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KINETICAL INVESTIGATIONS INTO ENCYMATIC INACTIVATION OF PENICILLIN G

BY

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Under the action of the enzyme penicillinase penicillins are converted into biologically inactive substances, the amide bond in the four ring presumably being opened on hydrolysis. In a previous paper (BRODERSEN 1947 a), it has been shown that, in all probability, different penicillinases of bacterial origin exist, since marked differences seem to prevail, on the one hand, between an enzyme studied by WOODRUFF and FOSTER (1945) and prepared from a grampositive, spore-forming air bacterium and, on the other hand, an enzyme which is produced by a gram-negative coli-like bacterium. The present work deals with the course of penicillin inactivation by the latter enzyme.

The investigations communicated in the above cited paper show that we here meet with a substance having the properties typical of enzymes. It has, however, not been demonstrated whether the accelerating effect of this substance on the inactivation of penicillin is due to catalysis or possibly is an ordinary stoichiometric reaction. In the latter case, the ratio between the quantity of the inactivator consumed and the quantity of penicillin inactivated is independent of the experimental conditions.

We shall, therefore, first investigate whether penicillinase has a catalytic effect.

Technique.

Penicillinase was applied in form of a culture filtrate of the previously described coli-like bacterium (BRODERSEN 1947 b). The cultivation procedure is described in the same paper.

The culture filtrate was adjusted to pH 6.7 and an equal volume of phosphate buffer (Sørensen) of the same pH was added. In the different experiments, to 5 ml of this mixture a small volume of a rather concentrated penicillin solution was added. In this

way, the hydrogen ion concentration and the salt concentration were the same in all experiments.

If not otherwise stated, the experimental temperature was 30° C. The temperature of the solution was adjusted prior to the addition of penicillin.

From the reaction mixture samples were drawn at intervals. In these samples, the inactivation process was interrupted by treatment with alcohol at room temperature for 10 minutes, as described earlier. Subsequently, the mixture was diluted to an alcohol content of c. 10 vol %. This alcohol concentration is without detectable influence on the penicillin determinations.

After end experiment, the penicillin activities were measured in the thus treated samples by means of the agar cup method. Generally, each sample was measured in one cup, only. In order nevertheless to obtain a reliable determination of the course of the curve, a considerable number of samples were drawn in each experiment. It might possibly appear more natural to confine oneself to a smaller number of samples and, instead, to determine each of them more accurately in different cups. However, the above procedure was preferred, because the relative change of the penicillin concentration frequently increases markedly towards the end of the experiment; thus, it was necessary to draw samples at rather short intervals in order to ensure a point in the last part of the curve.

Demonstration of the Catalytic Effect of Penicillinase

Fig. 6 shows the course of the inactivation of pure penicillin G-sodium salt "Glaxo" (in the following denoted as "preparation A"). The three curves correspond to three different initial concentrations. If the curves are displaced in such a way that the points with the same penicillin concentration fall on the same ordinate (Fig. 1), it appears that the curves are congruent. This means that for any penicillin concentration the reaction velocity is independent of the quantity of penicillin inactivated before the respective time. Disregarding the shape of the curves, this can be explained in two ways:

(1) Penicillin reacts stoichiometrically with a substance the molar concentration of which is high compared with even the highest penicillin concentration applied.

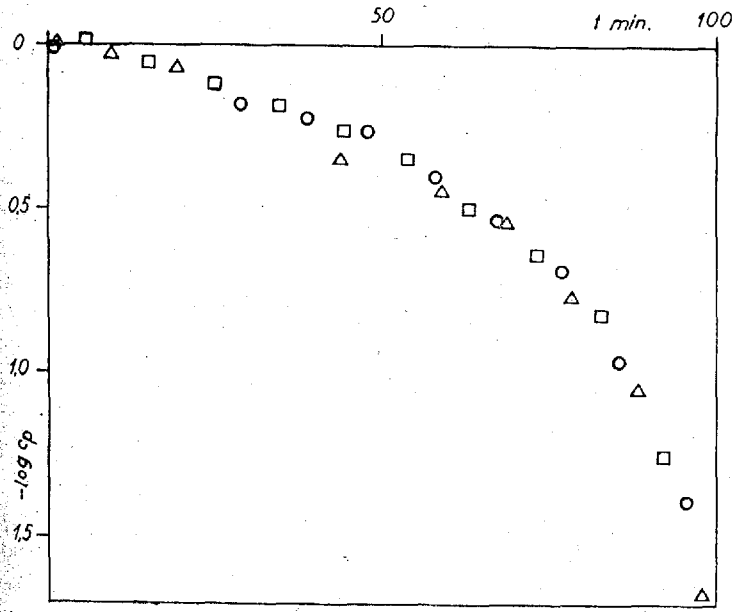

Fig. 1. The course of the reaction for the inactivation of preparation A (penicillin G "Glaxo"). At the start of the three curves the penicillin concentration is 0.208 mml.

○ Before t = 0, 0.408 mmol was inactivated.
□ — t = 0, 0.208 — — — .
△ — t = 0, 0 — — — .

(2) Penicillinase is a catalyst which does not change its catalytic activity during the process.

In the first case, we would expect to find a first-order reaction (pseudomonomolecular process). The curves obtained correspond approximately to a reaction of zero order, which is frequently found in enzymatic processes. This is in favour of the assumption that penicillinase is a catalyst.

Fig. 2 exhibits curves obtained in almost the same way as those represented in Fig. 1, however with another penicillin preparation (B) containing but c. 5 % sodium salt of penicillin G and, besides, biologically inactive substances. The inactivation of this preparation is a first-order reaction in accordance with assumption (1). On the basis of this assumption it seems, however, impossible to explain why the process occurs the slowlier, the higher the initial penicillin concentration. This observation

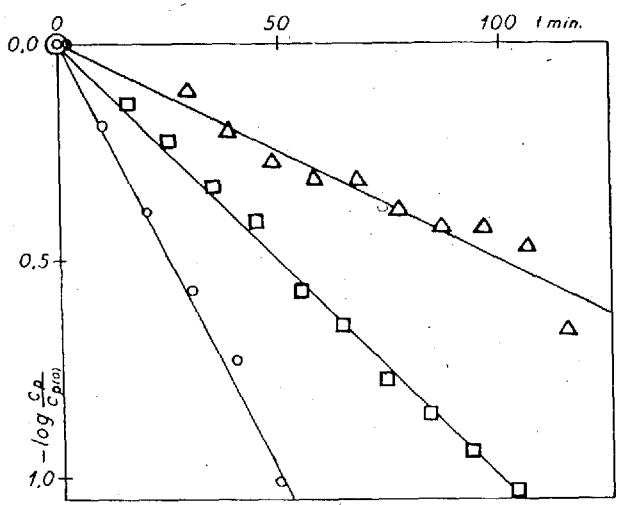

Fig. 2. The course of the reaction for the inactivation of preparation B (Leo-penicillin G, sodium salt, c. 5 %). At the start of the three curves the penicillin concentration is 0.027 mmol.
△ Before t = 0, 0.081 mmol was inactivated.
□ — t = 0, 0.027 — — — .
○ — t = 0, 0 — — — .

may, however, be explained when assuming the penicillinase to be a catalyst which is competitively inhibited by a substance present in the penicillin preparation. The impeding substance must then be present in a concentration so high that the amount of penicillinase bound to penicillin at any time is small as compared with the amount bound to the impeding substance. In this case, the slopes of the straight lines of Fig. 2 should be inversely proportional to the added quantity of the penicillin preparation. This condition is fulfilled in good approximation.

