J. Pharm. Pharmac., 1966, **18**, 801–808 Received July 15, 1966

Complexation of penicillins and penicilloic acids by cupric ion

W. A. CRESSMAN*, E. T. SUGITA, J. T. DOLUISIO AND P. J. NIEBERGALL

A kinetic method was developed to study the interactions between cupric ion and penicillins G and V. Complexation was found to occur with the intact penicillins, followed by rapid hydrolysis of the complex into the corresponding penicilloic acid-cupric ion complex. The logarithmic stability constants for the interaction between cupric ion and penicillins G and V were found to be 2·61 and 2·24, respectively. In addition, the method of continuous variation was used to determine the logarithmic stability constants for the cupric ion-penicilloic acid complexes. These were found to be 4·20 and 4·50, for penicilloic V and G acids respectively.

UNDER mildly acid conditions penicillins G and V have been shown to be catalytically hydrolysed by cupric ion, into the corresponding penicilloic acids, at rates much too rapid for possible intact penicillin-cupric ion interactions to be measured by standard complexation techniques (Niebergall, Hussar, Cressman, Sugita & Doluisio, 1966). Therefore, a kinetic method was developed for determining the association constants for these interactions. Since the penicilloic acids were found to be the only degradation products produced under the conditions used, it also appeared desirable to determine their interactions with cupric ion. This was done spectrophotometrically using the method of continuous variation.

Experimental

REAGENTS AND EQUIPMENT

All the chemicals used were of reagent grade. The penicillin V (1530 units/mg) was supplied by Wyeth Laboratories, Inc. and the penicillin G (1595 units/mg) by the Eli Lilly Co. All solutions were made in water that had been deionised after distillation. In all work involving potentiometric titrations, the water was subsequently degassed by boiling for 30 min.

Titrations were done automatically using the Radiometer TTT-lc titrator with the Radiometer Titragraph model SBR2c. Spectral curves were obtained using a Beckman model DB spectrophotometer with a Photovolt model 43 linear-log recorder. Single wavelength determinations were made using an Hitachi-Perkin Elmer spectrophotometer.

Penicilloic acid solutions were made by the hydrolysis of penicillins at pH 12 for 15 min at room temperature in the manner described by Rapson & Bird (1963). The penicilloic acid solutions were adjusted to pH 6·50 and either used or discarded within 4 hr of preparation.

From the Department of Pharmaceutics, The Philadelphia College of Pharmacy and Science, Philadelphia, Pa, U.S.A.

* Fellow of the American Foundation for Pharmaceutical Education.

This investigation was supported by the United States Public Health Service Research Grant AI-05321, from the National Institute of Allergy and Infectious Diseases.

COMPLEXATION BETWEEN INTACT PENICILLINS AND CUPRIC ION

Theory. One plausible explanation for the catalytic effect of cupric ion on the hydrolysis of penicillins to the corresponding penicilloic acids would be the rapid formation of a cupric ion-penicillin complex, which could then decompose rapidly into a penicilloic acid-cupric ion complex with the release of a proton. This would be indicated by:

$$P + C \underset{k_2}{\overset{k_1}{\rightleftharpoons}} Y \xrightarrow{k_3} B + H^+$$

in which P is the intact penicillin, C represents cupric ion, Y represents the penicillin-cupric ion complex and B represents the penicilloic acid-cupric ion complex. In subsequent discussions, the above symbols enclosed in brackets will indicate molar concentration. The appearance of product may be given by:

$$d[B]/dt = k_3 [Y] \quad .. \quad .. \quad .. \quad .. \quad (1)$$

If the equilibrium is established much more rapidly than the degradation of the complex into products, and the penicillin concentration is much greater than that of the cupric ion, so that it remains essentially constant, the equilibrium constant, K, may be given by:

$$K = \frac{[Y]}{P_0 [C]} \quad .. \quad .. \quad .. \quad .. \quad (2)$$

in which P_0 represents the initial penicillin concentration. Insertion of equation 2 into equation 1 results in:

