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Metal binding tendencies of various antibiotics

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A method to determine the presence of metal-drug complexes in dilute solutions is described. Using this method cycloserine was found to complex with cupric, nickelous, zinc and cobalt ions; streptomycin and novobiocin complexed with cupric ions; erythromycin complexed with cobalt ions, and chloramphenicol exhibited no metal binding tendencies. Various penicillins were found to interact with zinc and cupric ions. Preliminary investigations suggest that cupric ions, rather than simply complexing with penicillin as suggested by previous workers, promote the degradation of penicillin to penicilloic acid. Evidence is presented to confirm the presence of penicilloic acid in reaction mixtures initially containing penicillin G or V and cupric ions, and to establish that the reaction follows second order kinetics and ceases when all available cupric ion has been consumed. Good correlation was noted for these results and previous work which showed the effects of metal ions on the antibacterial properties of penicillin.

SINCE metal complexation may markedly change the physico-chemical properties of drugs, it is not surprising that, in certain instances, the *in vitro* stability (Guenther, 1950), as well as the *in vivo* distribution, storage, biotransformation and elimination of a drug may be influenced by the presence of metals (Beckett, 1958; Albert, 1960; Brock, 1962). It would appear, therefore, that a quantitative knowledge of the metal ion complexing tendencies of antibiotics would be of great value. Although investigations have been made previously, preliminary data from our laboratories showed discrepancies, in a few instances, with literature data. This investigation was made to expand earlier work, and to compare data for the possible interactions of chloramphenicol, erythromycin, cycloserine, streptomycin, novobiocin and various penicillins with certain divalent cations. The results both agree and disagree with those in some of the previous reports. The principal difference was with the penicillin G-cupric ion interaction reported by Weiss, Falab & Erlenmeyer (1957). We therefore discuss this in detail.

Experimental

REAGENTS AND EQUIPMENT

Methicillin sodium and oxacillin sodium were donated by Bristol Laboratories. Penicillin V potassium and penicillin G potassium were donated by Chas. Pfizer. Eli Lilly donated erythromycin and cycloserine. Novobiocin sodium, streptomycin sulphate, and chloramphenicol were donated by Upjohn, E. R. Squibb and Sons, and Parke, Davis respectively. The reagent grades of copper chloride ($CuCl_2 \cdot 2H_2O$), zinc chloride ($ZnCl_2$), nickel chloride ($NiCl_2 \cdot 6H_2O$), magnesium chloride ($MgCl_2 \cdot 6H_2O$), cobalt chloride ($CoCl_2 \cdot 6H_2O$), and calcium chloride ($CaCl_2 \cdot 2H_2O$) were obtained

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from the Mallinckrodt Chemical Works or J. T. Baker Chemical Company. Distilled, deionised water which was boiled for 1 hr to remove carbon dioxide was used. It had a specific conductance of $2{\cdot}50 \times 10^{-6}$ ohm^{-1} cm^{-1} or less, as determined by a Serfass conductivity bridge.

PROCEDURE

Thirty ml of a $1{\cdot}00 \times 10^{-3}$M solution of the antibiotic was diluted to 50·0 ml with water. The solution was then titrated potentiometrically, at $30 \pm 0{\cdot}2°$ with a standard solution of $2{\cdot}00 \times 10^{-2}$N sodium hydroxide at not greater than 0·4 ml increments. Absorption of carbon dioxide was shown not to be a factor, by duplicate titration on solutions both in the presence and absence of nitrogen. A Leeds and Northrup model 7401 pH meter or a Radiometer TTT-1 titrator was used to determine pH values.

To test for metal complexation, 15·0 ml of a $1{\cdot}00 \times 10^{-3}$M solution of divalent cation was mixed with 30·0 ml of the antibiotic solution. The resulting solution was diluted to 50·0 ml and titrated with standard sodium hydroxide solution in the manner described above.

With the penicillin salt derivatives and erythromycin, an equivalent amount of hydrochloric acid (1·50 ml of a $2{\cdot}00 \times 10^{-2}$N solution) was added, before dilution with water, to convert ionising groups to their acid form. In addition, with the penicillin salt derivatives, titrations were also made on solutions to which no acid had been added.