A closer derivation of the theory concerning the course of the process will be given below. Here it may only be stated that the curves considered are in good agreement with the assumption that the inactivated substance is a catalyst; on the other hand they cannot be explained if it is assumed that the inactivation is a stoichiometric reaction between penicillin and another substance.

Stability of Penicillinase under the Prevailing Experimental Conditions.

It is a rather general observation that enzymes are inactivated to a higher or lower degree during the process or already during storage under the experimental conditions. Since this is of utmost significance for the kinetical treatment of the reaction, we shall first investigate whether such an irreversible inactivation takes place in the case of penicillinase.

It results from the experiment plotted in Fig. 1 that irreversible inactivation is not very dominant in the first part of the process. In order to extend the investigation also to the last part of the process and, altogether, over a longer period of time, the following experiments have been performed.

To 5 ml penicillinase-buffer mixture was added penicillin preparation A dissolved in a very small volume of water to a concentration of 0.23 mmol[1]. After the lapse of 20 hours, this mixture was completely inactivated and preparation B was added to 0.27 mmol penicillin. Simultaneously, a control experiment was started in the following way: to another 5 ml penicillinase-buffer mixture the same amount of preparation B was added. The course of the reactions in these two glasses was studied and compared.

If, under these conditions, the penicillinase is stable for 20 hours, we should expect to find the same course of reaction in both glasses, while the process in the control experiment should occur more rapidly if a partially irreversible inactivation of the enzyme takes place.

For the last part of the experiment, preparation A could also have been used. However, it is possible that, during inactivation of the portion of preparation A added first, a small amount of competitively inhibiting substance was formed which, in view of the measuring uncertainty, did not manifest itself in the experiment shown in Fig. 1. A closer examination of the uncertainty shows that this could be the cause of an erroneous result of the experiment. If, on the other hand, preparation B is used, containing large amounts of competitively inhibiting substances, the

[1] Here, the penicillin concentrations are expressed in millimol per litre (mmol), since this proved to be more rational in chemical work than OU/ml. (1 OU/ml = 0.001795 mmol).

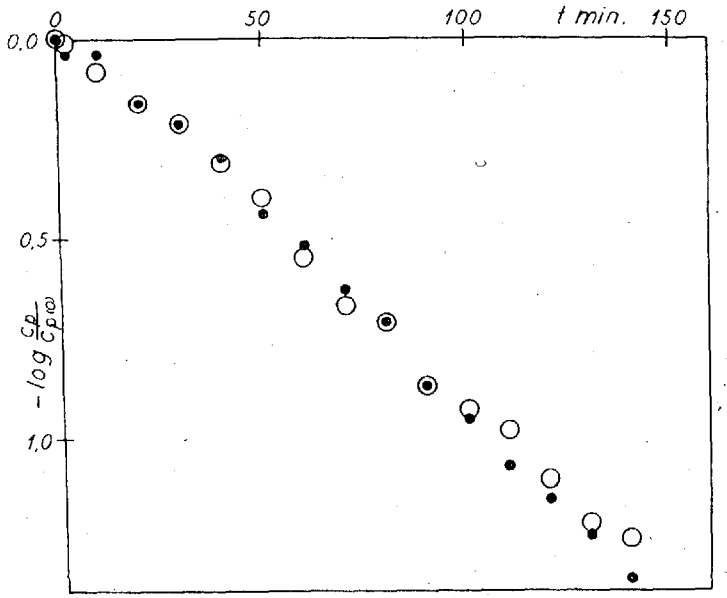

Fig. 3. The course of the reaction for the inactivation of preparation B after the same quantity of enzyme had inactivated a certain quantity of preparation A.
○ Before t = 0, 0.233 mmol of preparation A was inactivated.
● — t = 0, 0 — - — - — — .

effect of the inhibiting substance possibly formed in the first phase of the experiment will not be detectable.

The results of the measurements are shown in Fig. 3. The difference between the two curves appears to be very slight. Therefore, we shall in the following assume that penicillinase is stable throughout the experiment.

The Course of the Inactivation Process.

Theory.

On the basis of the orientating investigations outlined in the preceding section, we shall now deduce a formula for the course of the reaction with time and, then, compare this formula with the curves found experimentally.

In agreement with MICHAËLIS and MENTEN's theory (1913) we assume that the first step of the process is the formation of

an enzyme- and penicillin complex. This complex is subsequently converted and finally split into enzyme and reaction product.

$$P + E \rightleftharpoons PE \rightarrow P'E \rightleftharpoons P' + E \qquad (1)$$

The equilibrium reactions symbolized by double arrows are assumed to occur with velocities very high as compared with the velocity of the irreversible process which, thus, determines the rate of the reaction. If the equilibrium written on the right side is not completely displaced towards the right side, the reaction product exerts a competitive inhibition.

