resistant to hydrolysis by penicillinase (Doyle, Long, Nayler and Stove, 1961). The other class of penicillins known to be resistant to penicillinase, has been represented by methicillin in a series of similar studies (Citri and Garber, 1961; Garber and Citri, 1962).

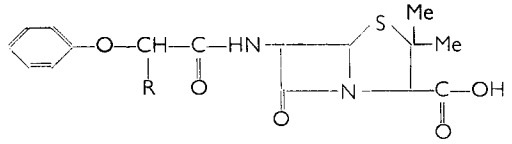
A comparison of properties common to both types of penicillins with those of hydrolysable penicillins will provide a basis for discussing the following questions.

What are the distinctive structural features of a resistant penicillin?

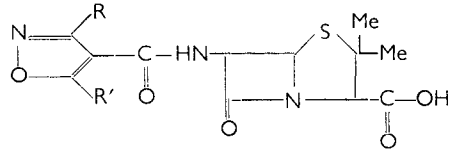
By what mechanism do such distinctive features protect the β-lactam ring from enzymic hydrolysis?

The first question can be answered by comparing the structural formulae representing (I), a class of hydrolysable penicillins (α-phenoxyalkyl penicillins), and both classes of resistant penicillins: a 4-isoxazolyl penicillin (II) and an aromatic disubstituted analogue (III).

I

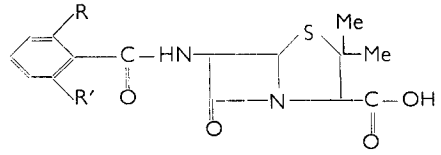


II



(In P-12, R=Ph, R′=Me)

III



(In methicillin R=R′=OMe)

The various penicillins differ, by definition, in the side-chain alone. Hence it is a modification in this moiety that imparts resistance to enzymic hydrolysis. It will be obvious from comparing the respective structures, that the side-chain of a resistant penicillin contains a ring situated in close proximity to the nucleus and substituted in both *ortho* positions. Some very interesting observations on the effect of various substituents in both groups of resistant penicillins, have recently been reported (Doyle, Long, Nayler and Stove, 1961).

In this paper we are concerned specifically with the second question, namely that of the mechanism by which the side-chain of a penicillin affects the action of penicillinase. Probably the most acceptable assumption is that the side-chain determines whether a penicillin can be properly accommodated on the active site of the enzyme. Although stated in rather general terms this is usually taken to imply that the active site is a rigid structure made to fit the substrate. Accordingly bulky substituents in the side-chain will prevent access of a substrate analogue to the active site.

Attempts to explain the properties of methicillin have been based on such a model (e.g. Knox, 1961), although there is no evidence to support it. On the other hand, there is a good evidence for an alternative concept. According to this concept, which may be regarded as a special case of the "induced fit" theory (Koshland, 1959), the alignment necessary for the activity of penicillinase depends on the flexibility of the active site, and on the orienting effect of the side-chain.

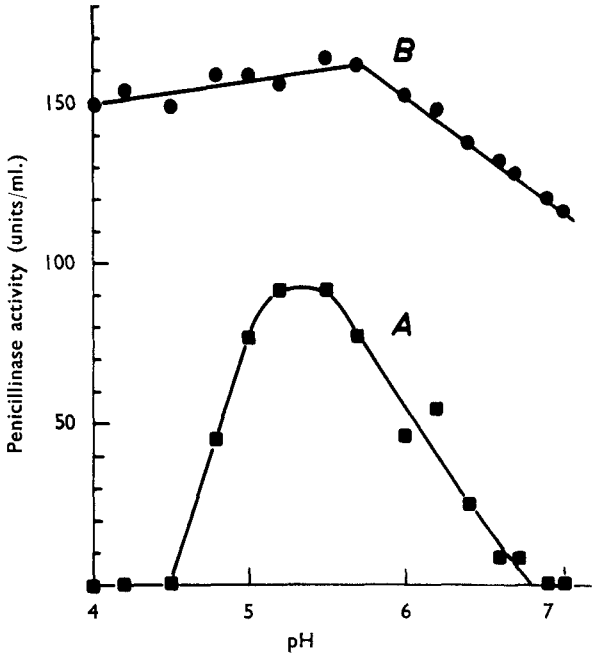


FIG. 7. Survival of penicillinase activity following heat treatment at varying pH. Aliquots of penicillinase (0·55 μg. of enzyme protein) in 0·05M buffer solutions of varying pH, exposed for 2 min. to 50° C. (A) in the presence of P-12 and (B) in its absence. Total volume—0·4 ml. Buffers used: phthalate—NaOH (pH 4·0–6·0), phosphate (pH 6·0–7·5). For assay of residual activity see Experimental Section.

The evidence has been presented and discussed in our previous reports (see refs.). We wish to emphasise that the effects of the isoxazolyl penicillin, used in this study, on the conformation of penicillinase are virtually identical with those observed previously with methicillin. In either case the change in conformation is reversed by hydrolysable penicillins.

The distinctive effect of compounds representing both classes of resistant penicillins on the conformation of the active site of penicillinase points to a common mechanism responsible for the resistance. The following is a tentative formulation of our conclusion concerning the nature of resistance to penicillinase. The enzymic activity of penicillinase depends on a definite and precise orientation of the active site. Such

orientation is imparted by some penicillins and distorted by others, according to the structure of the side-chain. Consequently, the former penicillins are classed as substrates, whereas the latter are relatively immune to enzymic hydrolysis.

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