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A PARTICULAR CONFORMATIONAL CHANGE OF *BACILLUS CEREUS* PENICILLINASE UNDER THE ACTION OF A NEW PENICILLIN ANALOGUE PYRAZOCILLIN

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SUMMARY

The effect of a new penicillin analogue, 1-(2,6-dichlorophenyl)-4-methyl-5-pyrazolyl penicillin, designated pyrazocillin, on penicillinase (penicillin amido-β-lactamhydrolase, EC 3.5.2.6) from *Bacillus cereus* 569/H has been studied. Pyrazocillin inhibits the enzyme according to a "mixed" type inhibition. The K_i value was $1.1 \cdot 10^{-2}$ M. The K_m value as calculated from the hydrolysis velocity of pyrazocillin was higher than $1 \cdot 10^{-2}$ M.

In addition to the competitive inhibition the effect of the time-dependent inhibition of pyrazocillin has also been examined in detail. It has been established that penicillinase incubated with pyrazocillin but in the absence of substrate was partially inactivated. This inactivation can be reversed by dilution, but in contrast to the effect of penicillin analogues already known, the inactivating effect is not reversed by the substrate. Moreover, the conformation of the enzyme, which has low catalytic activity when partially inactivated by preincubation with pyrazocillin, is stabilized by the substrate, while with higher substrate concentrations the catalytic activity is further decreased.

Based on this experimental evidence, the hypothesis of two substrate binding sites of the enzyme has been suggested.

INTRODUCTION

It is known that the conformation of penicillinase (penicillin amido-β-lactamhydrolase, EC 3.5.2.6), derived from *B. cereus* 569/H can be changed by various derivatives of penicillin. According to the detailed investigations of CITRI AND GARBER[1,2] and GARBER AND CITRI[3], the formation of the catalytic site of the enzyme is the result of a conformational change produced by the substrate, while various substrate analogues, depending on their molecular structures, induce conformational changes leading to a decreased catalytic activity. Conformational changes induced by substrate analogues proved to be reversible in the experiments referred to. GOUREVITCH *et al.*[4] have demon-

strated irreversible inactivation caused by substrate analogues in their experiments with a penicillinase from Staphylococcus. Experiments with penicillin analogues have thus far furnished valuable data towards elucidating the mechanism of action of penicillinase. In our experiments a new analogue, a penicillin derivative with a 1-(2,6-dichlorophenyl)-4-methyl-5-pyrazolyl side-chain, has been examined and some phenomena have been demonstrated which may be explained by a conformational change not observed with analogues previously studied.

MATERIALS AND METHODS

The sodium salt of pyrazocillin 1-(2,6-dichlorophenyl)-4-methyl-5-pyrazolyl penicillin was prepared in the Research Institute for Pharmaceutical Chemistry, Budapest (U.S.P. 3,376.288).

The potassium salt of penicillin G was a commercial preparation, in form suitable for injection, as manufactured by Biogal Pharmaceutical Works, Hungary.

Experiments were made with penicillinase produced by Strain 569/H of *B. cereus*. Purification of the enzyme and the assay method were as previously described[5,6].

RESULTS

From the studies of CITRI AND GARBER[1,2] and GARBER AND CITRI[3] it is known that the effect of penicillin analogues on the activity of penicillinase is competitive. At the beginning of our experiments the inhibition type of pyrazocillin was examined and the value of the "inhibitor constant" was determined. In these experiments penicillinase (2000 units) was incubated with different amounts of substrate and pyrazocillin in a reaction volume of 100 ml, which also contained 0.025 M phosphate buffer (pH 6.5) and 0.05% gelatin. A very short incubation time (20 sec) was chosen so that values closely approaching the initial velocity of the enzymic reaction could be measured (the rate of the reaction seemed to be linear up to 20–30 sec in preliminary experiments). Reciprocals of reaction velocities, as measured at different substrate and inhibitor concentrations, *vs.* reciprocals of substrate concentrations are shown in Fig. 1. The curves demonstrate that inhibition by pyrazocillin is not purely competitive but rather of some "mixed" type. The value of the inhibitor constant as calculated from our results was $1.1 \cdot 10^{-2}$ M, *i.e.* a value one order higher than that obtained by GARBER AND CITRI[3] for methicillin ($K_i = 1.8 \cdot 10^{-3}$ M). (Inhibitor constant is defined as the inhibitor concentration which increases the slope of the straight line 2-fold obtained from data of the uninhibited reaction in the above illustration).

