PENICILLIN. III. THE STABILITY OF PENICILLIN IN AQUEOUS SOLUTION[1, 2]

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The instability of penicillin in aqueous solution has been known since the time of its discovery by Fleming (1929). However, beyond the general recognition that excess acidity or alkalinity caused rapid inactivation, little work has been reported on the inactivation of penicillin over a wide pH range and at different temperatures. The maximum stability range of a barium salt of penicillin tested in aqueous solution by Abraham and Chain (1942) was between pH 5.5 to 7.5. Activity was retained at 2 C for several months, at 25 C for several weeks, and at 37 C for 24 hours, whereas most of the activity was lost in 30 minutes at 100 C. Rammelkamp and Helm (1943) studied the stability of penicillin during a 24-hour period at 5 C and 37 C in veal infusion broth at pH 2, 3, 4, 5, and 7.3. Although the original concentration of penicillin was low (0.625 Oxford units per milliliter), they were able to show rapid inactivation at pH 2 and 4 at both temperatures, partial inactivation at pH 5 and 37 C, and no inactivation at pH 4 and 5 at 5 C. Foster and Wilker (1943) conducted similar experiments in buffer solutions at pH 2.0, 2.6, 2.9, 4.8, 5.8, 6.8, 7.9, 9.4, and 10.3. The initial concentration of penicillin was only 0.168 Oxford units per milliliter. They concluded that penicillin is exceedingly labile in a medium below pH 4.8 or above 7.9, losing all activity in a matter of hours. The purity of the penicillin used by Rammelkamp and Helm and by Foster and Wilker was not given, but it is apparent from the low concentration (expressed as potency) that only partially purified preparations were used.

Previous experiments conducted by the authors with crude penicillin had indicated that the optimum stability in aqueous solution was between pH 5.6 and 6.1 instead of at pH 7.0, which had been previously considered as the stability optimum. Furthermore, it was expected that crude penicillin would prove to be less stable than the crystalline material, and examination of the data presented here substantiates this supposition.

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The present investigations differ essentially from the previous studies in that a crystalline sodium salt of penicillin was employed. The initial concentration was of sufficient potency that reasonably accurate determinations of the rate of decomposition could be made. The data cover a pH range of 2.0 to 11.0 and a temperature range of 0 C to 37 C.

MATERIALS AND METHODS

Two samples of penicillin were employed in this work. Both were prepared from crude penicillin obtained from submerged growth of *Penicillium notatum*, NRRL No. 832. A pure crystalline preparation of the sodium salt of penicillin was used for most of the determinations. In addition, the stability of a partially purified penicillin was compared with that of the crystalline salt at pH 2.0 only.

The buffer systems employed were those whose buffering capacities were near maximum for the desired hydrogen ion concentration. The systems used were (1) H_2SO_4-H_3PO_4-KOH (approximately 0.2 N) for pH 2.0 and 3.0; (2) McIlvaine's $C_6H_8O_7 \cdot H_2O$ (citric acid)-Na_2HPO_4 for pH 4.0 and 5.0; (3) K_2HPO_4-KH_2PO_4 for pH 5.5, 5.8, 6.0, 6.5, 7.0, and 7.5; (4) H_3BO_3-NaOH plus HCl for pH 8.0 and 9.0; and (5) H_3BO_3-NaOH plus NaOH for pH 10.0 and 11.0. Each buffer was carefully checked with a Beckman pH meter, and small amounts of chloroform and toluene were added to prevent growth of microorganisms.

Temperatures used in these investigations were 0, 10, 15, 24, 30, and 37 C. Constant temperature baths were employed for 24, 30, and 37 C. A well-insulated cold water bath was maintained at 15 C for tests at pH 2.0. For long-term experiments at 10 C, a bath was installed in a refrigerator maintained at that temperature. A large Dewar flask, filled with cracked ice and stoppered with cork, provided an excellent bath for 0 C studies.