The metal solution was titrated alone with the standard sodium hydroxide solution after diluting 15·0 ml of the divalent cation solution to 50·0 ml.

Results and discussion

The method of Doluisio & Martin (1963) was used to evaluate complexes between drugs and metal ions. In this, at least two titration curves are required. The first is the potentiometric titration of the drug (Curve I) and the second is the potentiometric titration of the same concentration of drug reacted with divalent cation (Curve II). A downward displacement of Curve II, due to metal displacement of a proton, serves as a qualitative test for metal complexation *providing* Curve II is not simply a summation of a metal hydroxo curve and Curve I. The greater the tendency of the metal to combine with a given co-ordinating agent, the greater the drop in pH. When the apparent metal complexation takes place in pH regions where metal hydroxo formation may also occur, a third potentiometric titration curve (Curve III), that of metal ions alone, is required. The sum of curves I and III must then be compared with Curve II. When Curve II deviates from the summation curve (Curve IV), it may be assumed that metal-drug complexation is occurring. This analysis is based on the assumption that when two simultaneously occurring reactions, metal hydroxo formation and proton dissociation of drug, are independent and non-interacting the total base consumed at a particular pH should equal the sum of the base consumed when each reaction is run separately. However, if interaction between metal and

drug occurs, deviations should be apparent. Fig. 1 illustrates the type of curves resulting when there is no interaction, or only weak interaction, between metal and drug. In these cases Curve II coincides with Curve IV

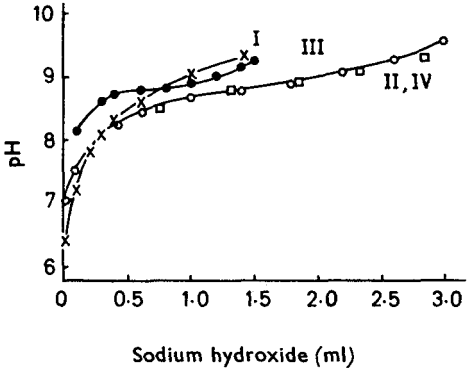

FIG. 1. Potentiometric titration of –●–● Co^{++}, –X–X– novobiocin sodium, –○–○– novobiocin sodium and Co^{++}; –□–□– summation of curves I and III. Titrant used was $2{\cdot}00 \times 10^{-2}$ N sodium hydroxide. Data indicate no complexation.

over the entire pH range investigated. Fig. 2 illustrates the type of curves resulting when simple mononuclear metal-drug complexes are formed. In these cases, Curve II is depressed from Curve I and bears no resemblance to either curves III or IV. The stoichiometric ratio of the drug to metal and the stability constants can be calculated from these data using the method described previously (Doluisio & Martin, 1963).

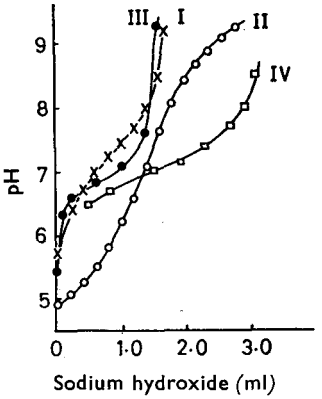

FIG. 2. Potentiometric titration of –X–X– cycloserine, –○–○– cycloserine and Cu^{++}, –●–● Cu^{++}; –□–□– summation of curves I and III. Titrant used was $2{\cdot}00 \times 10^{-2}$ N sodium hydroxide. Data indicate formation of a 1:1 and a 2:1 complex.

Table 1 summarises the metal complexing tendencies of erythromycin, cycloserine, novobiocin,chloramphenicol and streptomycin.