The kinetics of the hydrolysis of pyrazocillin (as the substrate) has also been examined. $1 \cdot 10^{-3}$ M pyrazocillin was incubated with penicillinase (2000 units/ml) in 0.05 M phosphate buffer (pH 6.5) containing 0.05% gelatin. Samples were withdrawn at various intervals and the amount of pyrazocillin decomposed was determined by iodometric titration. Results are given in Fig. 2 as a curve. It can be seen that the initial velocity of the reaction is relatively high but drops very rapidly (within a minute or so) and reaches a low and approximately constant value.

Using a very short incubation time (15 sec) the K_m value of pyrazocillin was determined. As calculated from the reaction velocities at various pyrazocillin concen-

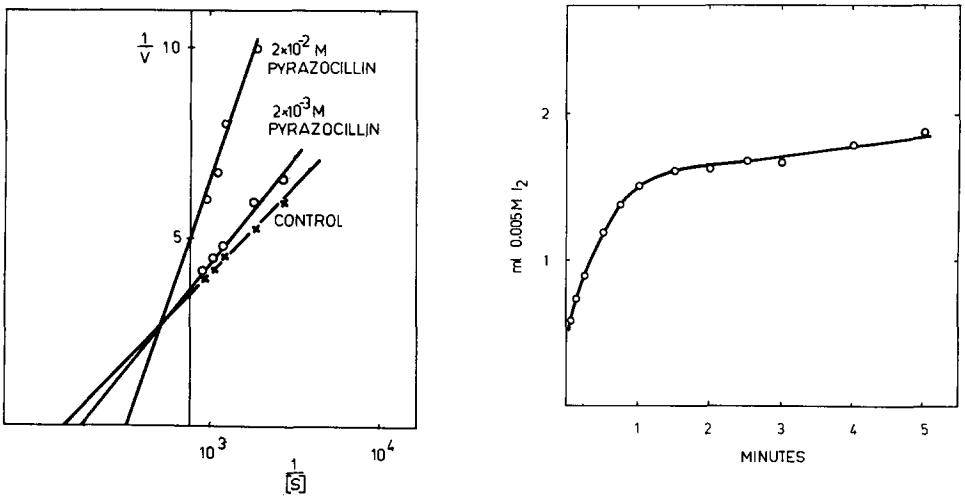


Fig. 1. Determination of the type of inhibition exhibited by pyrazocillin. In this experiment penicillinase (2000 units) was incubated at different substrate and pyrazocillin concentrations in a reaction mixture of 100 ml containing potassium salt of penicillin G, pyrazocillin, 0.025 M phosphate buffer (pH 6.5) and 0.05% gelatin. Incubation was carried out at 30° for 20 sec.

Fig. 2. Velocity of pyrazocillin hydrolysis by penicillinase. In this experiment penicillinase (2000 units/ml) was incubated in the presence of $1 \cdot 10^{-3}$ M pyrazocillin at 30° in 0.05 M phosphate buffer (pH 6.5) containing 0.05% gelatin. Consumption of iodine solution is plotted *vs.* time.

