[Chem. Pharm. Bull. 19(4) 730—736 (1971)]

UDC 546.56.09 : 615.33.011.5

Studies on the Stability of Drugs in Biological Media. III.[1] Effect of Cupric Ion on the Stability and Antibacterial Activity of Penicillins in Culture Medium[2]

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(Received September 26, 1970)

The effect of cupric ion on the stability and antibacterial activity of penicillins, benzylpenicillin and 2,6-dimethoxyphenylpenicillin, in phosphate buffer solution and phosphate broth kept at pH 5.0 was investigated. The degradation was found to obey first-order kinetics to produce the corresponding penicillenic acids, though the formation of penicilloic acid analogs have been postulated in other studies. Catalytic and complexing effect of cupric ion on the stability of penicillins was revealed in relatively lower and higher concentrations of the metal ion respectively, where an appreciable difference in the effect was noted between buffer solution and broth. Apparent stability constants for the proposed complex between penicillins and cupric ion were estimated to be log $K_{app}=2.65$ and 2.91 with benzylpenicillin and 2,6-dimethoxyphenylpenicillin, respectively.

The antibacterial activity against *Staphylococcus aureus* was not apparently affected by the addition of cupric ion, which indicates an apparent lack of correlation between the stability and the activity of the drug in broth. This is presumably because the probable enhancing effect of the metal ion on the cell wall permeability of the penicillins outweighs the other effects investigated.

Stability of penicillins in aqueous solution has been extensively investigated by a number of workers.[4-9] Schwartz has reviewed pharmaceutics of penicillins to discuss the major degradative reactions of the drugs in detail.[5b] He and his co-worker have reported catalytic participation of aminoalkylcatechols in the hydrolysis of penicillins, postulating a mechanism similar to that of several hydrolytic enzymes such as penicillinase.[9]

Recently Niebergall and his co-workers have reported that penicillins complexed with cupric ion undergo promoted hydrolysis in aqueous solution.[10] In these reports a kinetical analysis has been carried out by means of alkaline titration in order to substantiate the reaction mechanism which involves the formation of penicilloic acid analogs. In the presence of cupric ion, however, the possible participation of another degradation mechanism, such as the formation of penicillenic acid analogs, has not as yet been demonstrated. The primary purpose

1) Part II: K. Kakemi, H. Sezaki, K. Iwamoto, and Y. Sano, *Chem. Pharm. Bull.* (Tokyo), **17**, 1623 (1969).
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4) *a*) F.P. Doyle, J.H.C. Nayler, H. Smith, and E.R. Stove, *Nature*, **193**, 1091 (1961); *b*) F.P. Doyle, A. A.W. Long, J.H.C. Nayler, and E.R. Stove, *ibid.*, **193**, 1183 (1961).
5) *a*) M.A. Schwartz, A.P. Granatek, and F.H. Buckwalter, *J. Pharm. Sci.*, **51**, 523 (1962); *b*) M.A. Schwartz and F.H. Buckwalter, *ibid.*, **51**, 1119 (1962); *c*) M.A. Schwartz, *ibid.*, **54**, 472 (1965).
6) J.F. Gallelli, *Am. J. Hosp. Pharm.*, **24**, 425 (1967).
7) J.P. Hou and J.W. Poole, *J. Pharm. Sci.*, **58**, 447 (1969).
8) *a*) M.A. Schwartz, *J. Pharm. Sci.*, **57**, 1209 (1968); *b*) *Idem*, *J. Med. Chem.*, **12**, 36 (1969).
9) *a*) M.A. Schwartz, *J. Pharm. Sci.*, **54**, 1308 (1965); *b*) M.A. Schwartz and G.R. Pflug, *ibid.*, **56**, 1459 (1967); *c*) R.D. Kinget and M.A. Schwartz, *ibid.*, **57**, 1916 (1968); *d*) *Idem*, *ibid.*, **58**, 1102 (1969).
10) *a*) P.J. Niebergall, D.A. Hausar, W.A. Cressman, E.T. Sugita, and J.T. Doluisio, *J. Pharm. Pharmaol.*, **18**, 729 (1966); *b*) W.A. Cressman, E.T. Sugita, J.T. Doluisio, and P.J. Niebergall, *ibid.*, **18**, 801 (1966); *c*) W.A. Cressman, E.T. Sugita, J.T. Doluisio, and P.J. Niebergall, *J. Pharm. Sci.*, **58**, 1471 (1969).

