

The Inactivation Velocity of Penicillin G by Acids as a Function of Temperature

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In a previous paper¹, the inactivation velocity of penicillin G in acid solution was investigated at 30° C and at varying hydrogen ion concentrations. In the present work, the effect of a variation in temperature on this process is treated.

THEORY

In the paper mentioned above, the following equation is given for the relation between the rate of inactivation and the hydrogen ion concentration at constant temperature and salt concentration:

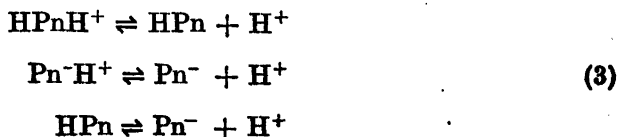
$$k = \left(\frac{k_+}{K_+} \cdot c_{\text{H}^+} + \frac{k_{\pm}}{K_{\pm}} \cdot K_0 \right) \frac{c_{\text{H}^+}}{K_0 + c_{\text{H}^+}} \quad (1)$$

At a constant hydrogen ion concentration, the terms on the right side of this formula, k_+ , k_{\pm} , K_+ , and K_{\pm} , K_0 are temperature dependent.

The dissociation constants vary with the temperature in the following way:

$$\frac{dK_+}{dT} = K_+ \frac{Q_+}{RT^2}; \quad \frac{dK_{\pm}}{dT} = K_{\pm} \frac{Q_{\pm}}{RT^2}; \quad \frac{dK_0}{dT} = K_0 \frac{Q_0}{RT^2} \quad (2)$$

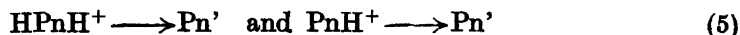
where Q_+ , Q_{\pm} , and Q_0 are the molar heat absorptions at the corresponding dissociation processes:



For the velocity constants k_+ and k_{\pm} the variation with the temperature is given by

$$k_+ = Z \cdot e^{-\frac{E_+}{RT}} \quad \text{and} \quad k_{\pm} = Z \cdot e^{-\frac{E_{\pm}}{RT}} \quad (4)$$

Each of these two equations refers to one of the two velocity determining processes, *viz.*



In (4), Z denotes the number of collisions between one penicillin molecule and water molecules per unit of time; E_+ and E_{\pm} denote the activation energies. This holds under the following suppositions:

The activation of the reacting penicillin molecules occurs exclusively by collision with water molecules, and the activation energy is not gathered from degrees of freedom other than the translatory one.

All collisions the energy of which surpasses the activation energy cause activation, irrespective of the orientation of the penicillin molecule at the moment of the collision.

All activated molecules are subjected to the irreversible process (5) before the activation energy has time to spread by new collisions with water molecules.

If these conditions are not fulfilled — and we can scarcely assume that they are — we obtain equations conformable to (4), where Z , E_+ , and E_{\pm} only should be attributed other values than the collision number and the activation energies.

Assuming that Z is independent of the temperature, we obtain

$$\frac{dk_+}{dT} = \frac{k_+}{T^2} \cdot \frac{E_+}{R} \quad \frac{dk_{\pm}}{dT} = \frac{k_{\pm}}{T^2} \cdot \frac{E_{\pm}}{R} \quad (6)$$

The equations (2) and (6) indicate the temperature dependence of the variable quantities of equation (1). From this, we find as an expression for the variation of the total decomposition velocity as a function of the temperature

$$E_A = -R \frac{d \ln k}{d \frac{1}{T}} = \frac{1}{k} \cdot \frac{k_+}{K_+} \cdot c_{\text{H}^+} \left[(E_+ - Q_+) \frac{c_{\text{H}^+}}{c_{\text{H}^+} + K_0} - Q_0 \frac{c_{\text{H}^+} \cdot K_0}{(c_{\text{H}^+} + K_0)^2} \right] \\ + \frac{1}{k} \cdot \frac{k_{\pm}}{K_{\pm}} \cdot c_{\text{H}^+} \left[(E_{\pm} - Q_{\pm} + Q_0) \frac{K_0}{c_{\text{H}^+} + K_0} - Q_0 \frac{K_0^2}{(c_{\text{H}^+} + K_0)^2} \right] \quad (7)$$

where E_A denotes the Arrhenius critical increment.