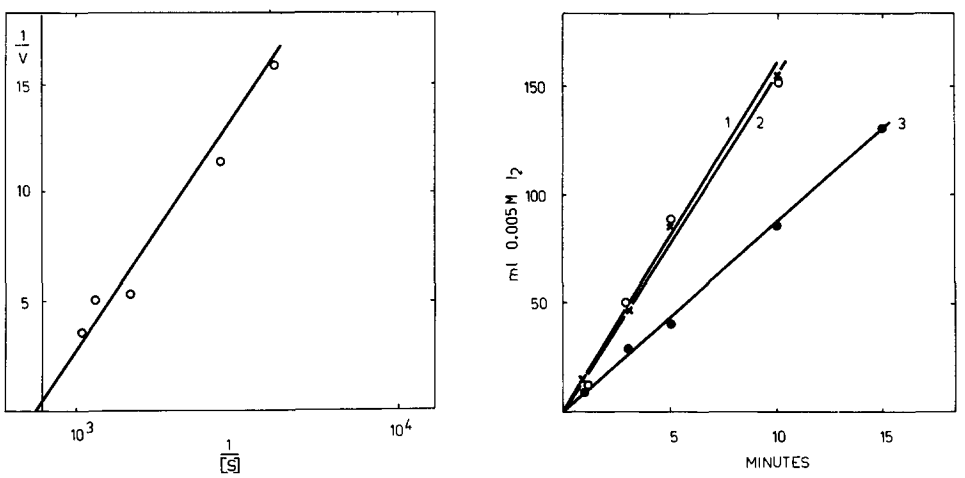


Fig. 3. Determination of K_m value of pyrazocillin. Penicillinase (2000 units/ml) was incubated with different pyrazocillin concentrations at 30° in 0.05 M phosphate buffer (pH 6.5) containing 0.05% gelatin.

Fig. 4. Effect of preincubation with pyrazocillin on the hydrolysis velocity of penicillin G. Reaction mixtures contained penicillinase (300 units/ml) in 0.1 M phosphate buffer (pH 6.5) containing 0.05% gelatin. Curve 1. Reaction mixture made up with potassium salt of penicillin G. (to 0.2 M final concentration) and incubated at 30°. Curve 2. Reaction mixture made up simultaneously with pyrazocillin to $2 \cdot 10^{-3}$ M (final concentration) and with potassium salt of penicillin G to 0.2 M (final concentration) and incubated at 30°. Curve 3. Preincubation at 30° for 5 min with $2 \cdot 10^{-3}$ M pyrazocillin (final concentration) then reaction mixture made up with potassium salt of penicillin G to 0.2 M (final concentration) and further incubated at 30°. 0-min value of this experiment was that taken at the instant of addition of potassium salt of penicillin G.

trations, it proved to be $1 \cdot 10^{-2}$ M or higher. Results of these measurements are shown in Fig. 3 in a double-reciprocal plot. This value, however, is higher by 2–3 orders than those obtained with penicillin ($4.8 \cdot 10^{-5}$ M)[7] or with competitive inhibitors studied earlier, *i.e.* methicillin ($4.6 \cdot 10^{-4}$ M) and oxacillin ($5.7 \cdot 10^{-4}$ M)[8].

The time-dependent inactivating effect of pyrazocillin was then studied by incubating penicillinase (300 units) with $2 \cdot 10^{-3}$ M pyrazocillin for 5 min at 30° in 0.1 M phosphate buffer (pH 6.5) containing 0.05% gelatin. 0.2 M penicillin G was then added and the incubation continued at 30° while the amount of penicillin decomposed was measured at intervals. In another test, pyrazocillin and penicillin G were added simultaneously, while in a third test pyrazocillin was omitted. Experimental results are shown in Fig. 4. The reaction velocity curves of the samples containing no pyrazocillin, and to which pyrazocillin and penicillin G were added simultaneously, are identical. The curves are linear and their slopes higher than that of the reaction velocity curve of the sample preincubated with pyrazocillin. This latter curve is also linear within the 15-min period of the experiment. From this experiment it could be concluded that preincubation with pyrazocillin caused an activity decrease which was irreversible by penicillin G. A prerequisite of this activity decrease was the preincubation with pyrazocillin, since on simultaneous addition of penicillin G no activity decrease could be observed.

Optimal conditions for the inactivation by pyrazocillin treatment were investigated. Fig. 5 shows the effect of pyrazocillin concentration on the inactivation after preincubation. Penicillinase (300 units) was incubated with different amounts of