of this paper is to evaluate the effect of cupric ion, one of the well-known metal ions which exhibit important roles in some biochemical reactions such as catalytic or complexing ones, on the stability and *in vitro* antibacterial activity of penicillins in aqueous biological medium.

Experimental

Materials——Potassium benzylpenicillin (Takeda Chemical Industries Ltd.), penicillin-G (PG), and sodium 2,6-dimethoxyphenylpenicillin (Meiji Seika Co., Ltd.), methicillin (MT), were used as received. All other chemicals used were of reagent grade. Solutions were prepared in water which had been degassed after glass distillation. Phosphate buffer solution (KH_2PO_4–Na_2HPO_4) and phosphate broth (Nissan) were made to be metal-free according to the method described previously.[11]

Identification of Degradation Products——Penicillenic acid analogs (PEA and MEA) were prepared by the method of Levine.[12] Penicilloic acid analogs (POA and MOA) were prepared in solutions by the hydrolysis of penicillins at pH 12 for 15 min at room temperature in the manner described by Rapson and Bird.[13] Under the same condition as in the kinetic runs, PG and MT were preparatively decomposed both in 0.05 M phosphate buffer solution (pH 5.0, $\mu=0.2$) and in 10-fold diluted phosphate broth (pH 5.0), with or without a trace amount of copper dichloride. Three parts of each sample were mixed with one part of methanol and the mixture was analyzed by means of thin-layer chromatography conducted in the same manner as described by McGilberay previously.[14] The results are shown in Fig. 1, where PG and MT with copper dichloride are expressed as product-1 and product-3, and without the copper compound as product-2 and product-4, respectively.

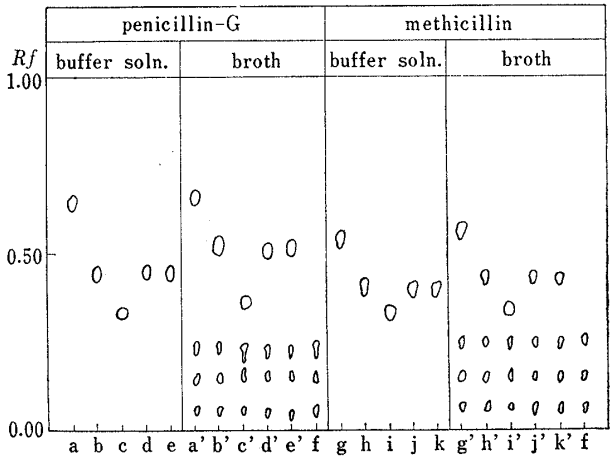

Fig. 1. Chromatoplate of Penicillins and Their Degradation Products

layer: Silica gel G
solvent: organic phase of isoamyl acetate–methanol–formic acid–water (65:20:5:10)
reagent: mixture of aqueous solutions of 10%-$FeCl_3$, 5%-$K_3Fe(CN)_6$, and 20%-H_2SO_4 (2:1:7)
compositions and *Rf* values:

PG: a(0.67), a'(0.68)	MEA: h(0.42), h'(0.45)
PEA: b(0.47), b'(0.55)	MOA: i(0.35), i'(0.36)
POA: c(0.35), c' (0.38)	product-3: j(0.41), j'(0.45)
product-1: d(0.47), d'(0.53)	product-4: k(0.41), k'(0.44)
product-2: e(0.47), e'(0.54)	broth blank: f
MT: g(0.56), g'(0.58)	