For each experiment with the crystalline sodium salt of penicillin, a carefully weighed sample in a volumetric flask was dissolved in buffer at the desired temperature to give a final concentration of 100 to 125 Oxford units per milliliter. The partially purified penicillin solution was transferred by pipette and diluted to approximately the same concentration. After taking a zero time sample, the penicillin buffer mixture was immersed in a bath held at the required temperature. Additional samples were taken at intervals throughout the experiment. Each sample withdrawn was immediately diluted with ice-cold phosphate buffer at pH 6.0 to stop penicillin inactivation and to provide suitable dilutions for assay. These were stoppered and refrigerated at 4 C until assayed in the afternoon or evening of the same day. The cylinder-plate method of Schmidt and Moyer (1944) was utilized for all assays, and the values from two or three plates were averaged to determine each point. Final pH values were determined on the penicillin buffer mixtures at the conclusion of each experiment.

EXPERIMENTAL RESULTS

The first inactivation studies were made at pH 2.0 and 0 C. A trial run was conducted to determine the approximate rate of inactivation and time

intervals for taking samples. After repeating the tests at pH 2.0 and 0 C, tests were conducted at 10 C and 24 C with approximately 24 samples taken

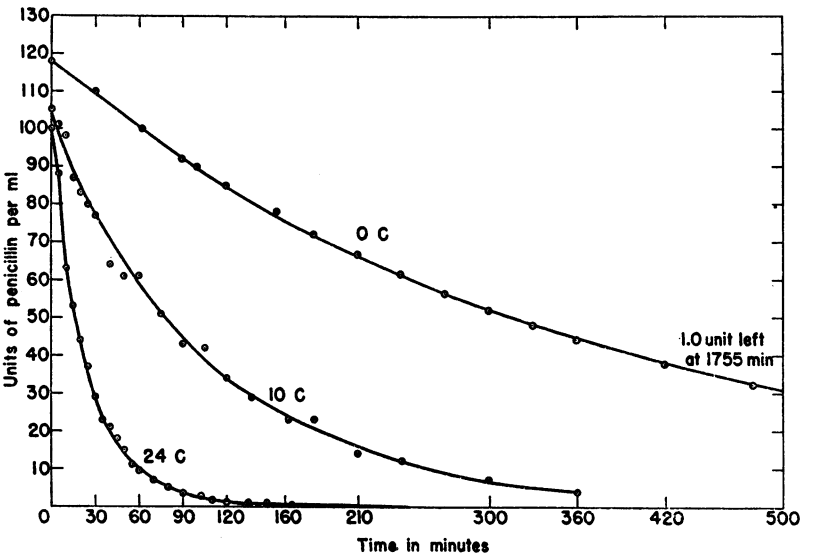

FIG. 1. DECOMPOSITION OF CRYSTALLINE PENICILLIN AT pH 2.0

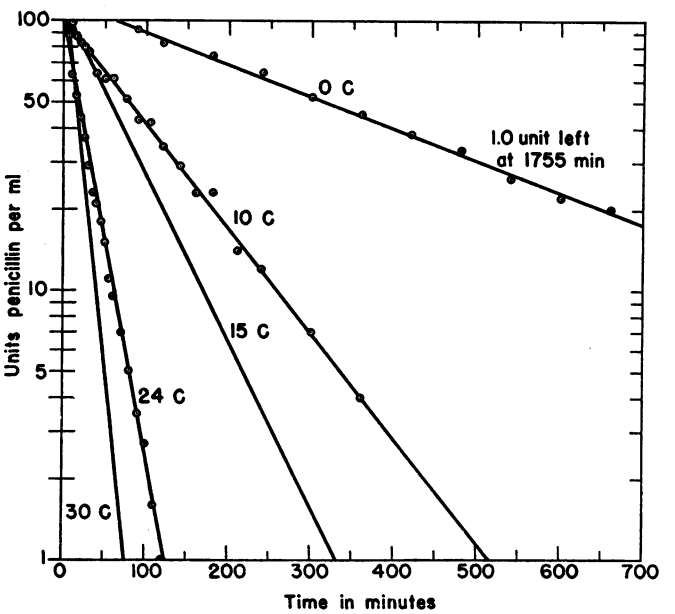

FIG. 2. EFFECT OF VARIOUS TEMPERATURES ON THE DECOMPOSITION OF CRYSTALLINE PENICILLIN AT pH 2.0

during each run. When the penicillin concentration is plotted against time, logarithmic curves are obtained (figure 1). If semilogarithmic paper is used, the points all fall on a straight line (within experimental error), as shown (figure

