EtOH saturated with NH_3 and the mixture was heated in a water bath for 1 hr. After partial evaporation of the solvent, the separated crystals were collected and recrystallized from water. Yield, 1.7 g. of m.p. 259~260°(decomp.). *Anal.* Calcd. for $C_5H_4O_2N_3Cl$: C, 34.63; H, 2.31. Found: C, 34.61; H, 2.27.

Preparation of Metal Chelate Compounds—A typical procedure of the preparation of metal chelate compound of (I) was as follows: 1.1 moles of metal acetate (Ni, Cu, Mn) in EtOH containing a few drops of AcOH was added to EtOH solution of 1 mole of (I), and the mixture was heated on a water bath for 30 mins. The precipitate that formed was collected, washed with hot water and EtOH, and recrystallized from glacial AcOH.

	Analysis (%)			
Formula	Calcd.		Found	
	C	H	C	H
$C_{10}H_4O_6N_4Cl_2Ni\cdot 3H_2O$	26.12	2.18	26.23	2.26
$C_{10}H_4O_6N_4Cl_2Cu\cdot {}^1/_2H_2O$	28.60	1.19	29.04	1.30
$C_{10}H_4O_6N_4Cl_2Mn\cdot 2H_2O$	27.40	1.83	26.89	1.86

Infrared spectra were measured with a Perkin-Elmer Model 21 double-beam spectrophotometer equipped with a NaCl prism and the ultraviolet spectra were obtained with a Shimadzu spectrophotometer Type QB-50 with 1-cm. quartz cells.

Summary

Infrared and ultraviolet absorption spectra of 4- and 5-carboxy-6-chloro-3-pyridazinol (I and II) and of their esters (I_E and II_E) were measured. It was found that (I) exists in a chelated form in its crystalline state and that (II) forms an intermolecular hydrogen bonding. Metal chelate compounds of (I) with nickel, copper, or manganese were reported. The preparation and ultraviolet absorption spectrum of 5-carbamoyl-6-chloro-3-pyridazinol (II_{Amide}) were described.

(Received May 15, 1958)

UDC 547.864.6—156 : 541.486 : 541.651

112. **Yoshinori Kidani**: Studies on Metal Chelate Compounds of Phenazine Derivatives. I. Spectrophotometric Studies on Copper Chelate Compounds of 1-Hydroxyphenazine and its Di-N-oxide.

(*International Christian University**)

Metal chelates were originally developed in the field of analytical chemistry and they recently have begun to play a very important rôle in the field of pharmaceutical sciences. One of the main reasons is that an enzyme is a kind of polymolecular chelate compounds and it has been found that chelating agents and metal chelates themselves are very closely related to the growth-inhibitory action of bacteria.

For example, 8-hydroxyquinoline (oxine), which had originally been used as an analytical reagent, shows antibacterial action and, inversely, some of the chemotherapeutics and antibiotics were found to be chelating agents.

Albert, Rubbo, and others[1] reported studies on oxine derivatives and they showed that metal chelates of organic compounds have a close relation to antibacterial action. When the growth-inhibitory action against *Staphylococcus aureus* was examined by adding a trace of iron or copper to oxine solution, the action increased markedly compared with that without the addition of metals. When hydroxyl in oxine was alkylated, or, when hydroxyl groups in oxine isomers are blocked except at the 8-position, they have no chelating ability and therefore such a compound did not show any effect from metals. From these results,

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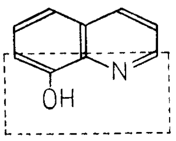
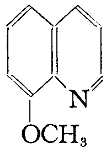
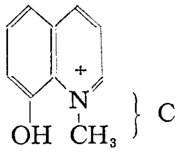
* 1500 Osawa, Mitaka-shi, Tokyo (喜谷喜徳).