Procedure for Kinetic Studies——Appropriate amount of penicillins and copper dichloride were dissolved in pH 5.0 phosphate buffer solution and pH 5.0 phosphate broth which were prepared to be metal-free as described previously. Initial concentration of the drugs were adjusted at 5×10^{-4}M unless otherwise specified. The reaction was carried out in duplicate in a volumetric flask which was immersed in a constant temperature bath kept at 37°. Aliquots were withdrawn periodically and analyzed spectrophotometrically with respect to PEA and MEA at 322 mμ and 332 mμ, respectively. Kinetical treatments were made in terms of residual penicillins by appropriate calculations. Effect of a few amino acids and ethylenediaminetetraacetic acid (EDTA) in the presence of cupric ion was studied in a similar manner.

Antibacterial Activity Tests——Twenty-four hours old culture of *Staphylococcus aureus* FAD 209 P was used as a test organism. Minimal inhibitory concentrations (MIC) were determined in triplet by the conventional tube dilution method. Each tube which contained 4 ml of phosphate broth including cupric ion in various concentrations was inoculated with 0.2 ml of suspension of the organisms (1 mg per ml) and added with 1 ml of drug solution which was prepared by a serial dilution. MICs were determined after 2 to 4 days incubation at 37°. All the procedures were carried out aseptically. Effect of varying drug-exposure times on a growing culture was tested in the same manner as described previously.[1]

Effect of Cupric Ion on Apparent Partition Coeffecient of Penicillins——This was carried out by a conventional manner in the system of phosphate buffer solution–isoamyl alcohol (1:1) at 5°. Drug concentration in aqueous phase was determined by hydroxamic acid method.[15]

11) W.S. Warning and C.H. Werkman, *Arch. Bioch.*, **1**, 303 (1942).
12) B.B. Levine, *Arch. Biochem. Biophys.*, **93**, 50 (1961).
13) H.D. Rapson and A.E. Bird, *J. Pharm. Pharmacol.*, **15** *Suppl.*, 222T (1963).
14) I.J. McGilberay and R.D. Strickland, *J. Pharm. Sci.*, **56**, 77 (1967).
15) *a*) J.H. Ford, *Anal. Chem.*, **19**, 1004 (1947); *b*) H.I. Henstock, *Nature*, **164**, 139 (1949).

Result and Discussion

Identification of Degradation Products

As shown in Fig. 1, the degradation products of PG an MT were merely penicillenic acid analog, PEA and MEA, respectively, under the conditions employed with or without a trace amount of cupric ion. Furthermore a number of reaction mixtures were scanned through the ultraviolet region to determine the possible presence of PEA and MEA, showing distinctively increasing absorbance at each λ_{max} (322 mμ and 332 mμ, respectively) with the increase of reaction interval and no shift of the spectra. Since the maximum absorbances of both drugs in an ultimate time did neither increase nor decrease and any other degradation products than PEA and MEA were not found by the thin-layer chromatography, the degradative reaction was postulated to proceed *via* the route II in Chart 1 which have been demonstrated by Schwartz and his co-worker.[5b]