In order to arrive at expressions which are less complicated and better suited for experimental checking, we shall examine especially the conditions prevailing at very low and very high hydrogen ion concentrations. For this purpose, equations (10) and (11) from the previously mentioned paper¹ will be used:

$$k = \frac{k_+}{K_+} \cdot c_{H^+} \quad \text{for } c_{H^+} \gg K_0 \quad (8)$$

$$k = \frac{k_{\pm}}{K_{\pm}} \cdot c_{H^+} \quad \text{for } c_{H^+} \ll K_0 \quad (9)$$

By inserting these conditions into (7) we obtain

$$-R \frac{d \ln k}{d \frac{1}{T}} = E_+ - Q_{+*} = E_{A_+} \quad \text{for } c_{H^+} \gg K_0 \quad (10)$$

$$-R \frac{d \ln k}{d \frac{1}{T}} = E_{\pm} - Q_{\pm} = E_{A_{\pm}} \quad \text{for } c_{H^+} \ll K_0 \quad (11)$$

where E_{A_+} and $E_{A_{\pm}}$ denote Arrhenius' critical increments for the two processes.

In other words, for low and high hydrogen ion concentrations we should find a linear relation between the logarithm of the velocity constant and the reciprocal of the absolute temperature, in agreement with Arrhenius' law, on the supposition that E_+ , E_{\pm} , Q_+ and Q_{\pm} are constant.

For hydrogen ion concentrations of the same order of magnitude as K_0 we obtain a curvilinear interdependence between the logarithm of the velocity constant and the reciprocal temperature. Here, — however, not at low and high hydrogen ion concentrations — Q_0 enters the expression for the temperature dependence. This is due to the fact that, in this case, the process consists of two parallel processes, viz. the inactivation of penicillin molecules with undissociated carboxyl groups and, on the other hand, the inactivation of penicillin ions where the hydrogen ion of the carboxyl groups is dissociated off. The two processes occur at different rates and, consequently, the total rate is changed when the ratio between the two reacting substances (*i. e.* their relative concentration) is changed. This ratio is determined by the dissociation constant K_0 which is temperature dependent according to equation (2) to a degree given by the magnitude of Q_0 . If Q_0 is 0, which cannot be excluded since we deal with a carboxyl group, K_0 will be independent of the temperature.

In this case, the velocity variation of the total process as a function of temperature will be due to the change in velocity of the single processes. Even if the single processes follow Arrhenius' law, the total process will not do so, unless the two quantities E_{A+} and $E_{A\pm}$ in equations (10) and (11) are equally large. Since, however, this condition is not fulfilled, as it will be seen in the experimental section of this work, the experimental checking of the validity of equation (7) at hydrogen ion concentrations of the same order of magnitude as K_0 cannot be performed on the basis of Arrhenius' law.

E_{A+} and $E_{A\pm}$ are accessible to experimental determination by means of equations (10) and (11). From (8) and (9) we can, by means of equations (2) and (6) find the terms k_+/K_+ and k_{\pm}/K_{\pm} at different temperatures. By experimental determinations of a series of corresponding values of T and k we may find E_A for different values of T . If the hydrogen ion concentrations are known in the single experiments, it will be possible in this way for each value of T to determine a relation between possible values of the two remaining terms of (7), viz. K_0 and Q_0 . If Q_0 is assumed to be independent of the temperature, the numerical value of Q_0 and, moreover, K_0 as a function of the temperature, can be determined. By inserting the values obtained into equation (12)

$$-R \frac{d \ln K_0}{d \frac{1}{T}} = Q_0 \quad (12)$$

the validity of (7) can be checked.

In practice, however, this procedure is not convenient, since it involves too much calculation work.

It is much more convenient to determine the value of K_0 at different temperatures directly from equation (1) which, for this purpose, has to be converted into

$$K_0 = -c_{H^+} \frac{\frac{k_+}{K_+} c_{H^+} - k}{\frac{k_{\pm}}{K_{\pm}} c_{H^+} - k} \quad (13)$$

c_{H^+} and k are determined at different temperatures and k_+ , K_+ and k_{\pm} , K_{\pm} are found at the corresponding temperatures from the formulae

$$\log \left(\frac{k_+}{K_+} \right)_{T_1} = \log \left(\frac{k_+}{K_+} \right)_{T_2} + \frac{E_{A+}}{R} \left(\frac{1}{T_1} - \frac{1}{T_2} \right) \quad (14)$$

$$\log \left(\frac{k_{\pm}}{K_{\pm}} \right)_{T_1} = \log \left(\frac{k_{\pm}}{K_{\pm}} \right)_{T_2} + \frac{E_{A\pm}}{R} \left(\frac{1}{T_1} - \frac{1}{T_2} \right) \quad (15)$$

Here, E_{A+} and $E_{A\pm}$ are determined experimentally by means of (10) and (11). R has the numerical value 4.571 cal, if log denotes Briggian logarithms. If we put $T_2 = 303.1$,

$$\log \left(\frac{k_+}{K_+} \right)_{T_2} = 0.80 \quad \log \left(\frac{k_{\pm}}{K_{\pm}} \right)_{T_2} = 1.32$$

From the previous work¹, the values 0.74 and 1.35 are obtained. The figures given here are the results of a more accurate determination⁶.