$$d[B]/dt = k_3 K P_0 [C] \quad .. \quad .. \quad .. \quad (3)$$

The molar concentration of cupric ion at any time is:

$$[C] = C_0 - [Y] - [B] \quad .. \quad .. \quad .. \quad (4)$$

in which C_0 represents the initial cupric ion concentration. Using equation 2 to replace [Y] in equation 4 and rearranging yields:

$$[C] = \frac{C_0 - [B]}{1 + KP_0} \quad .. \quad .. \quad .. \quad .. \quad (5)$$

Equation 5 can be inserted into equation 3 to give:

$$d[B]/dt = \frac{k_3 K P_0}{1 + K P_0} (C_0 - [B]) \quad .. \quad .. \quad (6)$$

Integration of equation 6 under the condition that at time zero, B = O;

$$\log (C_0 - [B]) = \log C_0 - \frac{k_3 K P_0 t}{2{\cdot}303 + 2{\cdot}303\, KP_0} \quad .. \quad (7)$$

Thus, the appearance of product should be first order with a slope, A, given by:

$$A = \frac{k_3 K P_0}{2{\cdot}303 + 2{\cdot}303\, K P_0} \quad .. \quad .. \quad .. \quad (8)$$

This equation can be inverted to give:

$$1/A = \frac{2{\cdot}303}{k_3 K P_0} + \frac{2{\cdot}303}{k_3} \quad .. \quad ... \quad .. \quad (9)$$

The first order rate constant for the appearance of product is thus obtained for a number of solutions in which C_0 is kept constant, but P_0 is varied, always keeping P_0 much greater than C_0. A plot of $1/A$ versus $1/P_0$ should give a straight line with a slope equal to $2{\cdot}303/k_3 K$ and an intercept equal to $2{\cdot}303/k_3$. Dividing the intercept by the slope would give the value for K.

Kinetic data. In the model proposed for the degradation process, equimolar amounts of hydronium ion and penicilloic acid-cupric ion complex would be produced. The proton release was therefore used to follow the kinetics of the reaction by the standard pH stat technique using solutions containing 1×10^{-4}M cupric chloride and penicillin concentrations ranging from 10 to 80×10^{-4}M at pH 5·50. The ionic strength was kept constant at 0·01 with potassium chloride. The reaction vessel used had a glass jacket which permitted the temperature to be kept at 30·00 $\pm$ 0·02°. The titrant was 0·0016N sodium hydroxide. The reaction vessel was continuously overlaid with a blanket of nitrogen throughout each determination. At the end of several determinations the solution was assayed for penicilloic acid content using the following modification of the method described by Pan (1954).

To 2 ml of a 0·20 M pH 5·50 acetate buffer containing 6×10^{-4}M cupric chloride add 4 ml of the solution to be assayed. The final concentration of penicilloic acid in the resulting 6 ml should not exceed 2×10^{-4}M. Add 1 ml of the Pan reagent and allow 200 sec for the colour to develop. Add 10 ml of distilled water and read the absorbance of the solution at 800 mμ in a 1 cm cell in exactly 100 sec.

In all instances in which solutions were assayed at the end of a kinetic run, the penicilloic acid content was within 3% of the theoretical 1×10^{-4}M. This was determined by comparing the absorbance of these solutions with that of solutions prepared to represent the reaction mixture at 100% reaction, i.e. 1×10^{-4}M cupric chloride and penicilloic acids, and an appropriate amount of penicillin, which had been simultaneously assayed using the modified Pan method. Finally a number of reaction mixtures were scanned through the ultraviolet region to determine the possible presence of penicillenic acids which absorb strongly at 322 mμ. The results of these scans verified our previous findings that penicillenic acids are not produced under these conditions.