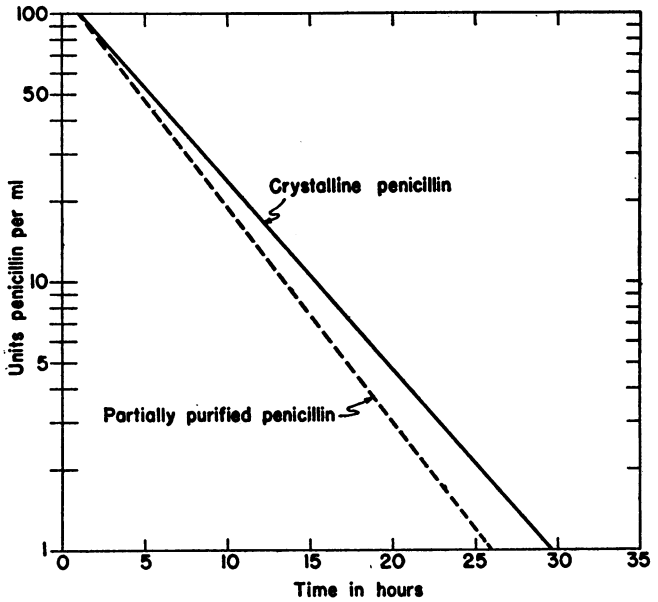

FIG. 3. DECOMPOSITION OF CRYSTALLINE AND PARTIALLY PURIFIED PENICILLINS AT pH 2.0—0 C

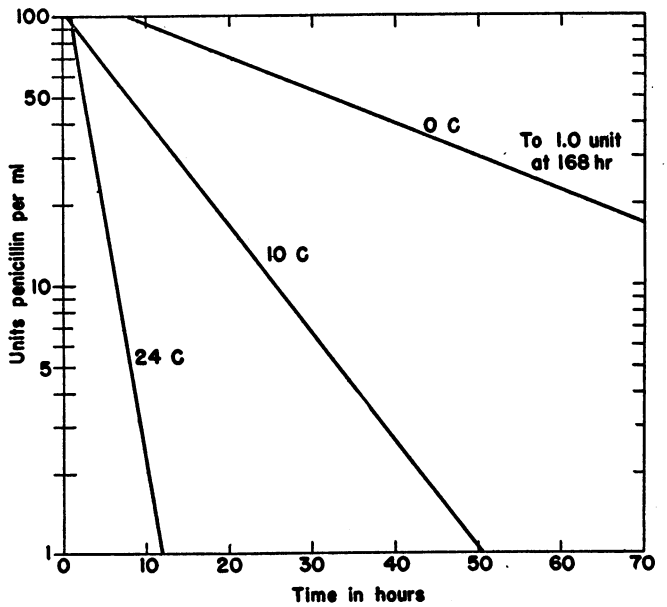

FIG. 4. DECOMPOSITION OF CRYSTALLINE PENICILLIN AT pH 3.0

2). The distribution of points for the slopes shown in this figure are considered representative of those obtained throughout the work. The effect of temperature is such that the times required to destroy 50 per cent of the penicillin at pH