Moreover, we must reckon with the presence of one or several other competitively inhibiting substances which compete with penicillin in reversibly combining with the enzyme, whereby the reaction velocity is decreased. A_1 denotes such a substance in the penicillin preparation, A_2 one in the enzyme preparation.

$$\left.\begin{aligned} A_1 + E &\rightleftharpoons A_1E \\ A_2 + E &\rightleftharpoons A_2E \end{aligned}\right\} \qquad (2)$$

Assuming that the molar concentration of the enzyme is small as compared with the concentrations of P, P', A_1, and A_2, the mass action terms corresponding to these equilibria can be written

$$K_P = \frac{c_P c_E}{c_{PE}} \qquad (3)$$

$$K_{P'} = \frac{c_{P'} c_E}{c_{P'E}} \qquad (4)$$

$$\left.\begin{aligned} K_{A_1} &= \frac{c_{A_1} c_E}{c_{A_1E}} \\ K_{A_2} &= \frac{c_{A_2} c_E}{c_{A_2E}} \end{aligned}\right\} \qquad (5)$$

For stoichiometric reasons, we obtain

$$c_{P'} + c_P = c_{P(0)}. \qquad (6)$$

The total concentration of free and reversibly bound enzymes is

$$C_E = c_E + c_{PE} + c_{P'E}. \quad (7)$$

Finally, we assume that the reaction velocity at any time is proportional to the concentration of the enzyme-penicillin complex

$$-\frac{dc_P}{dt} = k_{PE} c_{PE}. \quad (8)$$

After eliminating from equations (3)—(7) c_E, c_{PE}, $c_{P'E}$, c_{A_1E}, and c_{A_2E} we obtain

$$-\frac{dc_P}{dt}\left[1 + \frac{K_P}{c_P} + \frac{K_P}{K_{P'}}\left(\frac{c_{P(0)}}{c_P} - 1\right) + \frac{K_P}{c_P}\left(\frac{c_{A_1}}{K_{A_1}} + \frac{c_{A_2}}{K_{A_2}}\right)\right] = C_E k_{PE}. \quad (9)$$

For $t = 0$, we have $c_P = c_{P(0)}$, and by integration we obtain

$$\left.\begin{aligned} &c_{P(0)}\left(1 - \frac{c_P}{c_{P(0)}}\right)\left(1 - \frac{K_P}{K_{P'}}\right) + \\ &+ K_P\left(1 + \frac{c_{P(0)}}{K_{P'}} + \frac{c_{A_1}}{K_{A_1}} + \frac{c_{A_2}}{K_{A_2}}\right)\left(-\ln \frac{c_P}{c_{P(0)}}\right) = C_E k_{PE} t. \end{aligned}\right\} \quad (10)$$

This equation describes the relation between the time t and the penicillin concentration c_P.

The Course of the Reaction as a Function af the Enzyme Concentration.

Equation (10) shows that, as it is generally the case, inverse proportionality between the time and the total enzyme concentration must be expected if the other quantities in the formula are constant. In the following, this relation will be checked experimentally.

To this purpose, the enzyme concentration should be varied without simultaneously changing the salt concentration or the concentrations of inhibiting substances possibly present in the penicillinase solution. This is not feasible by changing the amount of culture filtrate in the reaction mixture, since the composition of the culture filtrate with respect to inhibiting substances, and partly also with respect to salts, is unknown. The most correct way of solving this problem would be to prepare the pure enzyme

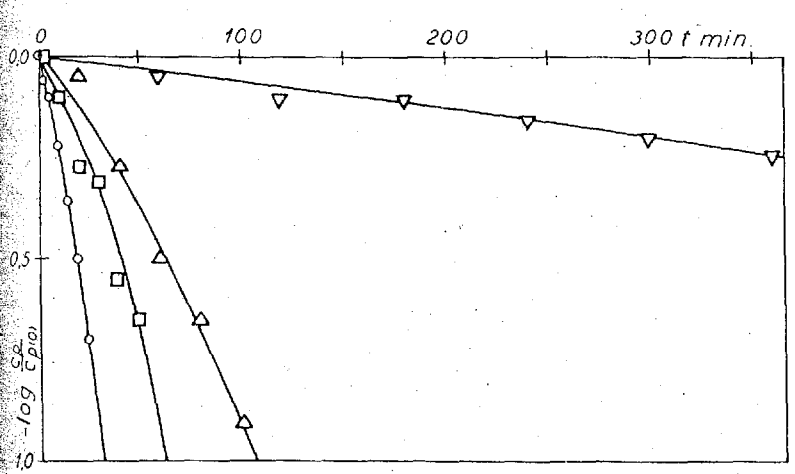

Fig. 4. The course of the reaction as a function of the enzyme concentration (penicillin G "Glaxo". $c_{P(0)} = 0.059$ mmol).

○ 2 ml culture filtrate + 0 ml boiled do. + 2 ml. buffer + 0.01 ml. penicillin solution.
□ 1 ml — — + 1 ml boiled do. + 2 ml. buffer + 0.01 ml. penicillin solution.
△ 0.5 ml — — + 1.5 ml boiled do. + 2 ml. buffer + 0.01 ml. penicillin solution.
▽ 0 ml — — + 2 ml boiled do. + 2 ml. buffer + 0.01 ml. penicillin solution.

However, numerous atterapts at a purification failed. Therefore, the following procedure had to be used. The culture filtrate was boiled so that the enzyme was destroyed. The solutions used for the experiments were then made up of different quantities of fresh and of boiled culture filtrate in such a way that the sum was kept constant. In order to obtain complete destruction of the enzyme, it was found necessary, however, to boil the filtrate for more than 20 minutes. During this procedure, a precipitate was formed in the solution. As it was unknown whether the removal of the substance forming this precipitate would affect the process, it was preferred to boil the culture filtrate for one minute, only, and to refrain from a complete destruction of the enzyme.

The enzyme concentration in the fresh culture filtrate will be denoted by c_{E_1} and the volume applied by V_{E_1}. The corresponding quantities for the boiled enzyme solution will be denoted by c_{E_2} and V_{E_2}, and the total volume of the reaction mixture we

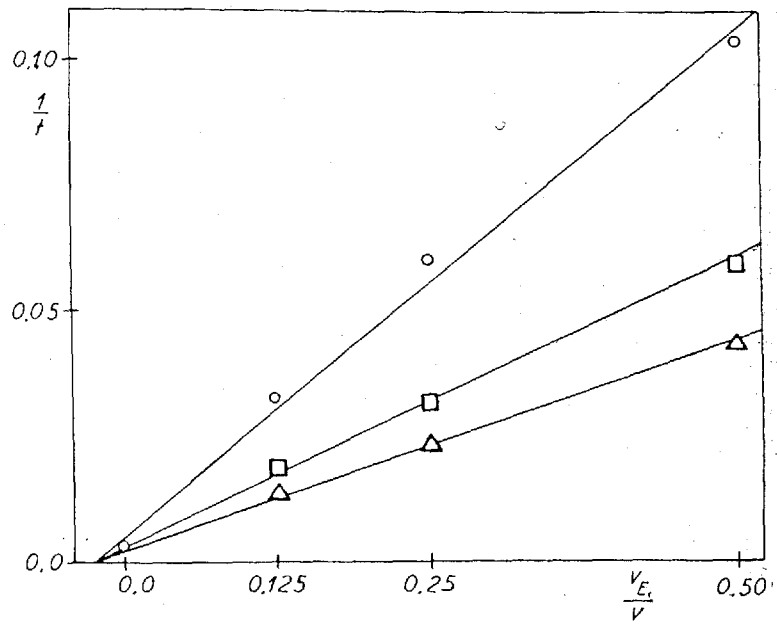

Fig. 5. Velocity variation as a function of the enzyme concentration. Abscissa: the fraction of the total volume of the reaction mixture made out by the non-boiled culture filtrate. Ordinate: reciprocal time elapsing to reach the following degrees of inactivation:

○ $-\log c_P/c_{P(0)} = 0.20$ mmol.
□ $-\log c_P/c_{P(0)} = 0.40$ — .
△ $-\log c_P/c_{P(0)} = 0.60$ — .

shall call V. For the sake of simplicity, equation (10) will be written

$$C_E k_{PE} t = f(c_P) \tag{11}$$

from which we obtain

$$\frac{1}{t} = \frac{k_{PE}}{f_{(c_P)}}(c_{E_1} - c_{E_2})\frac{V_{E_1}}{V} + c_{E_2}\frac{(V_{E_1} + V_{E_2})}{V}. \tag{12}$$

The reciprocal of the time necessary to obtain a certain degree of inactivation for constant $c_{P(0)}$, c_{A_1} etc. will be a linear function of the fraction of the total volume of the reaction mixture which is made out of the fresh culture filtrate. For different degrees of inactivation different slopes are obtained, but all lines should intersect the axis of abscissae at the same point.

The curves obtained for different enzyme concentrations are to be seen in Fig. 4. Fig. 5 illustrates the interdependence between

the reciprocal of the time necessary to reach a certain degree of inactivation and the fraction of the volume of the reaction mixture, which in the respective experiment made out the fresh culture filtrate.

The relation deduced from equation (10) appears to be fulfilled with good approximation.

Experimental Checking of the Theory concerning the Course of the Reaction.

The usual methods of checking graphically whether an enzymatic process occurs in agreement with MICHAËLIS' and MENTEN's theory (cf. VEIBEL 1942) are unsuited in the present case. This is a consequence of the poor relative accuracy with which the penicillin concentrations can be measured. On the other hand, penicillin can be determined in much lower concentrations than most other substances, a fact which should be utilized here. This may be done by the procedure described in the following.

Equation (10) can be transformed into

$$c_{P(0)} \left.\begin{aligned} &\frac{\left(1-\frac{c_P}{c_{P(0)}}\right)\left(1-\frac{K_P}{K_{P'}}\right)+\left(-\ln\frac{c_P}{c_{P(0)}}\right)\left(\frac{K_P}{K_{P'}}+\frac{K_P}{K_{A_1}}\cdot\frac{c_{A_1}}{c_{P(0)}}\right)}{C_E k_{PE}}+ \\ &+\frac{\left(-\ln\frac{c_P}{c_{P(0)}}\right)K_P\left(1+\frac{c_{A_2}}{K_{A_2}}\right)}{C_E k_{PE}}. \end{aligned}\right\} \tag{13}$$

Here, $c_{A_1}/c_{P(0)}$ is constant for one and the same penicillin preparation, and c_{A_2} is constant for one and the same enzyme preparation.

It is obvious that the time necessary to reach a certain degree of inactivation must be expected to be linearly dependent on the initial concentration of penicillin at a constant concentration of the enzyme.

Figs. 6 and 7 show the results of two series of experiments performed with the above discussed preparations A and B, respectively. For each preparation, the course of inactivation was

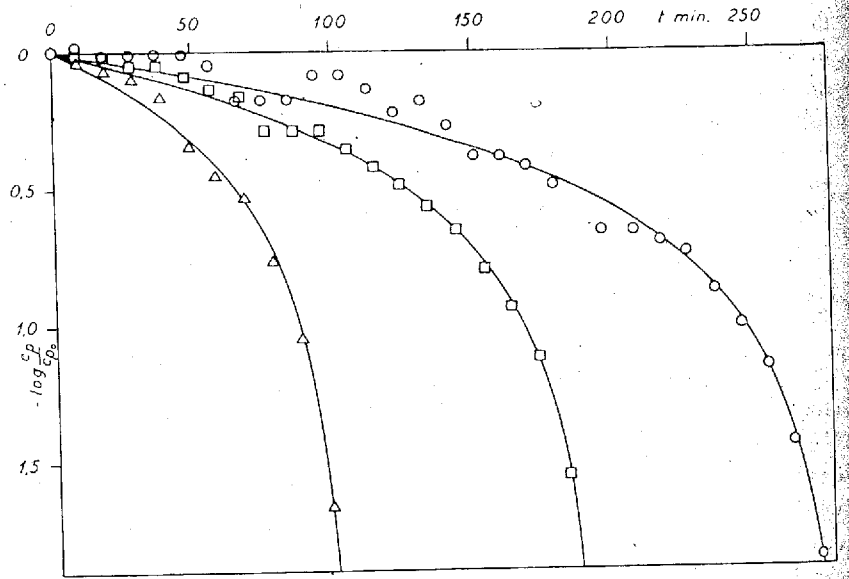

Fig. 6. The course of the enzymatic inactivation of pure penicillin G "Glaxo" (preparation A) at different initial concentrations of penicillin.
○ $c_{P(0)} = 0.625$ mmol.
□ $c_{P(0)} = 0.423$ — .
△ $c_{P(0)} = 0.217$ — .
The drawn curves are calculated.

determined with three different initial concentrations. Owing to the addition of different volumes of penicillin solution, the enzyme concentration is different from experiment to experiment. However, these differences are rather small, since the volume of the penicillin solution added never exceeded 6 % of the total volume. Correction was made for the presumable influence of the slight difference in enzyme concentration on the rate of the process by means of the formula deduced above. To this purpose, the times of the determinations are calculated in such a way that the points inserted refer to the enzyme concentration in the solution prior to the addition of penicillin. In both experimental series given here the same enzyme preparation was used. Thus C_E can be regarded as constant.