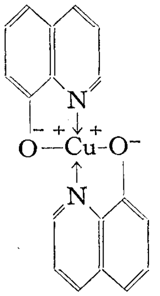
[1] A. Albert, D. Magrath: J. Biochem., **41**, 534(1947); A. Albert, S. D. Rubbo, R. J. Goldace, B. G. Balfour: Brit. J. Exptl. Pathol., **28**, 69(1947); S. D. Rubbo, A. Albert, M. I. Gibson: *Ibid.*, **31**, 4125(1950).

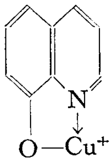






Albert, *et al.*[2] proposed the very interesting hypothesis that the 1:1 (oxine : Cu) metal chelate, which is called cation complex, is powerfully toxic against bacteria and that the 2:1 (oxine : Cu) metal chelate compound, a normal complex, is non-toxic.





Erlenmeyer, *et al.*[3] also reported results concerning such problems. They examined the influence of oxine and oxine-like compounds in structure with copper against tubercle bacilli. Copper was mainly used in these experiments and there was some cases in which a slight fortifying action was observed also in cobalt. The fortification of antibacterial power by copper as interpreted by Erlenmeyer, *et al.*[3] is that, first, the compound which has chelating ability makes a chelating bond with essential metals of enzyme and that, accordingly, the bond between enzyme and metal is instabilized. When copper is present, the instabilized metal of the enzyme is expelled and replaced by copper. Hence the enzyme is degenerated and consequently the intensification of antibacterial action is displayed.

Sijpesteijn, *et al.*[4] reported the results of oxine-copper chelate action against *Aspergillus niger*. They explained, in support of Albert's hypothesis, that the cation complex, 1:1 chelate, is supposed to be toxic and that the normal complex, 2:1 chelate itself, does not have any toxicity. They elucidated that, first of all, 2:1 chelate penetrates rapidly into the cells and is then transformed into the toxic 1:1 complex, which acts upon bacteria.

The author and the colleagues synthesized various phenazine derivatives and among them there are some which have oxine-like structure. Yosioka[5] examined the antibacterial action of these α-hydroxyphenazine derivatives, as well as the influence exerted by the addition of copper. The results obtained were in conformity with those of Albert and Erlenmeyer, *et al.*, an addition of copper exhibiting increased efficacy.

Ueno[6] also reported the effect of metallic ion on antitubercular agents.

In the present series of work, properties of copper chelate in solution were studied by spectrophotometric method and examinations were made to see what kind of chelates the oxine-like compounds, mainly α-hydroxyphenazine derivatives, will produce with copper ion. It is seen that α-hydroxyphenazine forms a fairly stable chelate with copper.

Emphasis was laid upon the question of whether there is any relationship between the stability of chelates and their antibacterial action, and accordingly by what mechanism

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[2] A. Albert: "Selective Toxicity," Methuen & Co., London.

[3] E. Sorkin, W. Roth, H. Erlenmeyer: Helv. Chim. Acta, **35**, 1736(1952); L. Müslin, W. Roth, H. Erlenmeyer: *Ibid.*, **36**, 886(1953); H. Erlenmeyer, J. Bäumler, W. Roth: *Ibid.*, **36**, 941(1953); H. Erlenmeyer: *Ibid.*, **37**, 636, 2010(1954).

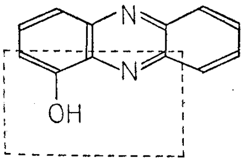
[4] A. K. Sijpesteijn, M. J. Janssen, H. M. Dekhuyzen: Nature, **180**, 5058(1957).

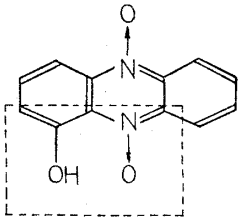
[5] Y. Kawakami, T. Takahashi, J. Yabe, I. Yosioka: J. Antibiotics, Ser. A, **8**, 51(1955); I. Yosioka, S. Uehara: Yakugaku Zasshi, **78**, 351(1958); I. Yosioka, T. Tanaka: *Ibid.*, **78**, 353(1958).

[6] T. Ueno: Yakugaku Zasshi, **76**, 825, 831, 839(1956).

phenazine-copper chelates exert their influence on bacteria. The results obtained are reported herein.