The values for K_0 found in this way may be applied to the determination of Q_0 by means of equation (12). The accuracy cannot be expected to be very high, since the differences in (13) can only be determined very roughly.

EXPERIMENTAL

The rate of inactivation of penicillin G at different temperatures was investigated by means of the method described previously.

Three series of experiments were performed, *viz.* one for $c_{H^+} \gg K_0$, one for $c_{H^+} \ll K_0$, and, finally, one where the hydrogen ion concentrations were of the same order of magnitude as K_0 . Since K_0 has the numerical value of ca. 10^{-3} , dilute hydrochloric acid, Sørensen's acetate-acetic acid, and Sørensen's glycine hydrochloric acid, respectively, were chosen as buffer solutions. To all buffers was added sodium chloride to a total molarity of 0.5. When choosing the hydrogen ion concentrations, not only the magnitude of K_0 had to be taken into consideration, but moreover the fact that the suitable range of temperature is limited, and that the velocity constants must lie within certain limits. Experiments below 0°C or above 100°C are unfeasible, while the limits for the measurable velocity constants are given by the still possible degrees of decomposition and the duration of the experiment. In practice, the degree of decomposition cannot be changed very much from experiment to experiment: a reduction of the degree of composition applied here would involve a lower accuracy of the measurements while, on the other hand, an essential increase was found impossible, because the quantities of penicillin available for the experiments were too small. The duration of the experiment, however, can be varied within rather wide limits; it is difficult to reduce the total duration of the experiment to less than 4 minutes since, in that case, the time and the temperature cannot be determined with sufficient accuracy. The upper limit for the duration of the experiments is given by the demand of sterility; it is difficult for many days to maintain sterility in a glass from which samples are drawn from time to time, especially at temperatures where micro-organisms

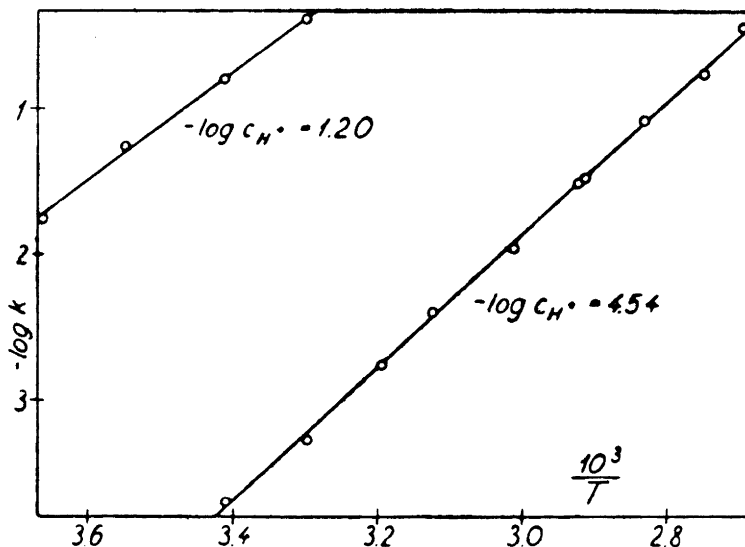


Fig. 1. Inactivation velocity at constant hydrogen ion concentrations as a function of temperature.

grow easily; the longest time applied was 6 days. The results obtained in two series of experiments are shown in fig. 1, where the logarithm of the velocity constants is plotted as a function of the reciprocal absolute temperature. The four straight lines which border the figure symbolize approximately the limitations mentioned above.

In order to check the validity of equation (7) the values for Q_0 and K_0 can now be inserted and the values of the left side of the equation can be calculated for different temperatures. This is, however, superfluous, since in the deduction of (7) no other theoretical suppositions were made than those already applied in the determination of K_0 and Q_0 . Thus, the test for the accuracy of the theory will consist only in an investigation of whether the values found for K_0 satisfy equation (12) within the limits of the experimental accuracy. In this connection, Q_0 can be considered independent of the temperature, because the values known for the heat of dissociation of carbonic acids do not vary more than what falls within the limits of the experimental accuracy in the range of temperature used for the velocity measurement of the present process.