TABLE 1. METAL BINDING TENDENCIES FOR VARIOUS ANTIBIOTICS

Antibiotic	Complex formation	Probable weak complex formation	No Apparent complex formation*
Chloramphenicol ..	—	—	Ca^{++}, Co^{++}, Cu^{++} Mg^{++}, Ni^{++}, Zn^{++}
Cycloserine	Cu^{++}, Ni^{++}	Co^{++}, Zn^{++}	Ca^{++}, Mg^{++}
Erythromycin	—	Co^{++}	Ca^{++}, Cu^{++}, Mg^{++} Ni^{++}, Zn^{++}
Novobiocin sodium ..	—	Cu^{++}	Ca^{++}, Co^{++}, Mg^{++} Ni^{++}, Zn^{++}
Streptomycin sulphate ..	Cu^{++}	—	Ca^{++}, Co^{++}, Mg^{++} Ni^{++}, Zn^{++}

* The potentiometric method is not suitable for the study of weak complexation and, thus, it is possible for weak complexes to form and not be detected.

Cycloserine. Cycloserine was found to form 1:1 (log $K_1 = 5{\cdot}5$) and 2:1 (log $K_2 = 4{\cdot}7$) complexes with cupric ion. The over-all logarithmic stability constant of 10·2 compares favourably with the value of 9·7 reported by Neilands (1956). Weak interactions were also noted with nickel, zinc and cobalt ions. These results agree with those reported by Neuzil & Breton (1958). There was no evidence of complexation occurring between cycloserine and either magnesium or calcium ions.

Chloramphenicol. There was no evidence of complexation between chloramphenicol and the divalent cations investigated. This is in agreement with the findings of Ujiie (1957), who demonstrated that the antibiotic activity of this antibiotic is not affected by zinc, copper, magnesium and calcium ions.

Streptomycin. Of the cations tested, streptomycin was found to complex only with cupric ion (log $K_1 = 4{\cdot}3$). Foye, Lange, Swintosky & others (1955) reported the formation of chelates with cupric, cobalt, magnesium and nickel ions in solutions that were approximately 0·1 molar with respect to metal ions and streptomycin. Evidence for chelate formation was a drop in pH during the formation, production of characteristic colours, decreased water solubility (except for magnesium), and absence of metal ions in solutions having a pH greater than 5·0. Metal hydroxo formation, however, could also partially explain these observations. Indeed, nickel and cobalt hydroxo compounds were present in the precipitates obtained. It was also stated that a 3:1 cupric ion-streptomycin chelate formed. However, using a potentiometric titration method, Zahn & Eisenbrandt (1964) found only a 1:1 cupric ion-streptomycin complex to be present. When solutions containing cupric ion-streptomycin in the ratio of 3:1 were studied, the 1:1 chelate formed, and was followed by a precipitate of copper hydroxo compounds. The conclusion formed was that cupric ion and streptomycin formed only a 1:1 chelate. Zahn & Eisenbrandt (1964) also found no evidence for a magnesium chelate.

Novobiocin. Of the metals tested only cupric ions were found to complex with novobiocin and even this interaction appeared slight. Brock (1962) reported a magnesium complex with novobiocin and has suggested that this interaction may affect the antibacterial activity of the drug.

Erythromycin. Of the metals tested only cobalt ions were found to complex with erythromycin and this interaction appeared slight.

PENICILLIN ANALOGUES

The penicillin derivatives exhibited similar results when titrated in the presence of divalent cations. No interactions were evident with calcium, cobalt, magnesium or nickel ions. Deviations in the titration curves were noted in the presence of cupric and zinc ions. However, the deviations varied depending on the time lapse between the preparation and titration of the test solutions. The nature of the zinc-penicillin interaction has not yet been investigated, but preliminary investigations suggest that cupric ions, instead of simply complexing, promote the degradation of penicillins.

Weiss & others (1957) investigated the interaction of cupric ions and penicillin G potassium and found log K_1 to be 4·8. These authors stated that the stability constant is not related to the acidity constant. They suggested that the β-lactam ring is involved in the complexation and that this ring, after 3 hr, is unaltered by the presence of cupric ions. Before this report, Guenther (1950) noted that cupric ions split penicillin G into penicillamine and corresponding cleavage products. His qualitative results indicated that the reaction proceeds rapidly via the penicilloic acid intermediate in mildly acid solutions.