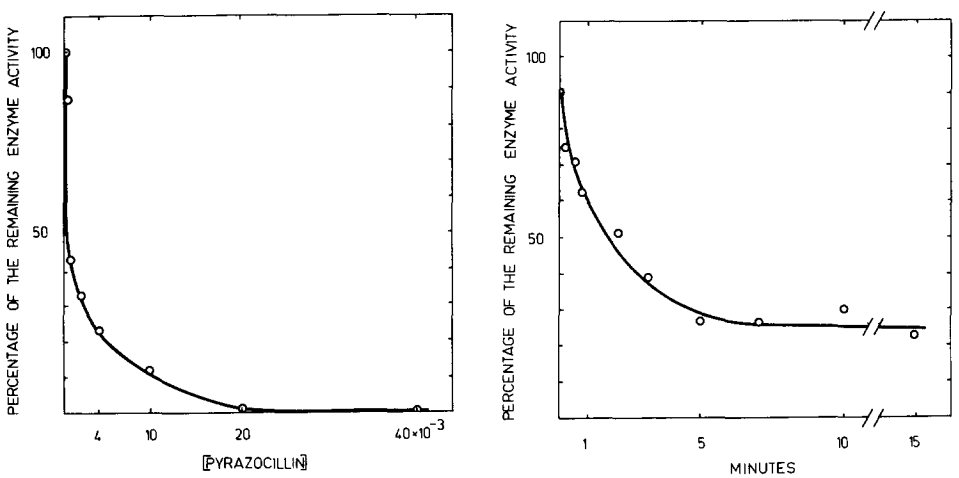


Fig. 5. Effect of pyrazocillin concentration on the inactivation of penicillinase. Penicillinase (300 units/ml) was incubated at different pyrazocillin concentrations for 5 min at 30° in 0.1 M phosphate buffer (pH 6.5) containing 0.05% gelatin. Then the mixture was made up with potassium salt of penicillin G to 0.2 M and incubated further for 5 min, after which the amount of penicillin decomposed was measured. Results were expressed as per cent of the activity of the control untreated with pyrazocillin.

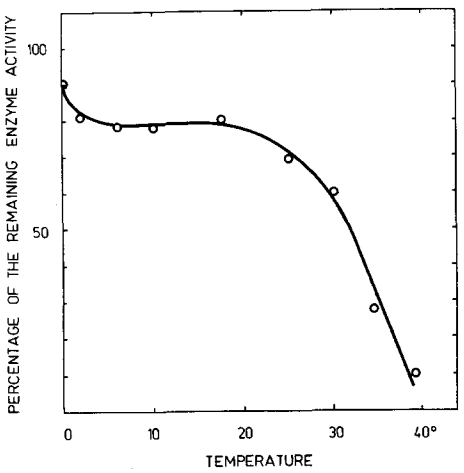
Fig. 6. Effect of duration of preincubation with pyrazocillin on the inactivation of penicillinase. Similarly to the conditions of the experiment illustrated in Fig. 5 preincubation was made with $2 \cdot 10^{-3}$ M pyrazocillin for different periods, and 5 min after the addition of potassium salt of penicillin G the amount of penicillin decomposed was measured. Results were expressed as per cent of the activity of the control untreated with pyrazocillin.

pyrazocillin for 5 min at 30° in 1 ml of 0.1 M phosphate buffer (pH 6.5) containing 0.05% gelatin. Subsequently, the mixture was made up with penicillin G to 0.2 M, and after a 5-min incubation the amount of penicillin decomposed was measured, allowing for the amount of pyrazocillin decomposed during preincubation. Results have been expressed as per cent of the activity of the control not pretreated with pyrazocillin. From the curve in the figure it can be seen that preincubation with $1 \cdot 10^{-2}$ M pyrazocillin caused a practically complete inactivation.

Preincubations were carried out with $2 \cdot 10^{-3}$ M pyrazocillin under similar conditions but for different periods of time. Results are summarized in Fig. 6. The inactivation reaction displayed first-order kinetics, and approx. 5 min were necessary to bring about the maximal inactivation possible with given concentration of pyrazocillin.

The temperature dependence of inactivation was also examined by pretreatment with $1 \cdot 10^{-3}$ M pyrazocillin for 5 min as was done previously. Results are given in Fig. 7. Inactivation was found to be highly temperature-dependent in the temperature range 20–40°.