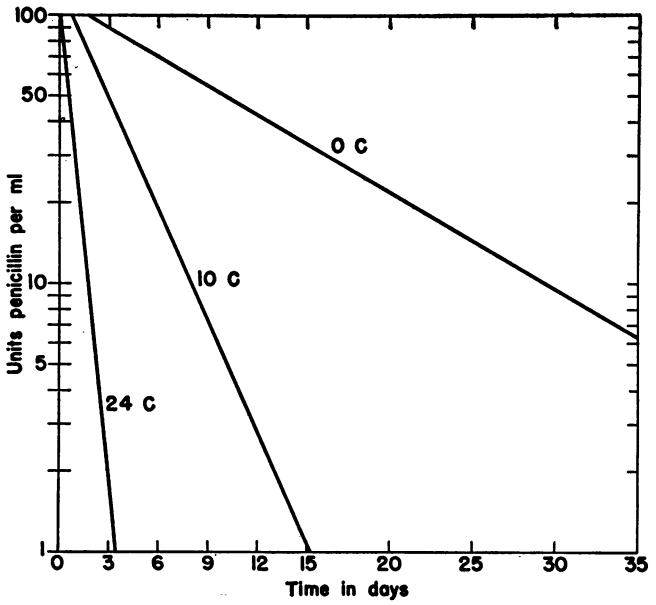

FIG. 5. EFFECT OF VARIOUS TEMPERATURES ON CRYSTALLINE PENICILLIN AT pH 4.0

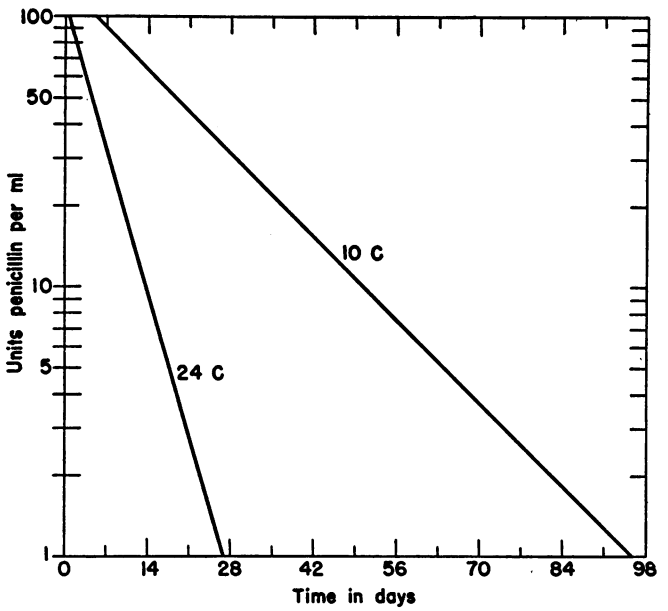

FIG. 6. STABILITY OF CRYSTALLINE PENICILLIN AT pH 5.0

2.0 are approximately 5 hours at 0 C, 77 minutes at 10 C, and 17 minutes at 24 C. Tests were run at 15 and 30 C, and the slopes for these temperatures are also shown in figure 2. The points used to determine them were purposely

omitted to avoid confusion. The additional information enabled one of us (Oleson) to derive certain mathematical formulae pertaining to the inactivation of crystalline penicillin at pH 2.0. This material may be found in a separate section of this paper.

A comparison of the stability of partially purified penicillin with that of the crystalline material was made at pH 2.0 and 0 C. Figure 3 shows the results of this comparison. The increased rate of destruction in the impure material is

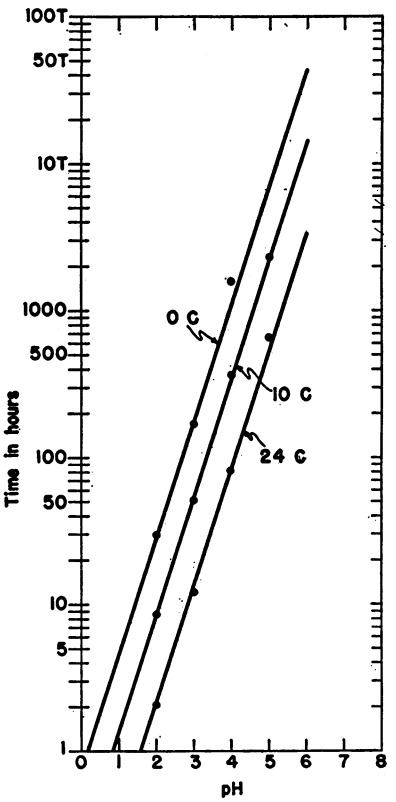

FIG. 7. TIME IN HOURS REQUIRED TO DESTROY 99 PER CENT OF CRYSTALLINE PENICILLIN AT 0, 10, AND 24 C PLOTTED AGAINST pH

probably due to the presence of certain impurities which react with penicillin, the curve representing the summation of this effect and that resulting from the acidity of the solution.