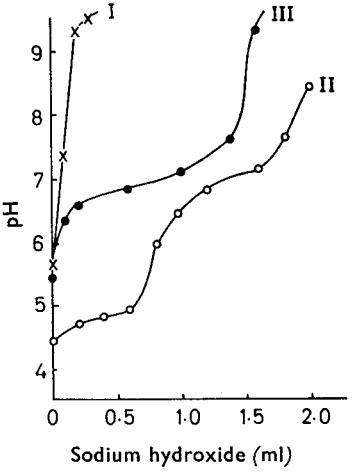

FIG. 3. Potentiometric titration of –X–X– penicillin V potassium, –○–○– penicillin V potassium and cupric chloride 30 min after mixing, and –●–●– cupric chloride. Titrant used was 2·00 × 10^{-2} N sodium hydroxide.

It was apparent, from our data, that when copper-penicillin interaction occurred, protons were displaced (Fig. 3). This proton displacement occurred after proton dissociation from the carboxyl group (pK_a = 2·8), and, as mentioned previously, was found to increase with time. This variation with time suggested that degradation was occurring. The

addition of disodium ethylenediaminetetra-acetic acid (EDTA) prevented this proton displacement.

The penicilloic acids, which form upon rupture of the β-lactam ring, give an intense blue colour with an arsenomolybdic acid-mercuric chloride reagent described by Pan (1954). A small amount of this reagent was added to aliquots of a pH 5·50 reaction mixture that initially contained equivalent amounts of penicillin G or V and cupric chloride. A blue colour, which intensified markedly with time appeared. When a similar reaction mixture was prepared without cupric ion, a very light blue colour appeared immediately. This colour did not intensify with time, and was attributed to a slight degradation of the penicillin caused by the reagent itself.

The ultraviolet absorption spectra of $3{\cdot}00 \times 10^{-4}$M solutions of penicillin G and V potassium in 0·12M acetate buffers at pH 4·00, 4·50, 5·00 and 5·50 were determined. Identical spectra were obtained for the four solutions. Solutions were made in the same buffers, and at the same penicillin concentrations, but containing an equimolar concentration of cupric chloride. The presence of the cupric ion did not immediately alter the spectra, which were determined by means of a Photovolt model 43 linear-log recorder attached to a Beckman model DB spectrophotometer.

Solutions containing $3{\cdot}00 \times 10^{-4}$M penicilloic V or G acid were made in the same acetate buffers. The spectra obtained were identical with the respective penicillin spectra down to 250 mμ. Solutions containing equimolar $3{\cdot}00 \times 10^{-4}$M amounts of penicilloic acid and cupric chloride were observed. The absorption spectra for penicilloic V acid-cupric ion

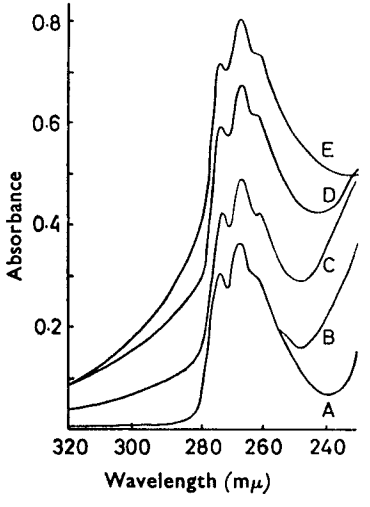

FIG. 4. Absorption spectra of A, penicilloic V acid $3{\cdot}00 \times 10^{-4}$ M; B, penicillin V $3{\cdot}00 \times 10^{-4}$ M both with and without equimolar concentrations of cupric ion; C, penicillin V and cupric chloride after 20 min; D, penicillin V and cupric chloride after 40 min; E, penicilloic V acid and cupric chloride immediately after mixing at pH 5·50, using 1 cm cells.

solutions show that the peaks at 273 mμ and 266 mμ, unlike those for the penicillin V-cupric ion solutions, rose markedly and were similar to spectral curves produced by penicillin V-cupric ion solutions after about 18·5 hr (Fig. 4). These data indicate that the conversion of penicillin V into penicilloic V acid in the presence of cupric ion could be followed at either 273 or 266 mμ. Parallel results were obtained with penicillin G at 245 mμ. The penicilloic acids were obtained by hydrolysis of penicillin at pH 12 for 20 min at room temperature (Rapson & Bird, 1963). These solutions were then adjusted to pH 6·50 and either used or discarded within 4 hr.