The experiments were performed in water- or oil thermostats. At higher temperatures (70—100° C) it was found difficult to maintain the same temperature in the reaction mixture and in the thermostat; this might be due to the circumstance that it was un-

feasible to apply stirring of the reaction mixture which had to be kept sterile with regard to the penicillin measurements. For the same reason, the thermometer could not be placed into the reaction mixture, but it was put into a glass containing the same amount of liquid, which was treated in the same way as the experimental glass. The temperature used in the calculations was an estimated mean of the temperature readings made in the course of the experiment. The uncertainty of the temperature determination was found to be less than 0.1°; as it will be shown later, this is a sufficient accuracy in view of the fact that the uncertainty of the determination of the velocity constants is rather high.

CALCULATION

The velocity constants were calculated according to the method described previously¹; therefore, this point will not be treated here. However, it is necessary to deal with the calculation of the hydrogen ion concentrations in the applied buffers as a function of the temperature.

In buffers of hydrochloric acid, the hydrogen ion concentration is equal to the hydrochloric acid concentration and is independent of the temperature. However, the hydrogen ion concentrations of glycine- and acetate buffers depend on the temperature, because the dissociation constants of the buffer substances are temperature dependent. In view of the fact that a direct measurement of the hydrogen ion concentration at the experimental temperature is encumbered with great difficulties, all reaction mixtures were cooled (heated) after the conclusion of the experiments to 20° C and the hydrogen ion concentration was determined at this temperature by means of the method described previously. The hydrogen ion concentration of glycine buffers at the experimental temperature can be calculated on the basis of Owen's² measurements of the dissociation constant of glycine at various temperatures. Owen's measurements comprise salt concentrations from 0.005 *M* to 0.04 *M*. Within this range the heat of dissociation seems to be independent of the salt concentration and, consequently, it can be assumed with the accuracy necessary here that the heat of dissociation also will have the same value for 0.5 *M* NaCl. The heat of dissociation however, varies considerably with the temperature, a fact which must be taken into account in the calculations. The highest temperature applied by Owen is 45° C. The hydrogen ion concentration in a glycine buffer at 50° C, was determined by extrapolation.

In the case of acetic acid, more detailed measurements are available; here, even the dependence of the heat of dissociation on the salt concentration over large concentration ranges is taken into consideration. Harned and Hickey³ have determined the dissociation constant of acetic acid in sodium chloride solutions at 0—40° C and found that the following equation holds:

$$-\log K_T = -\log K_{\max} + 5 \cdot 10^{-5} (T - T_{K_{\max}})^2 \quad (16)$$

Harned and Embree⁴ have shown that this equation is also valid over a somewhat larger range of temperature, at any rate if the salt concentration is zero. Since, however, Harned and Hickey found the constant $5 \cdot 10^{-5}$ to be independent of the salt concentration, it seems permissible to apply equation (16) in the temperature range 20—100° C. On the basis of the examples available in the literature concerning the application of this equation, it must be assumed that the error in this calculation of $\log K_T$ is less than 0.03. Harned and Hickey found for 0.5 *M* NaCl — $\log K_{\max} = 4.476$. The temperature, $T_{K_{\max}}$ where this maximum value is found, was determined by them to be 305.5° abs.

In the author's experiments, the concentrations of acetic acid and sodium acetate are the same, *viz.* 0.05 *M*. Since the hydrogen ion concentration at all the temperatures employed is low relative to these concentrations we obtain

$$-\log (c_{\text{H}^+})_T = -\log (c_{\text{H}^+})_{T_0} + 5 \cdot 10^{-5} [(T - 305.5)^2 - (T_0 - 305.5)^2] \quad (17)$$

from which the hydrogen ion concentrations may be calculated from values determined at 20° C, by inserting the value of T .

The velocity constants found in acetate buffer at different temperatures and hydrogen ion concentrations are converted to one and the same hydrogen ion concentration by means of equation (9), the supposition $c_{\text{H}^+} \ll K_0$ holding in these experiments. A corresponding conversion of the velocity constants to one and the same hydrogen ion concentration for the decomposition experiments performed in glycine buffer cannot be carried out directly, since here the hydrogen ion concentration is of the same order of magnitude as K_0 . On the other hand, the application of these latter figures does not require such a conversion.