The stability of the crystalline salt was tested at pH 3.0, 4.0, and 5.0 using 0, 10, and 24 C as the temperatures. For each unit rise in pH, the stability increased so markedly that the interval between taking of samples in the pH 5.0 series was increased to one day. Figures 4 and 5 show the slope of the lines for pH 3.0 and 4.0, respectively. After sampling at intervals for 25 days from the pH 5.0 and 0 C flask, the penicillin concentration was still above 100 units

per milliliter. This experiment was discontinued for that reason and accounts for the absence of a pH 5.0 and 0 C slope in figure 6. Some rather interesting data were obtained by plotting pH (on semilogarithmic, 5-cycle paper) against the time needed to destroy 99 per cent of the penicillin at 0, 10, and 24 C (figure 7). The slopes thus attained are parallel and give some indication of the stability at pH 6.0. On the basis of this information, it was not practical to make runs with pH 6.0 and temperatures of 0 and 10 C because the predicted times for 99 per cent inactivation were 42,000 and 14,500 hours, respectively.

Summary of mathematical data pertaining to decomposition of penicillin at pH 2.0

Previous reference has been made in this paper to the derivation of certain mathematical formulae by one of the authors. The data presented at this time are limited to a summary of this work since the mathematical calculations involve considerable detail.

The decomposition of penicillin at a constant pH of 2.0 appears to be a first-order irreversible reaction. The results may be expressed as follows:

$$\log_{10} \frac{C_0}{C} = K^1(\theta - \theta_0) \tag{1}$$

C_0 = concentration of penicillin at time θ_0.
C = concentration of penicillin at time θ.
K^1 = reaction rate constant.
C_0 and C may be expressed in any units.
θ is expressed as minutes.
K^1 has the dimension, min.^{-1}

The experimentally determined constant K^1 was obtained at pH 2.0 at 0, 10, 15, 24, and 30 C. The reaction rate constant was correlated with the Arrhenius equation to secure an equation with K^1 as a function of temperature. The result is equation (2).

At pH = 2.0

$$\log_{10} K^1 = -3818\ T + 11.05874 \tag{2}$$

T = temperature of reaction, °K (273 + t, ° C.).

Equation (2) may be used to calculate K^1 for any temperature (−10 C to +40 C) at pH = 2.0. The determined value of K^1 is then used in equation (1) to determine the extent of decomposition at any time, if the original concentration and time at which the reaction begins is known.

The rate of decomposition may be determined by calculating K^1 using (2) and substituting in the equation

$$\frac{dC}{d\theta} = -2.3026\ K^1C \tag{3}$$

where C is expressed as desired. In the work done it would be (units per milliliter) and since K^1 is (min^{-1}) then the rate is expressed dimensionally as units of penicillin per milliliter disappearing per minute.