To eliminate the non-catalysed degradation of the penicillins as a source of proton, several determinations were made using penicillin solutions (80×10^{-4}M), in the absence of cupric ion, but keeping the other conditions identical. Less than 2% of the penicillin G and none of the penicillin V degraded via the non-cupric ion catalysed route during the time interval used in this work.

PENICILLOIC ACID-CUPRIC ION INTERACTIONS

The method of continuous variations as modified by Woldbye (1955) was used. Beer-Lambert plots were obtained separately at 273 and 266 mμ for penicilloic V acid and cupric chloride, and at 245 mμ for penicilloic G acid and cupric chloride. These and all other determinations

were made in 0·034M acetate buffer at pH 5·50 at 30°. Equimolar stock solutions (1×10^{-3}M) of the penicilloic acids and of the cupric chloride were made. A series of solutions was prepared by adding (X)(V) ml of the penicilloic acid stock solution and (1-X)(V) ml of the cupric chloride stock solution to 30 ml of the acetate buffer. The final constant volume of these solutions is represented by V, and X is the fraction of the penicilloic acid stock solution. The difference between the absorbance of these solutions and the theoretical absorbance calculated from the Beer-Lambert plots was plotted against X conventionally.

The stability constants for 1 : 1 complexes were calculated by preparing solutions containing (X)(V) ml of a penicilloic acid stock solution and (1-X)(V) ml of a cupric chloride stock solution of a concentration different from that of the penicilloic acid stock solution. The solutions were added to 30 ml of the acetate buffer solution to give a total volume of 50 ml. The absorbance of each of these solutions was obtained at 273 mμ for penicilloic V acid and at 245 mμ for penicilloic G acid and then plotted against X. The value of X obtained at the maximum absorbance, X_{max}, was used in the following equation:

$$K = \frac{(r - 1)(1 - 2X_{max})}{(Cu^{++})(X_{max} + rX_{max} - 1)^2} \quad .. \quad .. \quad (10)$$

in which r is the ratio of penicilloic acid to cupric chloride in the stock solutions and the other terms have the meaning previously given. Two determinations were made using r values of 1·10 and 1·25 and a final chloride concentration of $4{\cdot}00 \times 10^{-4}$M.

Results

PENICILLIN-CUPRIC ION INTERACTIONS

The data in Fig. 1 demonstrate the first order dependency of the reaction, and the effect of P_0 upon the rate. In all instances the plots gave excellent results through more than three half lives. The rate constants, determined by least square methods, were used to determine the association constants according to equation 9. Fig. 2 shows representative plots of equation 9, indicating the validity of this method. The logarithmic association constants obtained in triplicate on penicillin G and penicillin V were 2·61 $\pm$ 0·01 and 2·24 $\pm$ 0·09 respectively.

PENICILLOIC ACID-CUPRIC ION INTERACTIONS

The spectral data for the method of continuous variation are shown in Figs 3 and 4, representing penicilloic G acid and penicilloic V acid, respectively. In both instances the lines intersect at X = 0·5, indicating a 1 : 1 complex, and justifying the use of equation 10 to obtain the association constants. The values for the logarithmic association constants from equation 10 were 4·20 $\pm$ 0·50 for penicilloic V acid and 4·50 $\pm$ 0·02 for penicilloic G acid.