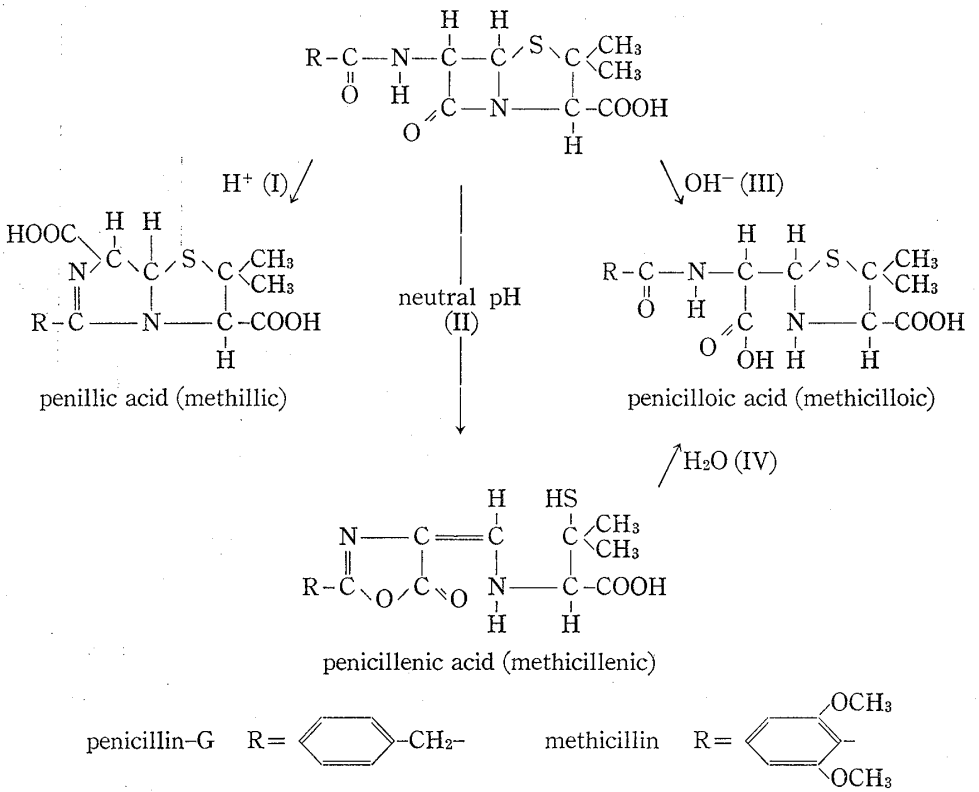

Chart 1. Major Degradative Reactions of Penicillins

Niebergall, however, has reported that penicilloic acid analogs are produced as a result of cupric ion catalyzed hydrolysis of penicillins.[10b,c] This incoincidence of the degradation products is possibly due to the difference in the experimental conditions such as reaction medium and concentration of cupric ion employed.

Effect of Cupric Ion on the Stability of PG and MT

The degradation of either drug showed to obey apparently first-order kinetics both in buffer solution and in broth as given in Fig. 2.

Effect of cupric ion concentration on the stability are demonstrated in Fig. 3 and 4.

In the case of buffer solution, a linear relationship between the concentration of cupric ion and the apparent rate constant (k_{app}) is obtained in the region of relatively lower concentration of the metal ion. As the concentration increased approximately over 5×10^{-7} M, ap-

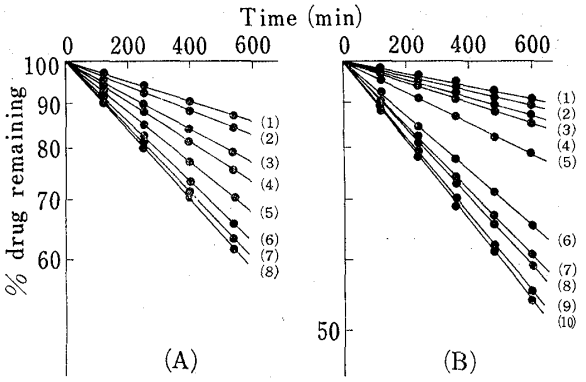

Fig. 2. Stability of Penicillin-G in Phosphate Buffer Solution (A) and in Phosphate Broth (B) at 37°

concentration of added cupric ion: in (A) (1) 0 M, (2) 3×10^{-8}M, (3) 5×10^{-8}M, (4) 1×10^{-7}M, (5) 1.6×10^{-7}M, (6) 2×10^{-7}M, (7) 3×10^{-7}M, and (8) 4×10^{-7}—1×10^{-5}M; and in (B) (1) 0 M, (2) 5×10^{-7}M, (3) 8×10^{-7}M, (4) 1×10^{-6}M, (5) 2×10^{-6}M, (6) 5×10^{-6}M, (7) 8×10^{-6}M, (8) 1×10^{-5}M, (9) 4×10^{-5}M, and (10) 8×10^{-5}—1×10^{-4}M