First, copper chelate of 1-hydroxyphenazine, which possesses one oxine-like functional group, a hydroxyl *peri* to a ring-nitrogen, was studied spectrophotometrically in ethanol solution. Its di-N-oxide also has a chelating ability and will be dealt with at the same time.





Experimental

All measurements were made under the following conditions:

1) **Apparatus**—a) pH measurements were made with a Tôyô-Rika glass electrode pH-meter, direct-reading type.

b) Measurements of absorbance were made with a Shimadzu Electro-spectrophotometer, Model QB-50, using glass and quartz cells of 10-mm. light path.

2) **Materials**—a) Solvent: EtOH was used as a solvent because phenazine derivatives are insoluble in water. The solvent was purified and dehydrated by conc. H_2SO_4, KOH-alkaline $AgNO_3$, CaO, and metallic Na, followed by distillation. Absence of copper ion in the solvent was ascertained by flame analysis made with a Shimadzu Flame Analysis apparatus.

b) Copper Acetate: $(AcO)_2Cu\cdot H_2O$ (special grade for analytical use). Absorption spectrum of this reagent in various concentrations in EtOH is shown in Fig. 1.

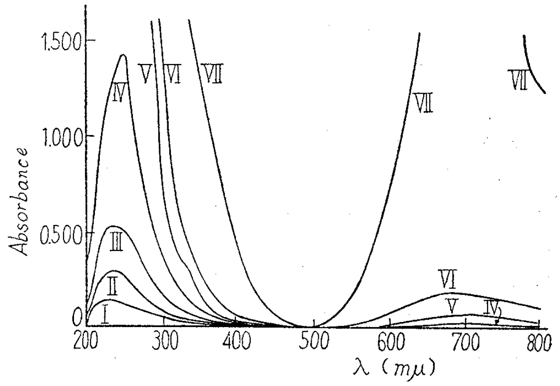


Fig. 1. Absorption Spectra of Copper Acetate (in EtOH)

I $5\times10^{-5}M$ II $1\times10^{-4}M$

III $2\times10^{-4}M$ IV $5\times10^{-4}M$

V $1\times10^{-3}M$ VI $2\times10^{-3}M$

VII Satd. solution

A $1\times10^{-4}\,M$ copper acetate solution was prepared by dissolving 1.99 mg. of the reagent in 100 cc. of EtOH. A saturated copper acetate solution in EtOH is almost the same as $1\times10^{-2}\,M$ EtOH solution. Solutions in other concentrations were prepared by dissolving the appropriate weight of the reagent in EtOH. As is shown in Fig. 1, absorption maxima are around 240 and 700 mμ which may be neglected in this series of study.

c) 1-Hydroxyphenazine: A $1\times10^{-4}\,M$ solution was prepared by dissolving 1.96 mg. of 1-hydroxyphenazine in 100 cc. of dehyd. EtOH.

d) 1-Hydroxyphenazine di-N-oxide: A $1\times10^{-4}\,M$ solution was prepared by dissolving 2.28 mg. of 1-hydroxyphenazine di-N-oxide in 100 cc. of dehyd. EtOH.

Procedure—All the measurements were carried out at room temperature.

1) Preparation of solution for absorption spectral measurements: One drop of saturated EtOH solution of copper acetate was added to 5 cc. of $1\times10^{-4}\,M$ EtOH solution of 1-hydroxyphenazine.

2) Varying procedure of pH: a) For the absorption spectral measurement, acidity or alkalinity was changed by adding a few drops of EtOH solution of AcOH or KOH, and change in pH of the solution was measured by a pH-meter. b) pH variation was effected by the following method: First, pH of the EtOH solution was changed by adding AcOH or KOH, dissolved in EtOH, as well as the buffer solution

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7) J. H. Yoe, A. L. Jones: Ind. Eng. Chem., Anal. Ed., **16**, 111(1944): A. E. Harvey, D. L. Manning: J. Am. Chem. Soc., **72**, 4488(1950).

of AcOH and AcONa beforehand, and the pH were measured by pH-meter. 5 cc. of $2\times10^{-4}\,M$ 1-hydroxyphenazine solution was diluted with 5 cc. of the properly variated solution whose pH was determined beforehand, and finally the pH was determined by pH-meter.