Finally, influence of pH on the inactivation during preincubation with pyrazocillin was investigated. Preincubation was carried out with $2 \cdot 10^{-3}$ M pyrazocillin for 5 min in phosphate buffers adjusted to various pH values. At the end of preincubation, at the same time as penicillin G was added, 0.1 M phosphate buffer con-



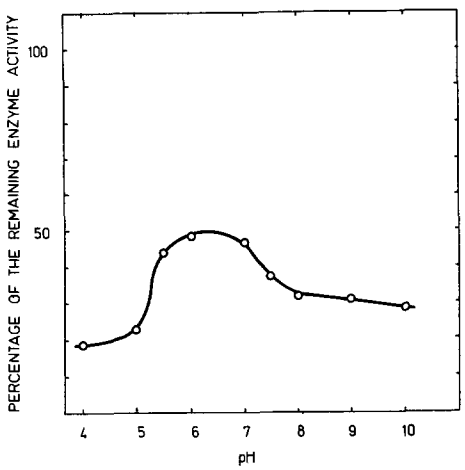


Fig. 7. Effect of temperature of preincubation with pyrazocillin on the inactivation of penicillinase. A 5-min preincubation was carried out with $1 \cdot 10^{-3}$ M pyrazocillin. Under similar conditions as in the experiment shown in Fig. 5 but with different temperatures in each trial. 5 min after the addition of potassium salt of penicillin G the amount of penicillin decomposed was measured and expressed as per cent of the value of the control untreated with pyrazocillin.

Fig. 8. Effect of pH value of preincubation on the inactivation of penicillinase by pyrazocillin. Preincubation with $2 \cdot 10^{-3}$ M pyrazocillin was carried out for 5 min at 30° in 0.1 M phosphate buffers adjusted to different pH values. After preincubation with potassium salt of penicillin G, calculated amounts of HCl and NaOH were simultaneously added to a 0.1 M phosphate buffer so as to maintain uniformly a pH value of 6.5 during incubation with potassium salt of penicillin G. After 5 min of incubation with potassium salt of penicillin G the amount of penicillin decomposed was measured and expressed as per cent of the activity of the control untreated with pyrazocillin.

taining HCl and NaOH was also added to the solution to prevent deviation of the pH from the optimal value of 6.5 during incubation with penicillin. Results are demonstrated in Fig. 8. In the optimal pH range of the enzyme (around pH 6) the inactivating effect of pyrazocillin proved to be minimal.

From the experiments illustrated in Fig. 4, it is clearly apparent that the inactivating effect of preincubation with pyrazocillin could not be reversed by penicillin G. Therefore the effect of dilution following preincubation on the inactivation was studied. For this purpose, penicillinase (300 units) was incubated with $2 \cdot 10^{-3}$ M pyrazocillin for 5 min at 30° in 0.1 M phosphate buffer (pH 6.5) containing 0.05% gelatin. After