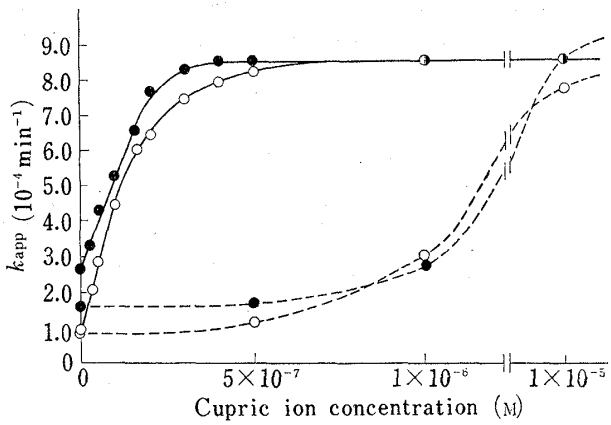

Fig. 3. Effect of Cupric Ion Concentration on the Stability of Penicillin-G (●) and Methicillin (○) in Phosphate Buffer Solution at 37°

Dotted lines indicate the results obtained in the case of phosphate broth.

parent effect of the ion was gradually reduced to attain a constant value. In the case of broth, on the other hand, the linearity was obtained up to a rather higher concentration region than in buffer solution. This effect in Fig. 4 is duplicated comparably with dotted lines in Fig. 3, where the effect of cupric ion in relatively lower concentration is apparently diminished. Since it could be expected that some sorts of amino acid included in broth protein would seize cupric ion to reduce its effective concentration, effect of a few amino acids and EDTA added to the reaction mixture of buffer solution was subsequently studied. As shown in Fig. 5, apparent degradation rate constant of PG was remarkably reduced by the addition of these compounds. A similar result was obtained in the case of MT. It is consequently considered that the possible uptake of cupric ion by broth protein might be responsible for the reducing effect like this, since the ranking order of the latter was the same as that of the affinity constants for the binding reaction.[16]

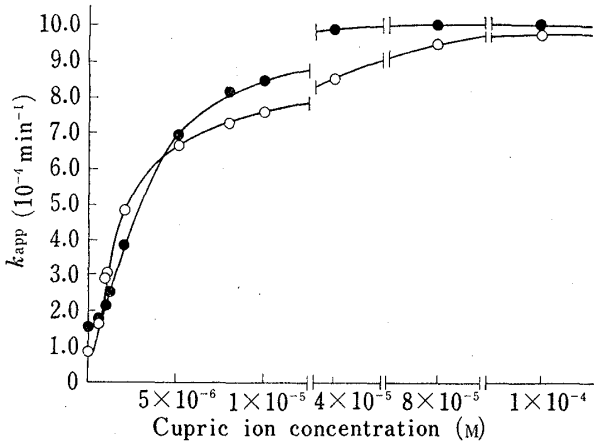

Fig. 4. Effect of Cupric Ion Concentration on the Stability of Penicillin-G (●) and Methicillin (○) in Phosphate Broth at 37°

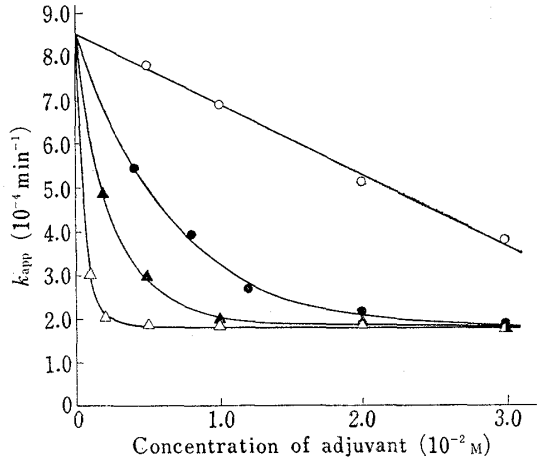

Fig. 5. Effect of Cupric Ion Complexing Agents on the Stability of Penicillin-G in Phosphate Buffer Solution at 37°

concentration of cupric ion: 1×10^{-6}M,
○: glycine, ●: tryptophan, ▲: histidine, △: EDTA