Molar Ratio Method⁽⁷⁾—To a certain amount of copper acetate (5 cc. of $1\times10^{-4}\,M$ EtOH solution) EtOH solutions of 1-hydroxyphenazine was added at a molar ratio, followed by dilution with EtOH to 10 cc.

Results

I. 1-Hydroxyphenazine-Copper Chelate

1) Absorption Spectra—In Fig. 2 are shown three kinds of absorption curves of 1-hydroxyphenazine, that is, in a $1\times10^{-4}\,M$ EtOH solution in acidic (pH 6.2), neutral* (pH 8.0), and alkaline (pH 9.9) reaction which are represented by solid lines, and the dotted line shows curves of their copper chelates. The curves of the reagent in acidic and neutral solution seem to be nearly the same but the one in alkaline solution is very different. The first absorption region which is characteristic in chelates is in the region of 560 mμ in neutral and acidic solutions. EtOH was used as the standard solution.

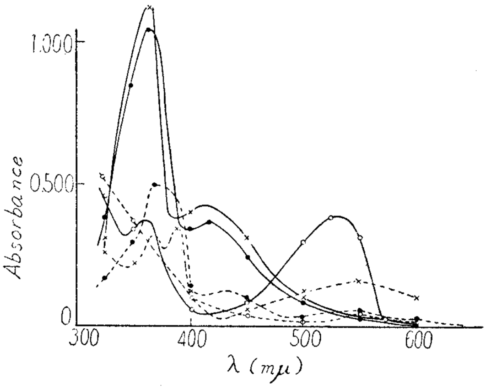

Fig. 2. Absorption Spectra of 1-Hydroxyphenazine-Copper Chelate ($1\times10^{-4}M$ 1-hydroxyphenazine)

	Reag.	Complex
Acidic	— • —	- - • - -
Neut.	— × —	- - × - -
Alkali.	— ∘ —	- - ∘ - -

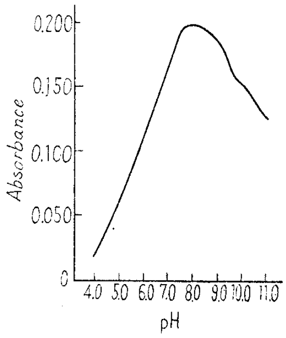

Fig. 3. Effect of pH Change on 1-Hydroxyphenazine-Copper Chelate
(Measured at 560 mμ. The solution contains $1\times10^{-4}M$ 1-hydroxyphenazine)

2) Effect of pH Change—The absorption of the copper chelate solution in various pH containing $1\times10^{-4}\,M$ of 1-hydroxyphenazine is shown in Fig. 3. The solution of the chelate shows the maximum absorption at a pH of about 7.3~8.2 and the absorption decreases markedly if the pH is increased or decreased, measured at 560 mμ.

3) Composition of the Chelate in Solution—Examination was made on the composition of copper chelate compound of 1-hydroxyphenazine in solution. Though the molar ratio of the 1-hydroxyphenazine-copper chelate is reported to be 2:1 from the result of elemental analyses,[8] this was reëxamined by spectrophotometric methods.

(i) Job's Continuous Variation Method[9]: The molar ratio of the 1-hydroxyphenazine-copper chelate in EtOH solution was determined at 560 mμ by Job's method at pH 8.0 as shown in Fig. 4. The experimental data clearly indicated that under these conditions a chelate containing 1-hydroxyphenazine and copper in 2:1 ratio is formed. No cation complex of 1:1 (Reag. : Cu), proposed by Albert, was detected by this method.

(ii) Molar Ratio Method⁽⁷⁾: Under the same conditions of EtOH solution at pH 8.0 and

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* The term "neutral" means, in this series of studies, an intermediate solution between acidic and alkaline EtOH solutions. The apparent pH of EtOH was 7.5~8.0.