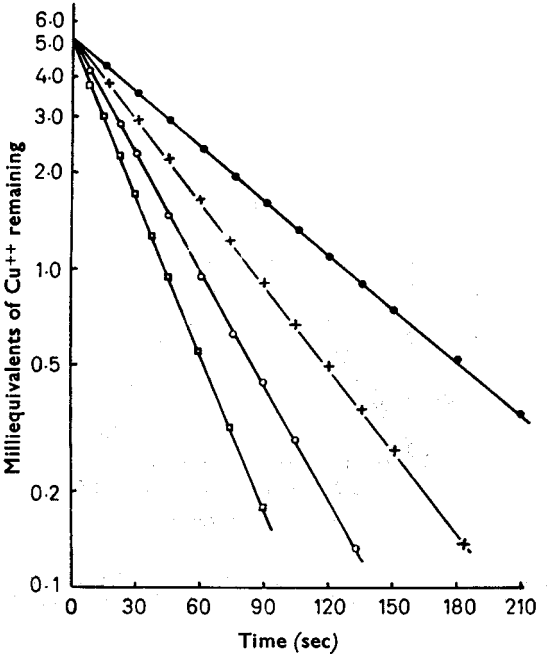


FIG. 1. First order dependency for the reaction between cupric chloride, 1×10^{-4}M, and penicillin V, -●-●- 10×10^{-4}M, -X-X- 16×10^{-4}M, -○-○- 30×10^{-4}M, and -□-□- 40×10^{-4}M, at pH 5·50, using 0·0016N sodium hydroxide as the titrant.

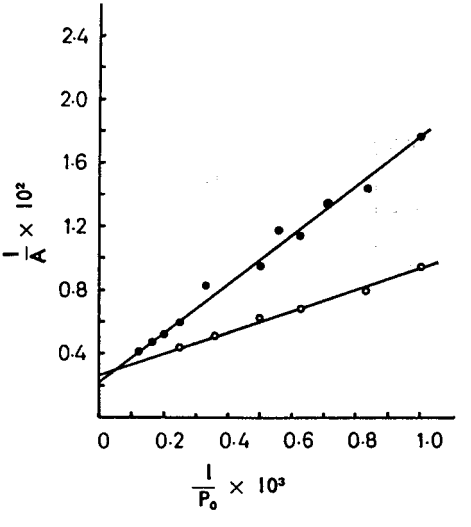


FIG. 2. Reciprocal of the apparent first order rate constant versus the reciprocal of the initial -●-●- penicillin V or -○-○- penicillin G concentrations, in solutions initially containing cupric chloride 1×10^{-4}M, at pH 5·50. Penicillin concentrations ranged from 10 to 80 $\times 10^{-4}$M.

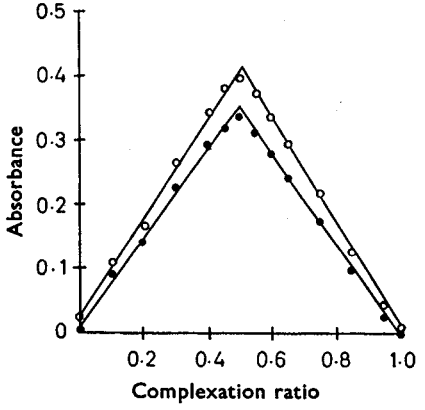


FIG. 3. Method of continuous variation to determine the complexation ratio between cupric ion and penicilloic V acid at 30° in 0·034M acetate buffer solutions at pH 5·50. Absorbance was recorded in 1 cm cells at –●–●– 273 mμ and –○–○– 266 mμ.

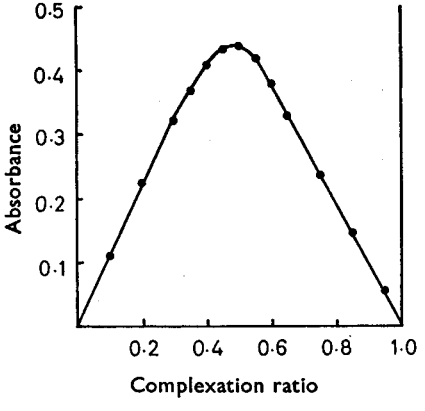


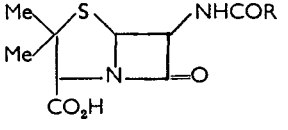
FIG. 4. Method of continuous variation to determine the complexation ratio between cupric ion and penicilloic G acid at 30° in 0·034M acetate buffer solutions at pH 5·50. Absorbance was recorded at 245 mμ using 1 cm cells.