16) *a*) C.B. Monk, *Trans Faraday Soc.*, **47**, 285, 292, 297 (1951); *b*) R.M. Izatt, W.C. Fernelius, and B.P. Block, *J. Phy. Chem.*, **59**, 80 (1955); *c*) A. Albert, *Biochem. J.*, **54**, 646 (1953); *d*) G. Schwarzenbach, G. Gut, and G. Anderegg, *Helv. Chim. Acta*, **37**, 937 (1954).

Catalytic effect of cupric ion is considered to be primary one in its lower concentration region and complexing effect is so in the higher region of the metal ion. The rate expression for the postulated situation, therefore, can be given as:

$$\text{rate}=(k_{app}-k_0)(P)_T=k_{Cu^{2+}}(Cu^{2+})_f(P)_f+k_c(P)_c \quad (1)$$

where k_{app}, k_0, and $k_{Cu^{2+}}$ are apparent, spontaneous, and catalytic rate constant, k_c is a rate constant for the moiety of complex, and $(P)_T$, $(P)_f$, and $(P)_c$ and $(Cu^{2+})_f$ represent the concentration of total, free, and complexed drug, and that of free cupric ion, respectively. Alternate expressions can be given when possible 1:1 complex formation is expressed as in Eq. 2:

$$A + B \rightleftarrows AB \quad (2)$$

$$(k_{app}-k_0)C_a=k_{Cu^{2+}}(C_b-C_c)(C_a-C_c)+k_cC_c \quad (3)$$

$$K_{app}=\frac{C_c}{(C_a-C_c)(C_b-C_c)} \quad (4)$$

where C_a and C_b are the added concentrations of A and B, respectively, and C_c and K_{app} represent the concentration and apparent stability constant of the complex at equilibrium. If C_c is negligibly small as compared with C_a, it can be approximately calculated by re-arranging Eq. 3.

$$C_c=\frac{k_{app}-k_0-k_{Cu^{2+}}C_b}{K_c-k_{Cu^{2+}}C_a}C_a \quad (5)$$

The value of k_0 was obtained graphically (Fig. 3) from the intercept and that of $k_{Cu^{2+}}$ was approximately caluclated from the slope of the linearity where C_c was considered negligibly small as compared with C_a and C_b in Eq. 3. Similarly k_c was calculated from the difference of rate constants between the intercept (k_0) and the plateau portion (k_{app}) where almost all amount of the drug was expected to be complexed with cupric ion. Subsequently, calculated C_C can be substituted in Eq. 4 to give an apparent stability constant of complex. Data in in Fig. 3 conformed well to the serial equations mentioned above as to give a clear-cut value of apparent stability constant.

These values calculated for the complexes of PG and MT with cupric ion are given in Table I.

TABLE I. Rate Constants and Apparent Stability Constants for the Reaction System of Penicillins and Cupric Ion

	Penicillin-G	Methicillin
k_0 (min^{-1})	2.73×10^{-4}	1.00×10^{-4}
$k_{Cu^{++}}$ ($min^{-1}M^{-1}$)	2.52×10^{3}	3.43×10^{3}
k_c (min^{-1})	5.81×10^{-4}	7.49×10^{-4}
K_{app} (M^{-1})	4.51×10^{2}	8.15×10^{2}
log K_{app} (M^{-1})	2.65 (2.63[a], 4.8[b])	2.91

a) Taken from the data reported by Niebergall and others (1969).
b) Taken from the data reported by Weiss and others (1957).