Table 1. Inactivation velocity in dilute hydrochloric acid. 0.5 *M* NaCl.

| $\frac{10^3}{T}$ | $-\log c_{\text{H}^+}$ | $-\log k$ 1/min. |
|------------------|------------------------|---------------------|
| 3,661 | 1.20 | 1.76 |
| 3,549 | 1.20 | 1.36 |
| 3,412 | 1.20 | 0.80 |
| 3,299 | 1.20 | 0.39 |

Table 2. Inactivation velocity in acetate - acetic acid. 0.5 M NaCl.

| $\frac{10^3}{T}$ | $-\log c_{H^+}$ calc. | $-\log k$ experimental | $-\log k$ calc. for $-\log c_{H^+} = 4.54$ |
|------------------|--------------------------|---------------------------|---|
| 3,410 | 4.54 | 3.70 | 3.70 |
| 3,298 | 4.54 | 3.28 | 3.28 |
| 3,195 | 4.54 | 2.76 | 2.76 |
| 3,125 | 4.55 | 2.41 | 2.40 |
| 3,012 | 4.57 | 1.99 | 1.96 |
| 2,924 | 4.60 | 1.58 | 1.52 |
| 2,915 | 4.61 | 1.55 | 1.48 |
| 2,833 | 4.65 | 1.19 | 1.08 |
| 2,755 | 4.70 | 0.92 | 0.76 |
| 2,698 | 4.76 | 0.66 | 0.44 |

Table 3. Inactivation velocity in glycine-hydrochloric acid. 0.5 M NaCl.

| $\frac{10^3}{T}$ | $-\log c_{H^+}$ 20° C exp. | $-\log c_{H^+}$ T° calc. | $-\log k$ | $-\log \frac{k_+}{K_+} c_{H^+}$ | $-\log \frac{k_+}{K_+}$ | $-\log K$ | $-\log k$ calc. |
|------------------|-------------------------------|------------------------------------|-----------|---------------------------------|-------------------------|-----------|--------------------|
| 3,531 | 2.90 | 2.94 | 2.77 | 3.04 | 2.69 | 2.60 | 2.80 |
| 3,410 | 2.97 | 2.97 | 2.30 | 2.60 | 2.16 | 2.85 | 2.29 |
| 3,298 | 2.98 | 2.95 | 1.79 | 2.15 | 1.63 | 2.80 | 1.77 |
| 3,198 | 2.98 | 2.93 | 1.30 | 1.73 | 1.13 | 2.80 | 1.28 |
| 3,094 | 2.97 | 2.91 | 0.89 | 1.33 | 0.65 | 3.00 | 0.83 |

RESULTS AND DISCUSSION

In tables 1 and 2 the results are shown of $c_{H^+} \ll K_0$ and of $c_{H^+} \gg K_0$, respectively. The converted velocity constants are plotted in fig. 1 which shows $-\log k$ as a function of $1/T$. The points fall with good approximation on straight lines. As it results from table 2, however, a curvilinear dependence is found between the logarithms to the non-converted velocity constants and $1/T$.

From the coordinates of the plotted points and by means of the equations (10) and (11) we obtain (using the method of least squares),

$$E_{A+} = 17\,570 \pm 340 \text{ cal/mole} \quad (18)$$

$$E_{A\pm} = 20\,980 \pm 220 \text{ cal/mole} \quad (19)$$

Since the mean errors of these two figures are so small relative to their difference, we may state with great certainty that the true values must be different.

$$\begin{aligned} \text{At } 30^\circ \text{C } -\log k &= 0.38 \pm 0.02 \text{ for } -\log c_{\text{H}^+} = 1.20 \text{ and} \\ -\log k &= 3.24 \pm 0.02 \text{ for } -\log c_{\text{H}^+} = 4.54. \end{aligned}$$

From previous experiments performed at 30°C we find $-\log k = 0.39$ and $-\log k = 3.21$, respectively, in satisfactory agreement herewith.

Finally, table 3 contains the results of the last series of experiments at hydrogen ion concentrations of the same order of magnitude as K_0 .

The values of $-\log K_0$ are calculated for each temperature by means of the equations (13), (14), and (15). The mean uncertainty of these figures can be estimated to be about 0.2, and if this is taken into consideration, we find that no noticeable variation of K_0 with temperature can be observed. This means that we can put $Q_0 = 0$.