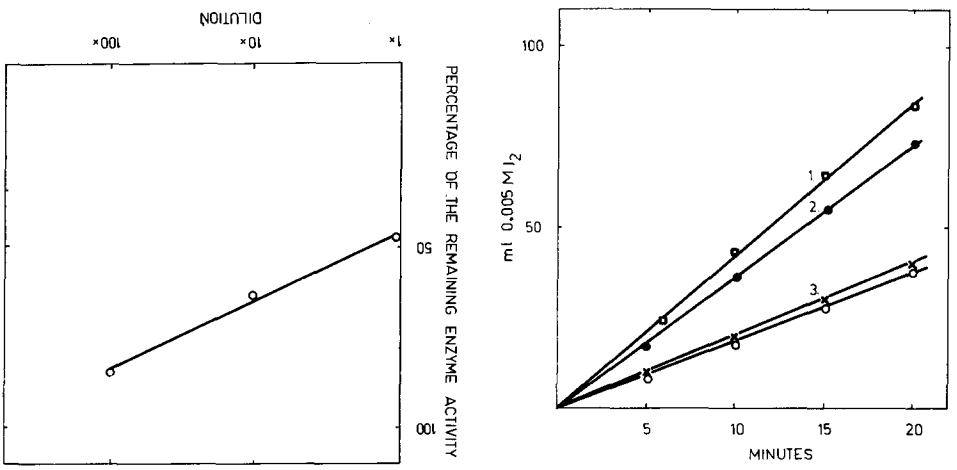


Fig. 9. Reversing action of dilution on the inactivation of penicillinase by pyrazocillin. Penicillinase (300 units/ml) was preincubated with $2 \cdot 10^{-3}$ M pyrazocillin for 5 min at 30° in 0.1 M phosphate buffer (pH 6.5) containing 0.05% gelatin. At the end of preincubation 1 ml of each reaction mixture was diluted with 0.05% gelatin solution and to each sample 60 mg potassium salt of penicillin G were added. After a 5-min incubation at 30° the amount of penicillin decomposed was measured and results expressed as per cent of the activity of the control untreated with pyrazocillin.

Fig. 10. Stabilizing effect of substrate on penicillinase inactivated by pyrazocillin. The figure shows substrate splitting kinetics of four penicillinase samples treated in various ways. (1) Control: penicillinase (300 units/ml) in 0.1 M phosphate buffer (pH 6.5) containing 0.05% gelatin, incubated at 30° with 0.2 M potassium salt of penicillin G. (2) A solution identical to that of the control penicillinase was treated for 5 min with $2 \cdot 10^{-3}$ M pyrazocillin and subsequently diluted 100-fold with 0.05% gelatin solution before adding potassium salt of penicillin G. (3) This sample differs from Sample 2 only in that it was not diluted. (4) This sample differs from Sample 3 only in that it was diluted 100-fold in the fifth minute of incubation with penicillin G.

the preincubation samples taken from the reaction mixture were diluted to various extents with 0.05% gelatin solution, 60 mg of penicillin G were added to each sample. After incubation for 5 min at 30°, the amount of penicillin decomposed was measured. Results are expressed as per cent of the activity of the sample not treated with pyrazocillin and are summarized in Fig. 9. Apparently the inactivating effect of pyrazocillin could have been reversed by dilution, *i.e.* diminishing the concentration of pyrazocillin.

Our next experiment was performed to examine whether this reversing action

of dilution could be counteracted by the substrate. In Fig. 10, the kinetics of substrate decomposition by the enzyme pretreated in four different ways is illustrated. Curve 1 shows the sample not treated with pyrazocillin (control); Curve 2 represents the substrate splitting under the influence of a similar amount of the enzyme as in the case of Curve 1 but pretreated with $1 \cdot 10^{-3}$ M pyrazocillin for 5 min and subsequently diluted 100-fold; Curve 3 shows a similarly pretreated ($1 \cdot 10^{-3}$ M pyrazocillin, 5 min) but undiluted sample. Finally, Curve 4 shows the activity of the enzyme pretreated with pyrazocillin for 5 min, the substrate then being added without dilution; after incubation with penicillin G for 5 min the reaction mixture was diluted 100-fold with 0.05% gelatin solution. The pattern of Curves 1, 2 and 3 corresponds to experiments presented earlier. However, Curve 4, having an identical slope with Curve 3, shows that if no dilution is made before adding the substrate (as in the case of Curve 2), but rather some time after interaction of enzyme and substrate, it has no reversing action on the inactivating effect of preincubation with pyrazocillin.

In earlier studies by CITRI AND GARBER[1,2] and GARBER AND CITRI[3] it was found that the substrate consistently antagonized the conformational changing effects of the analogues. Our case seems to be the first instance where this reversing effect does not occur. In fact, incubation with the substrate even counteracted the reversing action of dilution.

Considering these results, it can be suggested that the catalytic activity of the enzyme conformation that is induced by pyrazocillin could possibly also be decreased by the substrate itself. Therefore the dependence of enzyme activity on substrate concentration after pyrazocillin pretreatment was examined. Preincubation was carried out with $2 \cdot 10^{-3}$ M pyrazocillin as previously described, and the second 5-min incubation was started with different quantities of penicillin G, after which the amounts