Stability constants in broth could not be obtained in such a manner, since it was experimentally complicated to obtain a precise value of each parameter. The combined results pre sented by other investigators with respect to the system of PG and cupric ion are given in the parenthesis.[10c,17] Niebergall's and Weiss' values were obtained by the method of alkaline titration and ion exchange, respectively, and the former appears to be much closer to ours gained by different means of kinetical analysis.

Although a number of mechanisms and sites of cupric ion complexation(chelation) with penicillins have been postulated,[10,17,18] it might be considered most probable that the chelate formation between the side chain nitrogen and the beta lactam oxygen would place a severe strain on the lactam ring to result a fission itself.

Effect of Cupric Ion on *in Vitro* Antibacterial Activity of Penicillins

The *in vitro* and *in vivo* activity data of penicillins have been reported to reveal a close relation to the extent of binding to serum protein by Kunin and other investigators.[19,20] To avoid such effect of protein, 10-fold diluted phosphate broth was employed which was the same one as used in the stability test and was minimally satisfactory for the growth of organisms tested. The activity data are shown in Table II.

Table II. Comparison of Minimal Inhibitory Concentrations of Penicillins against *St. aureus* (FAD 209P)

Cupric ion concentration in phosphate broth (M)	Penicillin-G (M)	Methicillin (M)
0	4×10^{-7}	2×10^{-5}
1×10^{-7}	4×10^{-7}	2×10^{-5}
1×10^{-6}	4×10^{-7}	2×10^{-5}
1×10^{-5}	4×10^{-7}	1×10^{-5}

It is evident from the result that both drugs demonstrated the effect apparently independent on the concentration of cupric ion added to the culture media. MT was less active than PG, being coincident with the result described previously.[20] Recently Biagi and his co-workers have pointed out this difference in antibacterial activity against *St. aureus* with respect to the lipophilicity of the drug.[21]

The direct effect of the drug stability on the result of *in vitro* activity test was not apparently involved in this study, as compared with the case of some isoniazid derivatives reported previously.[1] These unexpected results might be explained by one or more concurrent participation of the following effects: 1) drug–exposure effect in the relatively early stage of test incubation, 2) spontaneous antibacterial activity of cupric ion, and 3) enhancing effect of cupric ion on the cell wall permeability of the drug.

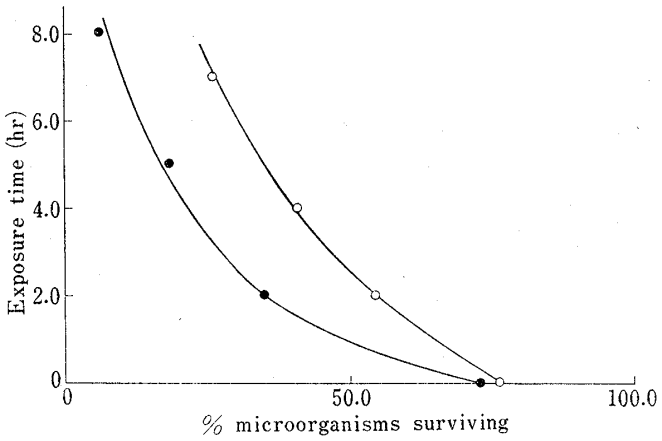

Fig. 6. Relationship between Exposure Time and Percentage of Microorganisms surving after Exposure to Penicillin-G (●) and Methicillin (○)

Firstly drug–exposure effect was studied in the same manner as conducted previously,[1] and the result is shown in Fig. 6.

17) *a*) A. Weiss and S. Fallab, *Helv. Chim. Acta*, **40**, 576 (1957); *b*) A. Weiss, S. Fallab, and H. Erlenmeyer, *ibid.*, **40**, 611 (1957).
18) J. Johnson, H. Clarke, and R. Robinson, "The Chemistry of Penicillin," Princeton University Press, Princeton, N. J., 1949, p. 421.
19) *a*) C. M. Kunin, *Clin. Pharmacol. and Therap.*, **7**, 166 (1966); *b*) *Idem*, *ibid.*, **7**, 180 (1966).
20) K.E. Price, A. Gourevitch, and L.C. Cheney, *Antimicrobial Agents and Chemotherapy*, **1966**, 670.
21) G.L. Biagi, M.C. Guerra, A.M. Barbaro, and M. F. Gamba, *J. Med. Chem.*, **13**, 511 (1970).