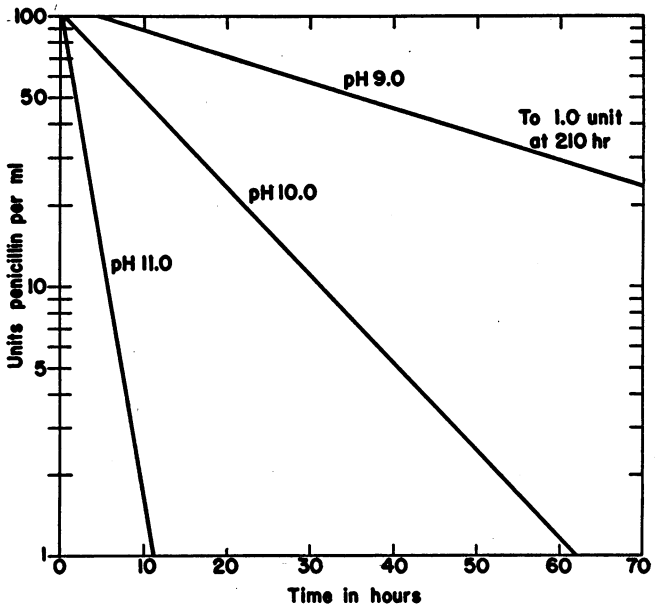

FIG. 8. STABILITY OF CRYSTALLINE PENICILLIN AT pH 9.0, 10.0, AND 11.0 (24 C)

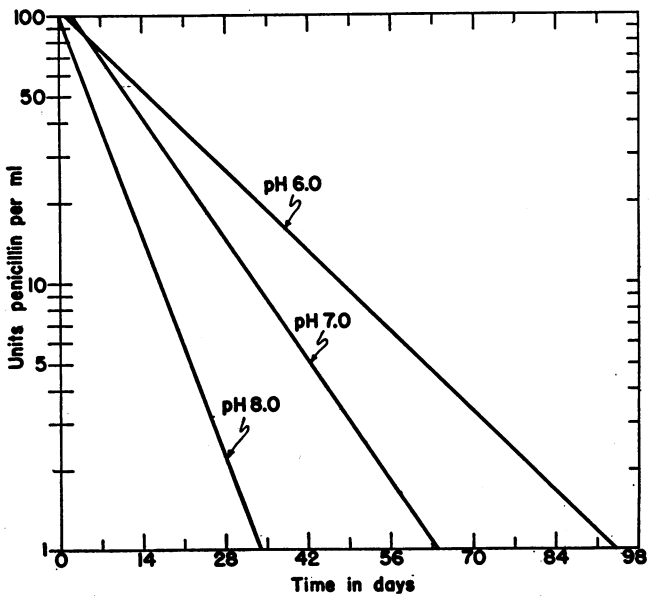

FIG. 9. STABILITY OF CRYSTALLINE PENICILLIN AT pH 6.0, 7.0, AND 8.0 (24 C)

Inactivation of crystalline penicillin at pH 6.0 to 11.0

Detailed investigations of the same nature as those from pH 2.0 to 5.0 were not possible in this phase of the work, due to the large numbers of assays necessary for other penicillin projects in the laboratory. The inactivation of penicillin at pH 9.0, 10.0, and 11.0 (24 C) is shown in figure 8.

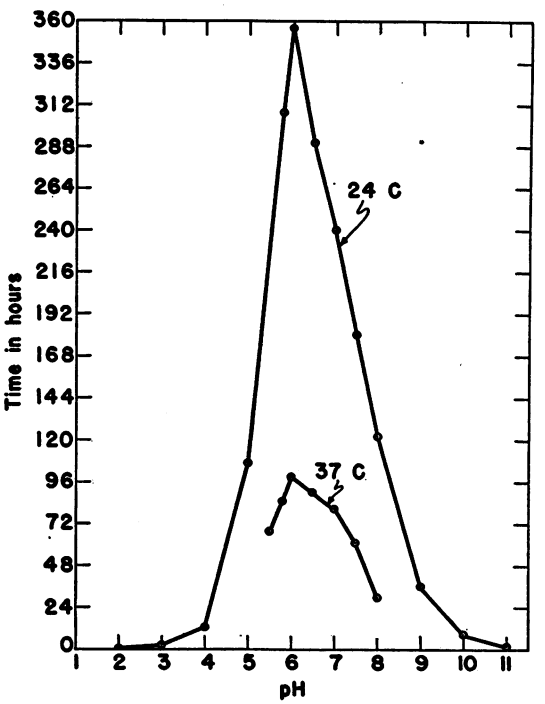

FIG. 10. EFFECT OF pH ON TIME REQUIRED TO DECOMPOSE 50 PER CENT OF CRYSTALLINE PENICILLIN IN AQUEOUS SOLUTION AT 24 AND 37 C