Discussion

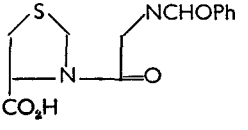
Adherence to first order kinetics through more than three half-lives, and the subsequent plots of the rate constants according to the proposed mechanism indicate the validity of the method for obtaining the association constants in question. Since the model and assumptions involved have been used successfully for years to obtain values for enzyme-substrate interactions, it would have been surprising indeed if the data did not fit. The major difference in our mechanism is a different balance equation for cupric ion, which unlike enzyme catalysts, is consumed in the reaction. The mechanism, however, cannot be firmly established solely on the basis of fit to complexation studies. Further kinetic analysis of this system is presently being undertaken.

The rate constants obtained indicate that the half lives for the cupric ion catalysed hydrolysis of penicillins V and G in unbuffered solutions at a pH 5·50 and ionic strength of 0·01, although varying with penicillin concentration, are approximately 50–100 sec. Thus any attempt to measure penicillin-cupric ion interactions via standard complexation methods which involve time periods greater than this are invalid.

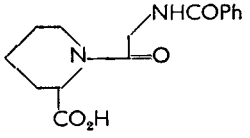
Weiss, Fallab & Erlenmeyer (1957), for example, measured the interaction between cupric ion and the compounds I-V in unbuffered solutions at an ionic strength of 0·1.



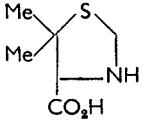
I



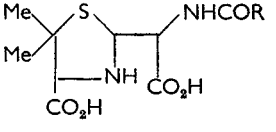
II



III



IV



V

Compounds II and III are acylated amino-acids structurally related to the penicillins I, while compound IV is structurallv related to the penicilloic acids, compound V.

The combined results for the work presented by Weiss & others (1957), and the data for this present study are given in Table 1. The value for the penicillin G-cupric ion interaction as given by the earlier work is much higher than those for the other structurally related acylated amino-acids, II and III, and higher than our values for the penicillins which, considering the ionic strength differences, are in close agreement with the values for compounds II and III. In fact, their value is not significantly different from our values for the penicilloic acids, or their values for compound IV which is more nearly related structurally to the penicilloic acids than to intact penicillin. The comparisons in Table 1 and the

TABLE 1. ASSOCIATION CONSTANTS FOR THE INTERACTION BETWEEN PENICILLINS AND RELATED COMPOUNDS WITH CUPRIC ION. DATA FOR THE PRESENT STUDY OBTAINED AT 30·00 ± 0·02° AT AN IONIC STRENGTH OF 0·01

	Compound	log K
I	Penicillin G	4·8*
II	*N*-Hippuryl-thiazolidin-4-carboxylic acid	1·8*
III	*N*-Hippuryl-pipecolinic acid	2·1*
I	Penicillin G	2·61
I	Penicillin V	2·24
IV	5,5-Dimethyl-thiazolidin-4-carboxylic acid	4·4*
V	Penicilloic V acid	4·20
V	Penicilloic G acid	4·50

* Taken from the data reported by Weiss & others (1957), at 2 ± 22° and ionic strength approximately 0·1.

very rapid rate of hydrolysis of the penicillins in the presence of cupric ion into the corresponding penicilloic acids, would suggest that the penicillin solutions used by Weiss & others had substantially degraded into the penicilloic acid during the complexation measurements.

References

Niebergall, P. J., Hussar, D. A., Cressman, W. A., Sugita, E. T. & Doluisio, J. T. (1966). *J. Pharm. Pharmac.*, **18**, 729–738.
Pan, S. C. (1954). *Analyt. Chem.*, **26**, 1438–1444.
Rapson, H. D. & Bird, A. E. (1963). *J. Pharm. Pharmac.*, **15**, *Suppl.* 222*T*–231*T*.
Weiss, A., Fallab, S. & Erlenmeyer, H. (1957). *Helv. chim. Acta*, **40**, 611–614.
Woldbye, F. (1955). *Acta chem. scand.*, **9**, 299–309.