The times found for certain constant degrees of inactivation as a function of the initial concentration are shown in Fig. 8. (The times inserted were found by interpolation between the

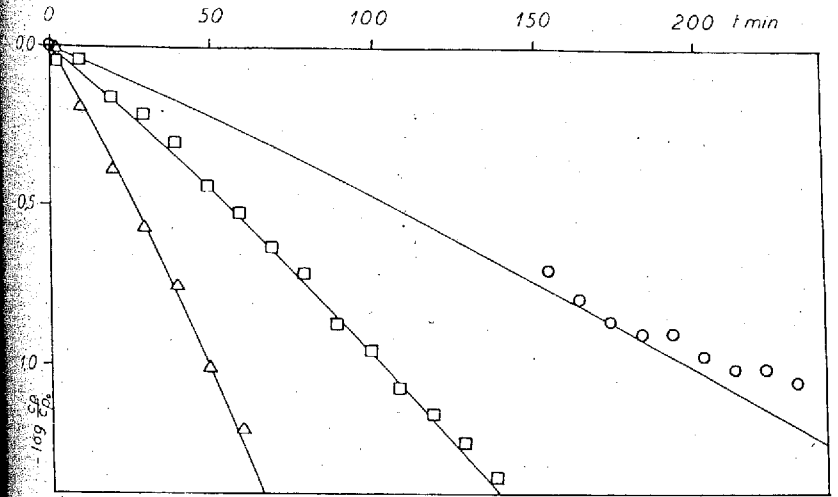

Fig. 7. The course of the enzymatic inactivation of a c. 5 % preparation of penicillin G (preparation B) at different initial concentrations of penicillin.
○ $c_{P(0)} = 0.0830$ mmol.
□ $c_{P(0)} = 0.0423$ — .
△ $c_{P(0)} = 0.0194$ — .
The drawn curves are calculated.

experimental points and not by reading from the curves; these curves are calculated and can first be drawn later.) Fig. 8 shows a linear interdependency between the time and the initial concentration, which is in agreement with equation (13). The lines are drawn in such a way that the parts between the origin and the points of intersection with the axis of ordinates are proportional to $-\ln c_P/c_{P(0)}$, which should be the case according to (13). The experimental uncertainty is too great as to permit a confirmation of the proportionality on the basis of these experiments. This support can, however, be obtained from a third series of experiments which will be discussed later.

Now, we shall investigate whether the slopes of the lines in Fig. 8 are in agreement with equation (13) which will be written as follows:

$$t = a c_{P(0)} + q \tag{14}$$

$$a = \frac{\left(1 - \frac{c_P}{c_{P(0)}}\right)\left(1 - \frac{K_P}{K_{P'}}\right) + \left(-\ln \frac{c_P}{c_{P(0)}}\right)\left(\frac{K_P}{K_{P'}} + \frac{K_P}{K_{A_1}} \cdot \frac{c_{A_1}}{c_{P(0)}}\right)}{C_E k_{PE}} \tag{15}$$

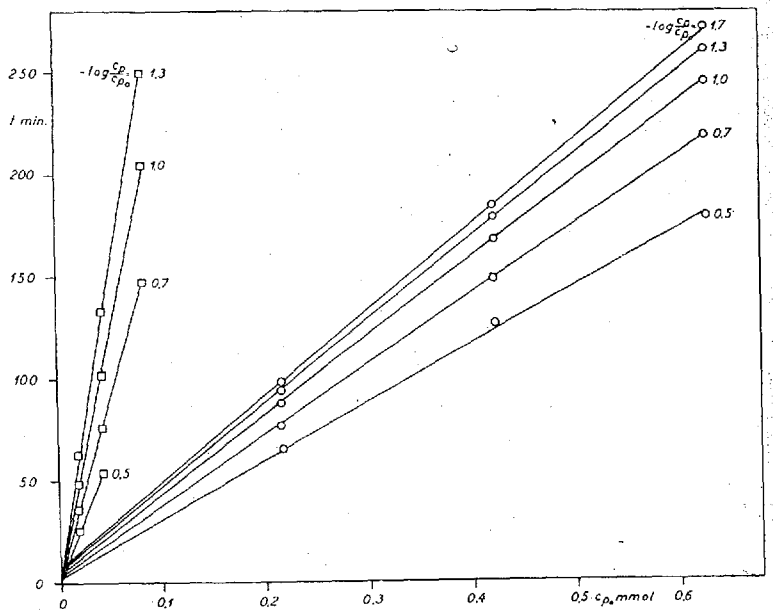

Fig. 8. The time elapsing to reach the given degrees of inactivation is seen to be linearly dependent on the initial concentration.
○ preparation A.
□ preparation B.

$$q = \frac{\left(-\ln\frac{c_P}{c_{P(0)}}\right) K_P \left(1 + \frac{c_{A_2}}{K_{A_2}}\right)}{C_E k_{PE}}. \qquad (16)$$

In Fig. 9, the ordinate $\alpha/(1 - c_P/c_{P(0)})$ is plotted against a function of the degree of inactivation. For the series with preparation A, this figure appears to be approximately independent of the degree of inactivation; the deviations found correspond to the experimental uncertainty. Thus, in this series of experiments, α is proportional to $1 - c_P/c_{P(0)}$. According to equation (15) this means that the second term in the numerator can be put equal to zero. Thus, we obtain

$$\frac{K_P}{K_{P'}} + \frac{K_P}{K_{A_1}} \cdot \frac{c_{A_1}}{c_{P(0)}} \simeq 0. \qquad (17)$$

Since both terms are positive it is seen that, in practice, each of them can be put equal to zero.

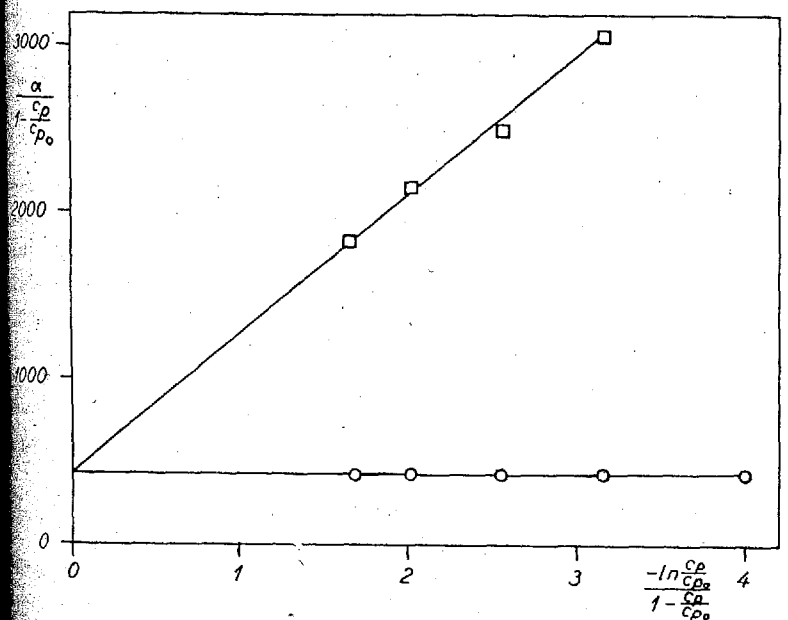

Fig. 9. Checking of formula (20).
○ preparation A.
□ preparation B.

$$\frac{K_P}{K_{P'}} \simeq 0, \qquad (18)$$

$$\frac{K_P}{K_{A_1}} \cdot \frac{c_{A_1}}{c_{P(0)}} \simeq 0. \qquad (19)$$

This does not mean, however, that K_P is very small as compared with $K_{P'}$, but only that the ratio between the two quantities is so small that, in these experiments, no inhibition of the enzyme caused by the inactivation product of penicillin can be detected. The experimental conditions can, of course, be changed in such a way that a more accurate investigation of a possible inhibition becomes feasible; it appears from equation (15) that the investigations should be extended to higher degrees of inactivation, which involves the use of larger amounts of penicillin. Such experiments could not be performed with the quantity of penicillin at our disposal.