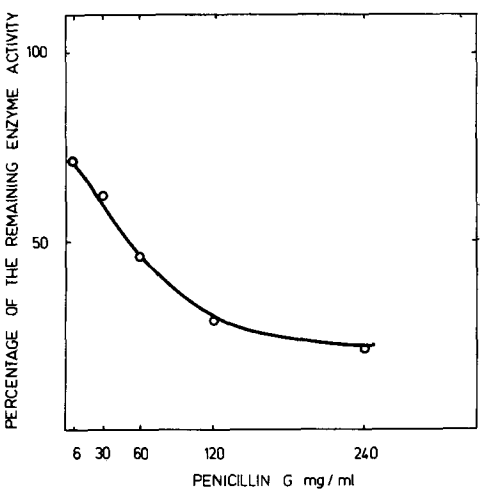


Fig. 11. Effect of various substrate concentrations on the activity of the enzyme partially inactivated by pyrazocillin. Penicillinase (300 units/ml) was incubated in 0.1 M phosphate buffer (pH 6.5) containing 0.05% gelatin with $2 \cdot 10^{-3}$ M pyrazocillin at 30°. It was further incubated for 5 min with the concentrations of potassium salt of penicillin G shown in the figure; the amount of penicillin decomposed was measured and expressed as per cent of the activity of the control untreated with pyrazocillin.

of penicillin decomposed were measured. Penicillin concentrations as chosen in preliminary experiments caused a linear decomposition for 5 min. The results obtained are shown in Fig. 11 and are expressed as per cent of the activity of the control not treated with pyrazocillin. It can be seen that increasing the substrate concentration strongly enhances the effect of preincubation with pyrazocillin, while on simultaneous addition of the substrate and pyrazocillin no inactivation can be observed.

Considering that with higher substrate concentrations the ionic concentrations of the mixtures studied were also higher, an experiment was carried out to see if the substrate inhibition as observed could not be attributed merely to an electrolyte effect. It was found that in concentrated electrolyte solutions (0.8–1.0 M phosphate) the inactivating effect of the substrate or of preincubation with pyrazocillin could not have been observed either. Consequently, Fig. 11 certainly shows a specific inhibitory action of the substrate.

DISCUSSION

Penicillin analogues which have been examined heretofore have proved to be able to inhibit competitively the action of penicillinase. As based on iodine sensitivity measurements, the analogues cause a reversible conformational change[1–3]. In only a single case—with a penicillinase from a Staphylococcus strain—has irreversible inactivation been demonstrated[4].

The effect of the new analogue we have investigated differs from those of the previous analogues in several respects. The K_m value of pyrazocillin proved to be considerably higher than those of the other penicillin derivatives studied thus far. The hydrolysis of pyrazocillin by penicillinase proceeds with a low velocity. It seems that the main cause of the slow hydrolysis is the inactivating effect of pyrazocillin which reveals itself within some minutes after commencement of incubation, depending on the circumstances. From the experiments illustrated in Figs. 2 and 5, it is obvious that the inactivating effect of pyrazocillin is time dependent, reaching a maximum after approx. 1 min of incubation. This enzyme inactivation could not be reversed by the substrate, but dilution before incubation with substrate could produce a reversion of inactivation.

The substrate exerts a special effect on the enzyme partially inactivated by pyrazocillin. As can be seen from Fig. 10, the substrate was able to stabilize the inactive conformation and could have hindered the reversion due to dilution. The experiment illustrated in Fig. 11, on the other hand, clearly demonstrates that the substrate not only stabilizes a less active conformation but is also able to decrease further the originally low activity in the case of increasing substrate concentration.

These results can be interpreted as indicating that the inactivating effect of pyrazocillin is not, or not exclusively, exerted on the active site but rather that it binds to some other part of the penicillinase molecule which is loosely connected with the catalytic site. The consequence of the conformational change due to this binding is the deformation of the active site and a diminution of catalytic activity. It may be assumed that the affinity of this second "penicillin-binding" site to pyrazocillin is greater than to penicillin G, and owing to this fact its effect cannot be reversed by an excess of substrate.

Based on this conception, it is also probable that in the conformation produced from the native enzyme under the influence of the substrate, this second site is inaccessible to pyrazocillin, since upon adding pyrazocillin and penicillin G simultaneously the phenomenon of irreversible inhibition could not have been observed (Fig. 4). The assumption of a second substrate binding site is at present only a hypothesis based on the available data but it would seem worthwhile to carry out further experiments to verify or reject it.

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