In a set of this experiment which treated samples at 0-time in the same way as others, instantaneous exposure of PG and MT were directly estimated to be merely 27 and 24%, respectively, and it seemed to require rather long period that these drugs exert almost all of the activity. Consequently it might be inadequate to anticipate the salutary effect of drug–exposure in an early stage of culture in the case of penicillins.

In the second place, the effect of cupric ion on the growth of organisms was examined by using the broth which contains cupric ion in various concentrations and excludes penicillins. Growth rate was analyzed nephelometrically in the same manner as in the exposure test. Degree of growth inhibition estimated was approximately 10% over 5×10^{-5}M of cupric ion, so this was not the case in the concentration region employed in the MIC test.

Finally, the possible effect of enhancement of cell wall permeability was evaluated from the data listed in Table III.

Table III. Effect of Cupric Ion on the Apparent Partition Coefficient of Penicillins to Isoamyl Alcohol at 5°

Concentration of added cupric ion (M)	Penicillin-G (ratio)	Methicillin (ratio)
0	0.410 (1.00)	0.394 (1.00)
1×10^{-6}	0.431 (1.05)	0.435 (1.10)
1×10^{-5}	0.467 (1.14)	0.456 (1.16)
1×10^{-4}	0.690 (1.68)	0.868 (2.20)
3×10^{-4}	1.125 (2.74)	1.572 (3.99)
5×10^{-4}	1.833 (4.47)	2.194 (5.57)

This partition experiment was carried out at 5° to eliminate the degradation of the drug. Apparent partition coefficient of penicillins increased considerably with the addition of cupric ion, the effect was more noticeable in MT than PG. Similar trend was found in the values of complex stability constant. Mode of action of penicillins against *Staphylococci* has been recognized that the drug interferes with transpeptidation responsible for the cross-linking of mucopeptide chains in the cell wall ploymer of the organisms,[22] and it has been reported, furthermore, that the relative activity is dependent upon the degree of lipophility of the compounds.[21] It is, therefore, reasonably explained that the apparent antibacterial activity of penicillins tested might be consequently enlarged by the addition of cupric ion owing to the corresponding enhancement of cell wall permeability. Ujiie has found that the antibiotic activity of penicillin against *M. pyogenes* var. *aureus* and *E. coli* by cup test was lost entirely in 3 to 6 hours by contact with cupric ion[23a] and Niebergall, in correspondance to this result, implied that cupric ion promoted the degradation of penicillin to penicilloic acid.[10a] However Ujiie has reported later that cupric ion did not inhibit the activity in the synthetic aqueous medium consisting of phosphates.[23b] These observations described above suggest that apparently antibacterial activity of penicillins may be enhanced by the addition of an appropriate amount of certain metal ions such as copper despite relative instability encountered in some cases.

22) *a*) D.J. Tipper and J.L. Strominger, *Proc. Nat. Acad. Sci., Wash.*, **54**, 1133 (1965); *b*) B.F. Erlanger and L. Goode, *Nature*, **200**, 183 (1967); *c*) H.J. Rogers, *ibid.*, **200**, 31 (1967); *d*) L.N. Johnson, *Proc. Roy. Soc. B.*, **167**, 439 (1967); *e*) J. Collins, *ibid.*, 167, 441 (1967); *f*) H. Felsenfeld and R.E. Handschumacher, *Mol. Pharmacol.*, **3**, 153 (1967); *g*) H.J. Rogers, *Biochem. J.*, **103**, 90 (1967).

23) *a*) H. Ujiie, *Nippon Saikingaku Zasshi*, **10**, 771 (1955); *b*) *Idem*, *ibid.*, **10**, 823 (1955).