TABLE 1

The effect of pH and temperature on the half-life of penicillin

(time in hours to inactivate 50 per cent)

pH	0 C	10 C	24 C	37 C
2.0	5.2	1.28	0.283	—
3.0	32.52	8.2	2.33	—
4.0	259.2	68.4	13.2	—
5.0	2,000*	458.4	107.2	—
5.5	—	—	—	67.2
5.8	—	—	318	85.2
6.0	—	—	356	99
6.5	—	—	290.5	90.0
7.0	—	—	252	81.6
7.5	—	—	180	61.2
8.0	—	—	122.4	29.8
9.0	—	—	36.0	—
10.0	—	—	9.7	—
11.0	—	—	1.7	—

* Estimated.

Stability tests were run at pH 6.0, 6.5, 7.0, 7.5, and 8.0 at 24 C. Samples were taken for dilution at 1-day intervals and 16 points were used to determine the slope of each line. Three of these slopes (for pH 6.0, 7.0, and 8.0) are shown

in figure 9. From these results it appeared quite certain that pH 6.0 was the optimum stability point for crystalline penicillin in aqueous solution. Before a stability curve at 24 C was drawn, tests were conducted with pH 5.5 and 5.8 at 24 C, and with pH 5.5, 5.8, 6.0, 6.5, 7.0, 7.5, and 8.0 at 37 C. Figure 10 shows the effect of pH on the time required to inactivate 50 per cent of a solution of crystalline penicillin at 24 C. The data available from the 37 C investigations (pH 5.5 to 8.0) are also included. A more complete summary of the effect of temperature and pH on the "half-life" of penicillin (time required to inactivate 50 per cent) is shown in table 1. These data show the rapid increase in stability of penicillin as the hydrogen ion concentration decreases (pH 2.0 to 6.0) with subsequent increase in decomposition as the concentration of hydroxyl ions becomes greater.

DISCUSSION

The authors hope that this work will greatly assist in the clarification of previous knowledge concerning the instability of penicillin. The program of work was originally outlined to include decomposition studies at pH 2.0 to 5.0 only. Consequently, more data were gathered concerning the stability in that pH range than at higher pH values. Reasonably accurate results were more easily attained when an inactivation experiment was completed in 1 to 5 days or less, since slight variations in pH or temperature were then less likely to occur. These sources of error were much harder to control in long-term experiments. Destruction of penicillin in the runs with pH 4.0 at 0 C and pH 5.0 at 10 C was so slow that accurate slopes were difficult to obtain within the time allowed for the experiments. Any microbiological method of assay is subject to certain errors, regardless of the competency of the investigator employing it. The cylinder-plate assay method of Schmidt and Moyer (1944), used in this work, may be in error 15 to 20 per cent. However, the writers, and the authors of the paper cited, are of the opinion that in the experiments reported here ± 8.0 per cent is a more likely error than 15 to 20 per cent. The assay errors in these investigations were greatly reduced by using two or three plates for each diluted sample so that 6 to 9 values were averaged for each point on a slope or curve. It is believed that the inactivation caused by the buffers in these experiments was brought about by excess hydrogen or hydroxyl ions and not by the presence of other ions in the systems. It is possible that different results might have been obtained with other buffers, but this phase was not investigated.

SUMMARY

A series of graphs has been presented to show the stability of a crystalline sodium salt of penicillin in aqueous solution at pH 2.0 to 11.0 at various temperatures. When compared with a solution of the crystalline salt at pH 2.0 and 0 C, a partially purified solution of penicillin under the same conditions was found to be less stable.

It appears that the destruction of penicillin in aqueous solution is a first-order irreversible reaction.

The time required to destroy a solution of crystalline penicillin at pH 2.0 between −10 C and +40 C, and the rate of destruction at any chosen point during that time, may be calculated by the use of formulae derived from the data presented in this paper.

ACKNOWLEDGMENT

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