From (19) it can be seen that the quantities of competitively inhibiting substances present in preparation A have not been so large that they could be detected. This is in good agreement with the statement of the manufactory that this preparation is a pure penicillin-G-sodium salt.

The chance of detecting a possible inhibition caused by the inactivation product of penicillin is smaller for other preparations containing larger or smaller concentrations of competitively inhibiting substances than for the pure preparation A. Consequently, for all experiments carried out we may put $K_P/K_{P'} = 0$. Then (15) can be converted into

$$\frac{a}{1-\frac{c_P}{c_{P(0)}}} = \frac{K_P}{K_{A_1}} \cdot \frac{c_{A_1}}{c_{P(0)}} \cdot \frac{-\ln\frac{c_P}{c_{P(0)}}}{1-\frac{c_P}{c_{P(0)}}} + \frac{1}{C_E k_{PE}}. \qquad (20)$$

Thus, we should expect to find a linear dependence between $a/(1 - c_P/c_{P(0)})$ and $(-\ln c_P/c_{P(0)})/(1 - c_P/c_{P(0)})$. The part on the axis of ordinates gives the reciprocal of $C_E k_{PE}$, a figure which must be expected to be constant for one and the same enzyme preparation independent of the concentration of inhibiting substances in the penicillin preparation.

From Fig. 9 this is seen to be fulfilled for both preparations A and B.

From Figs. 8 and 9, we find

For enzyme preparation I:
$$\begin{cases} C_E k_{PE} = 0.00233 \text{ mmol/min.} \\ K_P\left(1+\frac{c_{A_2}}{K_{A_2}}\right) = 0.004 \text{ mmol.} \end{cases}$$

For penicillin preparation A:
$$\frac{K_P}{K_{A_1}} \cdot \frac{c_{A_1}}{c_{P(0)}} \sim 0.$$

For penicillin preparation B:
$$\frac{K_P}{K_{A_1}} \cdot \frac{c_{A_1}}{c_{P(0)}} = 1.96.$$

The curves drawn in Figs. 7 and 6 are calculated from these figures by means of equation (13). There is very good agreement between these curves and the points found experimentally.

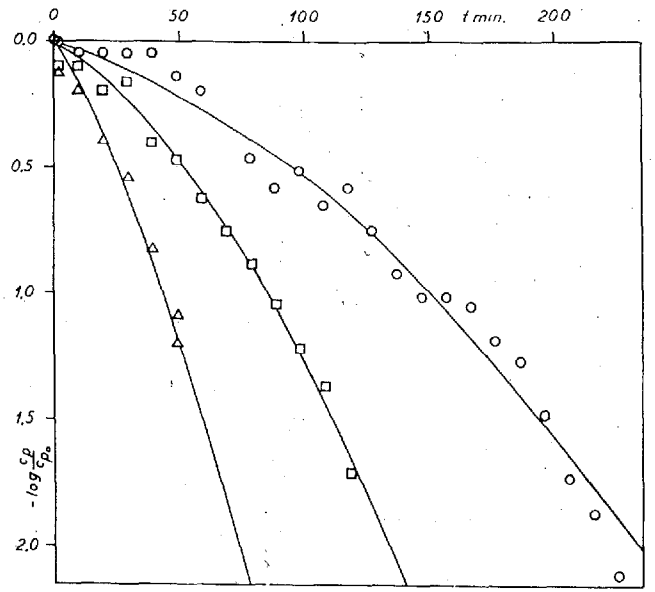

Fig. 10. The course of the enzymatic inactivation of a c. 25 % preparation of penicillin G (preparation C), employing an enzyme preparation (II) different from those applied until now.
○ $c_{P(0)} = 0.235$ mmol.
□ $c_{P(0)} = 0.1190$ — .
△ $c_{P(0)} = 0.0470$ — .

As mentioned above, it is still left to investigate whether the form of the last term in equation (13) can be verified experimentally. In both series of experiments performed up to the present, the numerical values of this term are too small as to make these experiments suitable for such an investigation.

Higher values of the last term in equation (13) were found when another enzyme preparation was applied which was prepared in the same way as the preparation discussed above, but from another charge. Fig. 10 illustrates the course of inactivation of an American commercial preparation of penicillin-G-sodium salt (preparation C, presumably c. 25 %) found when applying this last mentioned enzyme preparation. In Fig. 11 are plotted the times necessary to reach certain degrees of inactivation as a function of the initial concentration of the penicillin. Also here, the linear relation is seen to be valid. The lines in the figure

are drawn in such a way that the best possible agreement with the points is obtained. The ordinates (q) found for the points of intersection of the lines with the axis of ordinates should, according to (16), be proportional to $-\ln c_P/c_{P(0)}$. This is seen from Table 1 to be fulfilled with an approximation of the order of magnitude of the measuring uncertainty.

Thus, also the last term in (13) is in agreement with the experiments.

From this series of experiments, we find in the same way as above

For enzyme preparation II

$$\begin{cases} C_E k_{PE} = 0.0027 \text{ mmol/min.} \\ K_P\left(1 + \frac{c_{A_2}}{K_{A_2}}\right) = 0.020 \text{ mmol.} \end{cases}$$

For penicillin preparation C

$$\frac{K_P}{K_{A_1}} \cdot \frac{c_{A_1}}{c_{P(0)}} = 0.28.$$

The curves drawn in Fig. 10 are calculated on the basis of these figures.

As compared with the penicillin content, the content of competitively inhibiting substances in preparation C is seen to be smaller than in preparation B, which is in good agreement with the high purity of preparation C.

For the enzyme preparation II, the quantity $K_P(1 + c_{A_2}/K_{A_2})$ is five times as large as for the enzyme preparation I. It appears improbable that the two values of K_P should be so different. Both preparations are prepared from one and the same strain of bacteria and under approximately the same cultivation conditions; therefore, it can scarcely be assumed that we here have to do with two chemically different enzymes. Hydrogen ion- and salt concentrations have also been approximately equal in both preparations. For an explanation of the experimental results it is, thus, necessary to assume that the divergency is due to different values of c_{A_2}.