To establish the order of the reaction with respect to each of the reactants, a series of solutions was prepared containing the same amount ($3{\cdot}00 \times 10^{-4}$M) of penicillin G or V potassium, but varying the initial amounts of cupric chloride from 0·50 to $3{\cdot}00 \times 10^{-4}$M. The change in absorbance for these solutions was measured as a function of time, using a Beckman model DB spectrophotometer with the cell compartment thermostated at 32°. The initial rates, obtained graphically from plots of absorbance against time were plotted against cupric chloride concentration. The resulting linear relationship indicated a first order dependence upon cupric ion. The experiment was repeated, keeping the cupric chloride concentration constant at $3{\cdot}00 \times 10^{-4}$M while varying the penicillin concentration from 0·13 to $3{\cdot}00 \times 10^{-4}$M. The plots of initial rate against penicillin concentration were also linear, indicating a first order dependence upon penicillin concentration.

In view of the spectral differences between penicillin V and penicilloic V acid in the presence of cupric ion, we decided to conduct kinetic studies by following the change in absorbance at 273 mμ. The percent penicillin V remaining at any time can be obtained using the general method of Frost & Pearson (1961), and the following:

$$A_0 = c\,a \quad .. \quad .. \quad .. \quad .. \quad .. \quad (1)$$

$$A_0 = c\,(a-x) + c'\,x \quad .. \quad .. \quad .. \quad (2)$$

$$A_\infty = c'\,x = c'\,a \quad .. \quad .. \quad .. \quad ... \quad (3)$$

in which A_0 is the absorbance of a solution containing equimolar amounts of penicillin V potassium and cupric chloride at initial concentration a, assuming that any absorbance contribution due to the cupric chloride would be negligible, c is the molar absorptivity of penicillin V, x is the amount of penicilloic V acid formed, A_∞ is the absorbance of the solution after complete conversion to penicilloic V acid and c′ is the molar absorptivity of penicilloic acid in the presence of an equimolar amount of cupric ion. Thus we find that:

$$\%\ \text{Penicillin V remaining} = \frac{a-x}{a} = \frac{A_\infty - A_t}{A_\infty - A_0} \times 100 \quad .. \quad ... \quad (4)$$

The metal caused degradation of the penicillins is generally spoken of as the "metal catalysed" degradation. If this were true, by the classical definition for catalysis, the reaction should follow pseudo first order

kinetics. In this event, a plot of the logarithm of the absorbance terms in equation 4 against time would yield a straight line.

Solutions containing equimolar 3×10^{-4}M penicillin V and cupric chloride at pH 4·00, 4·50, 5·00 and 5·50 were made in 0·12M acetate buffers. The value for A_∞ was obtained using a solution containing equimolar 3×10^{-4}M amounts of penicilloic V acid and cupric chloride. The change in absorbance of the solutions was followed at 273 mμ, at $30 \pm 0{\cdot}1°$.

In all instances, first order plots of the data showed marked curvature, indicating that cupric ion was being consumed in the reaction. The reaction would therefore be expected to follow second order kinetics, and the following equation would apply for this system:

$$\frac{A_\infty - A_0}{A_\infty - A_t} = a\,k\,t + 1 \qquad .. \qquad .. \qquad .. \quad (5)$$

in which t represents time, k is the second order rate constant and the other terms have the meaning previously given. The data plotted according to equation 5, as shown in Fig. 5, indicate a second order