[8] I. Murase: Research Rept., Dept. Chem., Kyushu University, **1**, 225(1953).

[9] P. Job: Ann. chim., [10] **9**, 113(1928); W. C. Vosburgh, G. R. Cooper: J. Am. Chem. Soc., **63**, 437 (1941).

at a wave length of 560 mμ, the molar ratio of 1-hydroxyphenazine to copper was 2 : 1 (Fig. 5), as by the former method. The measurements were made by changing the conditions in order to find a cation complex of 1:1 ratio, but no agreeing data were obtained. The results obtained at pH 6.0 and at a wave length of 370 mμ, which is the second absorption region in acidic medium, is shown in Fig. 6. In this case, sharp breaks are observed in the ratio of 1:1 and 2:1, and although it is too bold to conclude the presence of a cation comlpex at this wave length, it could be a sign of the formation of 1:1 cation complex.

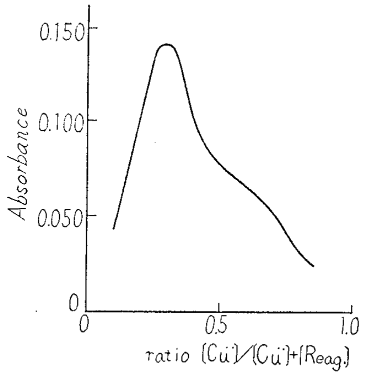

Fig. 4. Absorption Curve of 1-Hydroxyphenazine-Copper-Chelate
(Job Method—Measured at 560 mμ and at a pH of 8.0. Total concentration, $1\times10^{-4}M$)

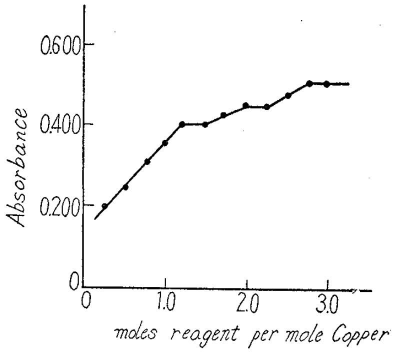

Fig. 5. Absorption Curve of 1-Hydroxyphenazine-Copper Chelate
(Molar Ratio Method—Measured at 560 mμ and at a pH of 8.0. The concentration of copper is $1\times10^{-4}M$)

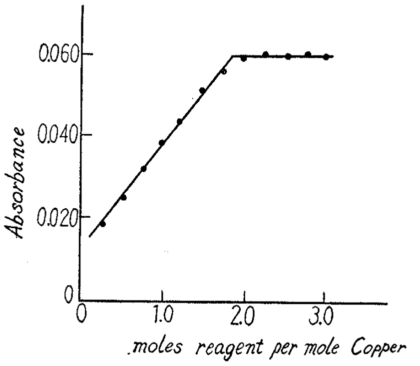

Fig. 6. Absorption Curve of 1-Hydroxyphenazine-Copper Chelate
(Molar Ratio Method—Measured at 370 mμ at pH 6.0. The concentration of copper, $1\times10^{-4}M$)

II. 1-Hydroxyphenazine Di-N-oxide-Copper Chelate

This oxide differs a little from oxine-like structure, but it also has a functional group capable of forming a six-membered chelate ring.

1) Absorption Spectra—In Fig. 7, the absorption of both 1-hydroxyphenazine di-N-oxide and its copper chelate compound in a concentration of $1\times10^{-4}M$ are shown. The curves of reagent in acidic and neutral media are