Herewith, the presence of a competitively inhibiting substance in the enzyme solutions is made probable.

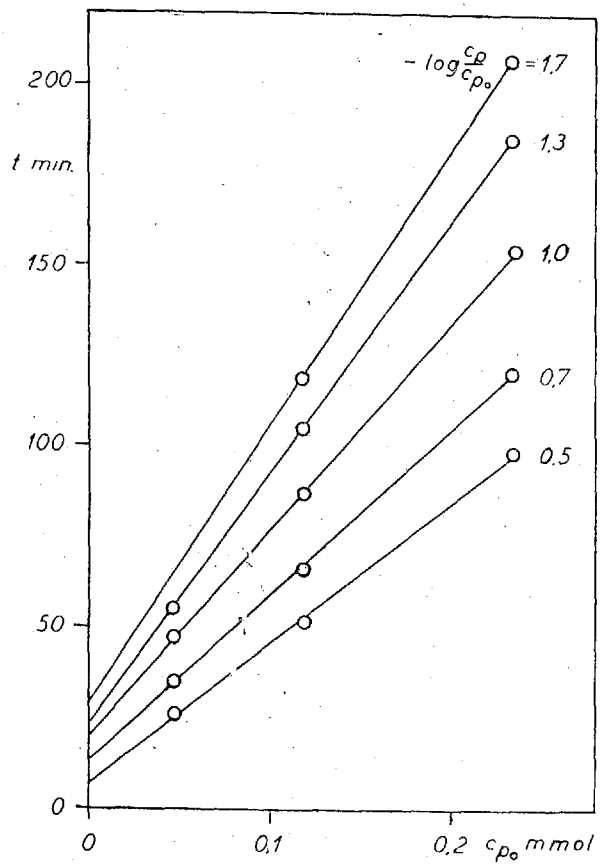

Fig. 11. The time elapsing to reach the given degrees of inactivation in Fig. 10. Here, the lines are seen to intersect the axis of ordinates at a higher point than in Fig. 8. This can be explained by assuming that the enzyme preparation (II) used contains a competitively inhibiting substance in higher concentration than does the enzyme preparation I.

Table 1 (cf. Fig. 11).

$-\log \frac{c_P}{c_{P(0)}}$	q	$\frac{q}{-\ln \frac{c_P}{c_{P(0)}}}$
1.7	30	7.2
1.3	24	8.0
1.0	20	8.5
0.7	13	8
0.5	7	6

The Effect of the Temperature on the Enzymatic Process.

In consequence of the deductions made above, equation (13) can be simplified to

$$t = c_{P(0)} \left\{ \frac{\left(1 - \frac{c_P}{c_{P(0)}}\right) + \left(-\ln \frac{c_P}{c_{P(0)}}\right) \frac{K_P}{K_{A_1}} \cdot \frac{c_{A_1}}{c_{P(0)}}}{C_E k_{PE}} + \right. \left. \frac{\left(-\ln \frac{c_P}{c_{P(0)}}\right) K_P \left(1 + \frac{c_{A_2}}{K_{A_2}}\right)}{C_E k_{PE}} \right\} \quad (21)$$

The experiments discussed in the following were performed with enzyme preparation I. Therefore, the numerical values of the last term of (20) are rather small compared with the first term on the right side. Since it can scarcely be assumed that the relative change with temperature of the last term is much larger than the relative change of the first term, it will be admissible in a first approximation to neglect this last term.

Using a penicillin preparation without detectable content of inhibiting substances (preparation A), (21) can further be simplified to

$$t = c_{P(0)} \frac{1 - \frac{c_P}{c_{P(0)}}}{C_E k_{PE}} \quad (22)$$

Here, only k_{PE} is dependent on temperature. According to this equation, we should expect to find the course of the reaction to change with temperature in such a way that t for constant $c_P/c_{P(0)}$ is changed at a given change in temperature by a constant factor, independent of the degree of inactivation.

In the experiment shown in Fig. 12 this condition appears to be fulfilled in good approximation. Thus, the change in the course of the reaction with temperature can be characterized only by the change of the time elapsing until a given degree of inactivation is reached.

It becomes clear from the course of the curves that the most accurate expression for the temperature dependence is obtained

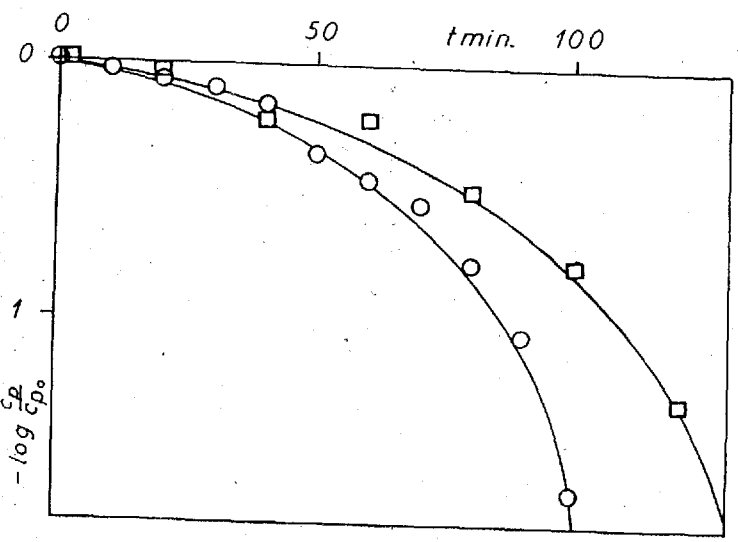

Fig. 12. The course of the reaction on inactivation of penicillin preparation A with enzyme preparation I.
○ 30° C.
□ 20° C.
$c_{P(0)} = 0.217$ mmol.