The Committee on Medical Research and the Medical Research Council⁵ give $-\log K_0 = 2.8$. The values found in the present experiments are in good agreement with this statement; it should, however, be kept in mind that the present experiments were performed in $0.5 M$ NaCl, while the Anglo-American values must be supposed to be valid for an ionic strength zero.

Obviously, the procedure outlined above is not suited for a determination of Q_0 . The significance of the above account lies, thus, only in the fact that for the practical calculation of the rate of inactivation of penicillin by means of equation (7) we can put $Q_0 = 0$ within the range of temperature investigated here, *i. e.* 10° — 50°C .

$$E_A = -R \frac{d \ln k}{d T} = E_{A+} + (E_{A\pm} - E_{A+}) \frac{\frac{k_{\pm}}{K_{\pm}} K_0}{\frac{k_{\pm}}{K_{\pm}} K_0 + \frac{k_+}{K_+} c_{\text{H}^+}} \quad (20)$$

Since k_{\pm}/K_{\pm} and k_+/K_+ are dependent on the temperature, also E_A will be dependent on the temperature if c_{H^+} is of the same order of magnitude as K_0 . E_A will, however, always lie between E_{A+} and $E_{A\pm}$, because the numerical value of the fraction will always be between 0 and 1. At a given hydrogen ion concentration we find from equation (20) and equations (14) and (15) that E_A approaches $E_{A\pm}$ at very high temperatures and, inversely, that E_A approaches E_{A+} at very low temperatures, since $E_{A\pm} > E_{A+}$.

In order to investigate the magnitude of the numerical variation of E_A within a narrower temperature range, we put $c_{\text{H}^+} = K_0$. From equation (20) we obtain, applying the values for the constants given above,

$$\begin{aligned} \text{at } 10^\circ \text{C } E_A &= 19\,900 \text{ cal} \\ \text{, } 50^\circ \text{C } E_A &= 20\,390 \text{ ,} \end{aligned}$$

The difference between these two values is of the same order of magnitude as the uncertainty with which E_A may be determined experimentally, and hence it will be permissible within this temperature range to reckon with a constant value of E_A .

At 30°C we obtain, by inserting the constants into (20)

$$E_A = -R \frac{d \ln k}{d \frac{1}{T}} = 17\,570 + \frac{3410}{1 + 200 c_{\text{H}^+}} \quad (21)$$

The two first series of experiments are performed at hydrogen ion concentrations which are supposed to be so high and so low, respectively, relative to K_0 that E_A approximately will assume its lowest and its highest value. Equation (21) offers a possibility of examining whether these suppositions are fulfilled with sufficient accuracy. By inserting we obtain

$$\begin{aligned} E_A &= 17\,820 \text{ cal for } -\log c_{\text{H}^+} = 1.20 \\ E_A &= 20\,960 \text{ cal for } -\log c_{\text{H}^+} = 4.54 \end{aligned}$$

The difference between these values and those found experimentally is considerably smaller than the mean uncertainties and, correspondingly, the above mentioned suppositions can be assumed to be fulfilled with sufficient approximation.

In order to estimate the applicability of equation (21) to the calculation of the velocity constants at different temperatures, $-\log k$ was calculated for the experimental conditions prevailing during the experiments of table 3. As a basis of the calculations were used the velocity constants at 30°C read from the curve given previously⁶. The results are shown in the last column of the table, from which it appears that the calculated values are in good agreement with those found experimentally, if it is taken into account that the mean uncertainty of the experimental determination of $\log k$ is ca. 0.02—0.04.

SUMMARY

On the basis of previous investigations into the velocity of inactivation of penicillin by acids as a function of the hydrogen ion concentration and, moreover, of a theory for the reaction mechanism, an equation for the inactivation

velocity at different temperatures is deduced (7). For high and for low hydrogen ion concentrations, this equation is reduced to expressions conformable to Arrhenius' equation.

By measurements of the velocity of the process at different temperatures, Arrhenius' critical increments are determined for high and for low hydrogen ion concentrations. These terms are shown to have somewhat different numerical values and to be independent of the temperature within the range investigated.

On the basis of velocity determinations at intermediate hydrogen ion concentrations, it is shown that, within the accuracy necessary here, the heat of dissociation for the acid dissociation of the penicillin molecule can be assumed to be zero. From this, an equation (21) is deduced which can be applied to the calculation of the rate of inactivation at different hydrogen ion concentrations and at temperatures between 10° and 50° C. The velocity constants determined by means of this equation are in agreement with those found experimentally.

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