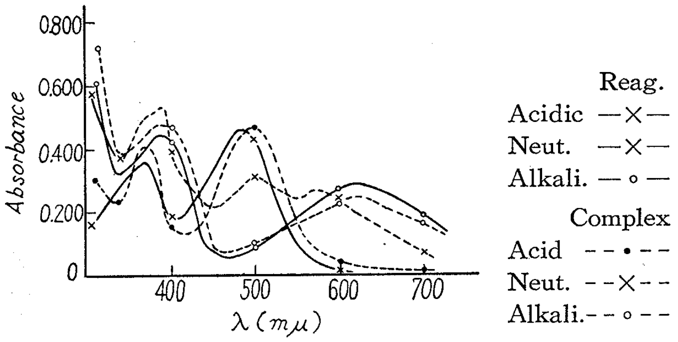

Fig. 7. Absorption Spectra of 1-Hydoxyphenazine Di-N-oxide-Copper Chelate.
(The solution contains $1\times10^{-4}M$ 1-hydroxyphenazine di-N-oxide)

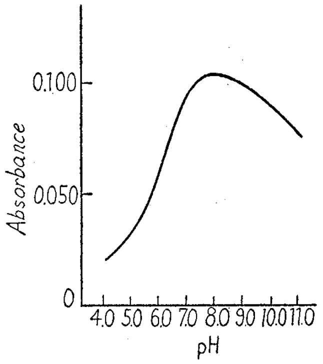

Fig. 8. Effect of pH Change on 1-Hydroxyphenazine Di-N-oxide-Copper Chelate
(Measured at 600 mμ. The solution contains $1\times10^{-4}M$ 1-hydroxyphenazine di-N-oxide)

almost the same but the one in alkaline medium shows a marked difference. The curves of copper chelate in acidic and neutral solutions are different. The first maximum absorption is at 500 mμ in acidic, and 580 mμ in neutral solution.

2) **Effect of pH Change**—The solution of the chelate shows maximum absorption at a pH of about 7.8∼8.5 and the absorption decreases markedly when the pH is increased or decreased, as shown in Fig. 8, measured at 600 mμ.

3) **Composition of Chelate in the Solution**—i) Job's Continuous Variation Method: By this method, composition of 1-hydroxyphenazine di-N-oxide to copper was 2:1 at pH 8.0 and 600 mμ, and 1:1 at pH 6.0 and 690 mμ (Fig. 9).

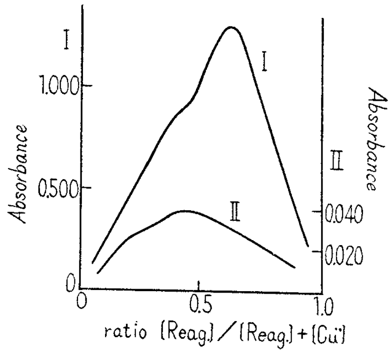

Fig. 9. Absorption Curves of 1-Hydroxyphenazine Di-N-oxide-Copper Chelate (Job Method)
(I) 600 mμ and pH 8.0; $2\times10^{-4}M$
(II) 690 mμ and pH 6.0; $1\times10^{-4}M$

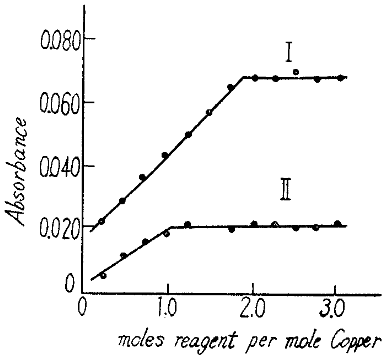

Fig. 10. Absorption Curves of 1-Hydroxyphenazine Di-N-oxide-Copper Chelate (Molar Ratio Method)
(I) 600 mμ and pH 8.0
(II) 690 mμ and pH 6.0
Concentration of copper, $1\times10^{-4}M$.

ii) Molar Ratio Method: Under similar conditions, the same results were obtained, as shown in Fig. 10. The ratio was 2:1 at 600 mμ in neutral solution and 1:1 at 690 mμ in acidic medium.

4) **Degree of Dissociation** (α)[10] **and Dissociation Constants (K) of the Chelate Compounds**—From the spectrophotometric measurements, the degree of dissociation and dissociation constants of chelate compounds were determined by the following calculations.