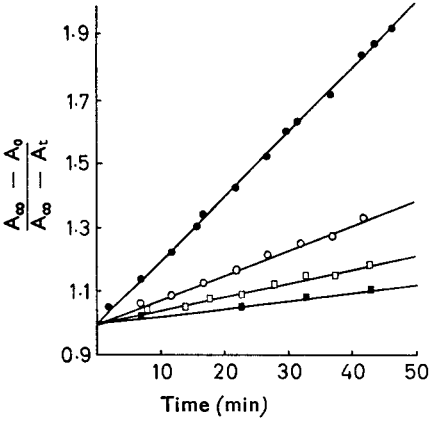

FIG. 5. Second order plots for reaction mixtures consisting of equimolar $3{\cdot}00 \times 10^{-4}$ M penicillin V potassium and cupric chloride at -■-■- pH 4·00, -□-□- pH 4·50, -○-○- pH 5·00, -●-●- pH 5·50 at 32°.

overall reaction. It should be noted that a further test of the suitability of the plots would be the observation that the intercept of this equation should be equal to 1·00. A *t*-test was made on all of the intercepts, which had been calculated through the method of least squares. In all instances, the intercepts did not differ significantly from the expected value at the 95% confidence level.

The rate constants were evaluated by the method of least squares, and found to increase markedly with pH within the range studied. The half lives for the reaction mixtures at the concentration used were found to

vary between 50–120 min depending upon pH. It should be emphasised that the kinetic data are introduced at this point only to demonstrate the order of the reaction, and to help estimate the speed of the reaction. Rate constants are not shown, since the runs were made at only one concentration of reactants, and only the appearance of penicilloic V acid was followed.

Since the reaction was shown to be second order, it was felt that it would be reasonable to assume that cupric ion is not catalysing the reaction, in the classic sense, but is promoting it, appearing as a penicilloic acid-cupric ion complex. The distinction between the terms "catalysed" and "promoted" may shed some light on the mechanism by which additives such as citrates, phosphates and EDTA stabilise penicillin solutions. Since it is well known that heavy metal ions inactivate the penicillins, these metal ion (cupric) chelators have been added to penicillin solutions to prevent deterioration by removing free metal ion which may have been introduced as a contaminant. It has also been suggested (Schwartz & Buckwalter, 1962) that their principal effect is to buffer the solutions to a pH range where the degradation rate is minimal. We prepared solutions containing penicillin V and cupric chloride in the ratio of 10:1, in a pH 5·50 0·12M acetate buffer. The reaction should have ceased when 10% of the penicillin V had been degraded, since the pH used gives excellent stability in the absence of cupric ions. The reaction, as followed at 273 mμ, apparently ceased with 90·6% penicillin V remaining (calculated using equation 4). Thus it would appear that in solutions containing trace amounts of metal ion, the effect of citrates would be primarily one of a buffering action.

Another interesting development during these investigations was the effect of cupric ion upon the re-arrangement of both penicillins G and V into their corresponding penicillenic acids. At pH 5·50 in 0·12M acetate buffer at 32°, penicillin G (3×10^{-4}M) in the absence of cupric ion develops a marked 322 mμ peak after 25 hr and increasing up to 72 hr which is characteristic of the penicillenic acids; in the presence of an equimolar amount of cupric chloride, the 322 mμ peak does not appear. The absorbance at 322 mμ in the presence of cupric ion is due to the spectral shift accompanied by the hydrolysis of the penicillin into the penicilloic acid. The same spectral shift, with no 322 mμ peak can be seen in Fig. 4 for penicillin V (3×10^{-4}M) in 0·12M acetate buffer at pH 5·50, although in the absence of cupric ion, a peak does appear. Thus it appears that the mechanism of degradation, as well as the rate appear to be influenced by the presence of cupric ions.

Our results compare well with those obtained by Ujiie (1957), who found that magnesium and calcium ions did not affect zones of inhibition produced by penicillin. Our results showed that these ions do not interact with penicillin. Ujiie (1957) also found that cupric and zinc ions decreased the zones to almost zero; our findings were that cupric ions promoted the degradation of penicillins to penicilloic acids, and that zinc interacted with penicillin. In the present investigation EDTA prevented the cupric ion promoted degradation of penicillins G and V while

Ujiie (1957) found that EDTA removes the zone decreasing effect of cupric ion.

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