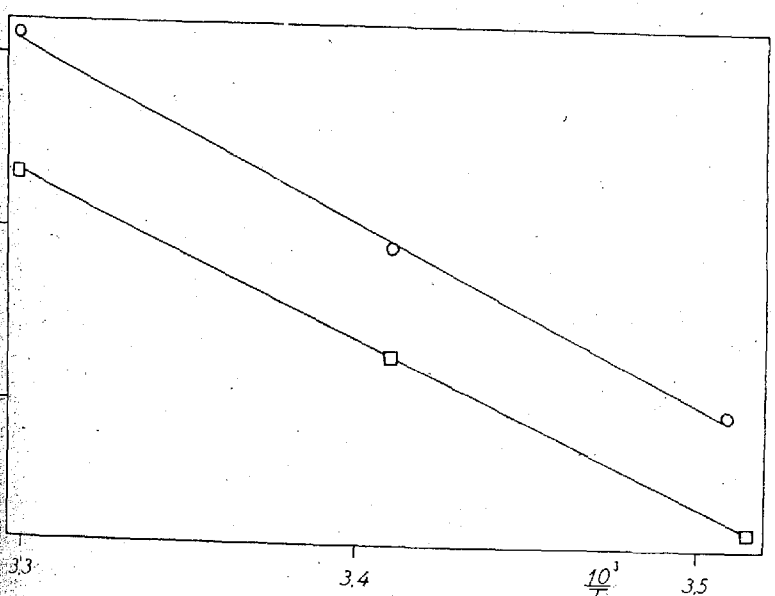

Fig. 13. The logarithm of the velocity of the enzymatic process (ordinate) as a function of the reciprocal of the absolute temperature (abscissa).
○ preparation A.
□ preparation B.

by measuring the time elapsing till a given degree of inactivation is reached, for example, the degree of inactivation corresponding to a point in the last steep part of the curve, since here the relative uncertainty of the time found is least.

Fig. 13 shows the results of three experiments of this kind. The logarithm of the reciprocal time is plotted as ordinate and the reciprocal absolute temperature as abscissa. In agreement with Arrhenius' law, the interdependence between these magnitudes is linear. For Arrhenius' critical increment, we find

$$E_A = -R \frac{d\ln\frac{1}{t}}{d\frac{1}{T}} = -R \frac{d\ln k_{PE}}{d\frac{1}{T}} = 4650 \text{ cal/mol.} \quad (2$$

This figure holds for the buffer and for the hydrogen ion concentration (phosphate + c. $^1/_2$ m NaCl, $c_{H^+} = 10^{-6.7}$) applied here. In other buffers other numerical values are to be expected.

From these experiments, no conclusions can be drawn as to how great a fraction of the change according to changing temperature is due to a change in the hydrogen ion- and the hydroxyl ion concentration and how much is caused by a change in velocity of the velocity determining process proper. Consequently, the magnitude of the activation energy of the velocity-determining process in the conversion of the molecule PE is still unknown. Even if this fact is taken into consideration, it seems to be clear that the activation energy of the enzymatic process should be lower than the activation energy of the spontaneous conversion of penicillin, for which $E_A = 22700$ cal/mol (BRODERSEN 1947 b). This is also in agreement with theoretical expectations, since the effect of the enzyme as a catalyst actually consists in a reduction of the activation energy whereby the velocity of the reaction is increased.

The experimental finding of the slight temperature dependence of the penicillin conversion under the action of penicillinase thus lends further support to the assumption that the process is of catalytical nature.

Finally, experiments were performed on the temperature coefficient of the inactivation of the impure preparation B. The

results of these experiments are comprised in Fig. 13; also under these conditions, the process follows Arrhenius' law. We find

$$E_A = 4200 \text{ cal/mol.}$$

According to equation (21), we might expect to find that the value of E_A differs considerably from the value found for the pure preparation, since here also the temperature will influence K_P and K_{A_1}. The difference found between these two values is, however, not large enough to be regarded as significant if the experimental uncertainty is taken into consideration. Thus, K_P and K_{A_1} seem to change almost proportionally. The result is not especially surprising if we keep in mind that the two equilibrium reactions to which these two constants refer must be assumed to be chemically related.

Discussion.

From the above account it seems safe to conclude that the penicillin inactivating substance studied here acts as a catalyst. The first part of the process is a reversible formation of an enzyme-penicillin complex which undergoes an irreversible decomposition and is split into a free enzyme and a biologically inactive conversion product of penicillin. This enzyme can, moreover, combine reversibly with substances other than penicillin, which are found both in the enzyme preparation and in less pure penicillin preparations. As it was shown above, the experimental results are in perfect agreement with the theoretical expectation.

Penicillinase can presumably be classified as an amidase since, according to Anglo-American investigations, the amide bond in the four ring is hydrolyzed in the penicillin molecule (The Committee on Medical Research and the Medical Research Council 1945). Several other amidases have been detected in the bacteria and it is not impossible that penicillinase is identical with one of them. Even if this is not the case, it would be natural to assume that penicillinase plays a role as an enzyme in these bacteria. It is rather improbable that strains of bacteria which have never been exposed to penicillin should contain an enzyme the only function of which is to inactivate penicillin. This other process

which is catalyzed by penicillinase in the bacteria will be in-
hibited in the presence of penicillin, since part of the penicillinase
will be bound to penicillin and, thus, be ineffective in the other
process. In this connection, we remember TURNER's et al. (1943)
statement that penicillin inhibits the splitting of urea with urease
which also is an amidase.

Now, it may be assumed that the inhibiting effect of penicillin
on the growth of the bacteria is a blocking of an amidase
necessary for the normal growth of the bacteria. If this holds
it should also be expected that the substances competitively
inhibiting penicillinase detected above should have the same bac-
teriological effect as has penicillin. This is not the case and
therefore, the explanation of the mode of action of penicillin
should possibly be searched in other fields. On the basis of the
above discussion it will, however, appear appropriate in future
investigations into the action of penicillin to pay special attention
to the amidases of the bacteria.

Summary.

The course of reaction for the enzymatic inactivation of some
penicillin G preparations is investigated as a function of peni-
cillin- and enzyme concentration and temperature.

It is shown that the penicillinase acts as a catalyst.

The enzyme is shown to be stable during the process at 30°
and pH 6.7.

A relation is deduced for the course of the reaction as a
function of the concentrations of penicillinase, penicillin, and
competitively inhibiting substances, applying MICHAËLIS and MEN-
TEN's theory.

Good agreement is prevailing between this relation and the
experimental curves, if it is taken into consideration that both
the less pure penicillin preparations and the enzyme preparations
contain competitively inhibiting substances.

The temperature dependence is shown to be in agreement
with expectations.

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References.

BRODERSEN, R., Acta pathol. et microbiol. 1947a, *24*, 383.
— R., Acta chem. 1947b, *1*, 403.
Committee on Medical Research and the Medical Research Council. Nature 1945, **156**, 766.
MICHAËLIS, L. and MENTEN, M. L., Biochem. Z. 1913, **49**, 333.
TURNER, J. C., HEATH, F. K. and MAGASANIK, B., Nature 1943, **152**, 326.
VEIBEL, S., Ingeniøren 1942, Nr. 11. K. 9—14.
WOODRUFF, H. B. and FOSTER, J. F., J. Bact. 1945, **49**, 7.

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ON THE SYSTEMATIC CHANGES OF THE ECCENTRICITIES OF NEARLY PARABOLIC ORBITS

BY

ERIK SINDING

KØBENHAVN
I KOMMISSION HOS EJNAR MUNKSGAARD
1948