From the molar ratio method,[7] they are calculated from equation (1) when no consideration of the disturbing effect of the reagent is taken into account:

$$\alpha=\frac{A_m-A_s}{A_m} \quad \cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots(1)$$

where A_m is the maximum extinction obtained from the horizontal portion of the curve, indicating that all the copper is present in the chelate compound, and A_s is the extinction at the stoichiometric molar ratio of 1-hydroxyphenazine or its di-N-oxide to copper in the chelate.

Supposing that a ligand (L) and a metal (M) form a chelate compound, ML_n, then

$$\begin{array}{llll} M & + \quad nL & \rightleftharpoons \quad MLn \cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots(2) & \\ 0 & \quad\; 0 & \quad C & \text{initial concentration} \\ C\alpha & \quad\; nC\alpha & \quad C(1-\alpha) & \text{equilibrium concentrations} \end{array}$$

C is the total concentration of the chelate in moles/L., assuming that no dissociation takes place. The equilibrium constant may be written as

$$K=\frac{(\alpha C)\cdot(n\alpha C)^n}{C(1-\alpha)} \cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots(3)$$

where n indicates the ratio.

The degree of dissociation (α) and dissociation constants (K) of copper chelate of

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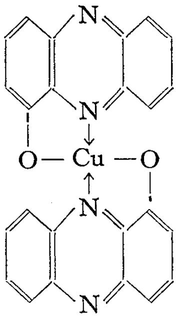
[10] M. Ishidate, Y. Yamane: Yakugaku Zasshi, **77**, 389(1957).

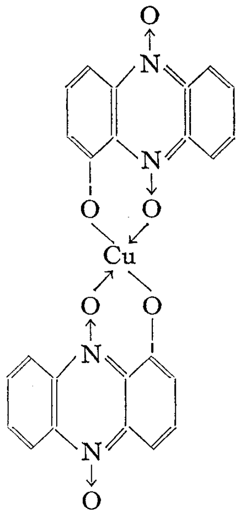
1-hydroxyphenazine and 1-hydroxyphenazine di-N-oxide, calculated from the formulae (1) and (3) are shown in Table I.

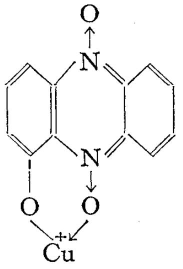
TABLE I.

Compound	α	Reag.: Cu	$-\log K$	pH
1-Hydroxyphenazine	0.34	2:1	8.68	8.0
1-Hydroxyphenazine di-N-oxide	0.18	1:1	5.89	6.0
	0.30	2:1	8.62	8.0

5) **Structure of the Chelates**—From the results obtained, the structures represented below are proposed. As represented below, 1-hydroxyphenazine-copper chelate forms a fairly stable five-membered, 2:1, normal chelate compound, and cation complex was not detected clearly. In acidic medium, the presence of a cation complex may be supposed. On the contrary, its di-N-oxide-copper chelate, showed both normal and cation complexes, forming a six-membered ring.







The author wishes to express his deep gratitude to Prof. M. Ishidate of the University of Tokyo for his encouragements and helpful advices. The author is also very grateful to Prof. T. Sakaguchi of the University of Chiba for his valuable suggestions and encouragements. A part of the expenses for this work was defrayed by the Grant in Aid for Scientific Research from the Ministry of Education to which the author's thanks are due.

Summary

The properties of copper chelate compound of 1-hydroxyphenazine, which possesses one oxine-like functional group to form a chelate, as well as the composition of the chelate in solution and its dissociation constant were studied by the spectrophotometric method. 1-Hydroxyphenazine-copper chelate forms a five-membered ring compound in a neutral medium. 1-Hydroxyphenazine di-N-oxide also has a chelating functional group which seems to be a little different from the former compound, and the properties and composition of copper chelate were also investigated. The copper chelate of its di-N-oxide forms a six-membered chelate ring.

(Received July 12, 1958)

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11) Y. Yamane: Yakugaku Zasshi, **77**, 393(1957); T. Sakaguchi, K. Taguchi, S. Fukushima, N. Obi: *Ibid.*, **78**, 179(1958).