[CONTRIBUTION FROM THE MEDICINAL CHEMISTRY BRANCH, CHEMICAL CORPS MEDICAL LABORATORIES]

Model Reactions of Phosphorus-containing Enzyme Inactivators. IV.[1a] The Catalytic Activity of Certain Metal Salts and Chelates in the Hydrolysis of Diisopropyl Fluorophosphate[1b]

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It was found that certain metal salts, in particular those containing copper(II), accelerate the hydrolysis of diisopropyl fluorophosphate (DFP). The effect of copper(II) salts, at a *p*H above 7, can be greatly enhanced by complex formation with certain nitrogen-containing bases. The copper complexes of various amino acids, imidazole, ethylenediamine (en), *o*-phenanthroline and 2,2′-dipyridyl have been found to be highly active as catalysts for the hydrolysis of DFP. The most effective activator thus far encountered is the Cu(II)-2,2′-dipyridyl chelate. With bidentate donor groups such as dipyridyl and ethylenediamine the 1:1 chelates Cu(II)–dipyridyl and Cu(II)–en are much more effective catalysts for the hydrolysis of DFP than Cu(II)–dipyridyl$_2$ and Cu(II)–en$_2$, respectively. In the case of monodentate donors such as imidazole, maximum activity is reached at the Cu(II)/imidazole ratio 1:2, and remains at that level with further addition of the heterocyclic base. The product of Cu(II) and a tetradentate donor, such as ethylenediaminetetraacetic acid, is a poor catalyst for the hydrolysis of DFP. Maximum activity probably is limited to those complexes in which firmly bound ligands do not completely fill the first sphere of the copper(II) ion. Some aspects of the mechanism of action are discussed.

Recently it has been shown that imidazole and histidine slightly increase the rate of hydrolysis of diisopropyl fluorophosphate (DFP) and of diethyl fluorophosphate (DEFP).[1a] Mazur[2] previously had discovered a powerful enzymatic catalyst for the hydrolysis of dialkyl fluorophosphates in plasma, red blood cells and tissues. One might be inclined to conjecture that the active group of this "fluorophosphatase" could be related in some way to an imidazole or histidine containing constituent of a protein highly activated by the special structure features of the surroundings. Speculations on the various factors which might influence the activity of imidazole and its derivatives as catalysts for the hydrolysis of DFP in approximately neutral solution led to an investigation of the influence of metal ions on the effectiveness of these heterocyclic bases. With the discovery of a strong synergistic effect in certain instances, the study was broadened to cover a wider range of metal complexes.

The hydrolysis of DFP yields two moles of acid, *i.e.*, one mole of HF and one mole of $(i\text{-PrO})_2PO(OH)$. The rate of this reaction may be determined by measuring the production of acid at a constant *p*H. In this study, two methods were used for this determination. (a) The acid produced in an unbuffered solution was neutralized by continually adding dilute NaOH to maintain a constant *p*H in a Beckman Autotitrator. (b) CO_2 production in a sodium bicarbonate–CO_2 buffer was measured by the manometric Warburg method, described in earlier papers.[1,3] The reaction volume remains constant in the latter case, whereas in method (a) the solution is diluted to some extent by the addition of base during the course of the hydrolysis. The rates of reaction are given in terms of "half-life" ($L_{1/2}$) which is the time in minutes required for 50% hydrolysis. The expression "half-time" used in our earlier publications will be reserved for phosphorylation reactions.

(1) (a) III: T. Wagner-Jauregg and B. E. Hackley, Jr., THIS JOURNAL, **75**, 2125 (1953). (b) Some of the results of this paper were presented at the 125th Meeting of the American Chemical Society at Kansas City, Mo., March, 1954. A brief abstract has been published in *Federation Proc.*, **12**, No. 1, 284 (1953), under the title: Models for Fluorophosphatase Reaction.

(2) A. Mazur, *J. Biol. Chem.*, **164**, 271 (1946).

(3) B. J. Jandorf, T. Wagner-Jauregg, J. J. O'Neill and M. A. Stolberg, THIS JOURNAL, **74**, 1521 (1952).

1. Effect of Metal Compounds.—The rate of dephosphorylation of energy-rich phosphorus compounds like phosphocreatine, acetyl phosphate, phosphoglyceryl phosphate[4] and phosphoamino acids[5] is greatly accelerated in the presence of molybdate. Comparison of the titration curves for the hydrolysis of DFP at *p*H 7.4, at 29°, in the absence and presence of molybdate (curves I and II of Fig. 1), shows that sodium molybdate also markedly accelerates the hydrolysis of DFP.[6]

It has been demonstrated by Bamann, *et al.*,[7] that the hydrolysis of glycerophosphates, at slightly alkaline *p*H, is markedly accelerated in the presence of various metal salts, particularly those of the rare earths, *e.g.*, lanthanum. We found that lanthanum chloride had no catalytic effect upon the hydrolysis of DFP in bicarbonate–CO_2 buffer, at a *p*H of 7.6.[8] Under the same conditions, ferrous sulfate, palladous chloride and chromic chloride were inactive and cobaltous chloride and nickel sulfate only slightly active. One might conclude that the lack of activity could be due to the formation of insoluble carbonates. However, in a freshly prepared mixture of cupric sulfate and bicarbonate–CO_2 buffer at *p*H 7.6, the hydrolysis of DFP was distinctly accelerated in spite of the formation of a precipitate. Figure 2 demonstrates that 25 μmoles of $CuSO_4$ increased the initial rate of hydrolysis of 10 μmoles of DFP by a factor of almost 4.

2. Effect of Metal Complexes. (a) Copper.—It can be seen from Fig. 2 that the effect of 25 μmoles of $CuSO_4$ on the hydrolysis of 10 μmoles of DFP is almost the same as that of 50 μmoles of imidazole.

(4) Compare F. Lipmann, "Advances in Enzymology," **I**, 112 (1941); F. Lipmann and L. C. Tuttle, *J. Biol. Chem.*, **153**, 571 (1944).

(5) T. Winnick and E. M. Scott, *Arch. Biochem.*, **12**, 201 (1947).

(6) Using the manometric technique, the half-life of 10 μmoles of DFP in the presence of 50 μmoles $Na_2MoO_4 \cdot 2H_2O$ in a total volume of 2.2 ml. of $NaHCO_3$–CO_2 buffer at 30°, at *p*H 7.6, was found to be 48 minutes. In the absence of molybdate the $L_{1/2}$ of DFP is approximately 2 days.

(7) E. Bamann and M. Meisenheimer, *Ber.*, **71**, 1711, 1980 (1938); E. Bamann, *Angew. Chem.*, **52**, 186 (1939); *Chemiker-Zeitung*, **76**, 3 (1952); E. Bamann and E. Nowothy, *Chem. Ber.*, **81**, 451, 455, 463 (1948); E. Bamann, E. Nowothy and E. Heumueller, *Arch. Pharmazie*, **283**, 4 (1945).

(8) Experiments run in the absence of bicarbonate were difficult to interpret for lack of good reproducibility. An appreciable acid production was observed at *p*H 7.6 in the control experiment with $LaCl_3$ in the absence of DFP.

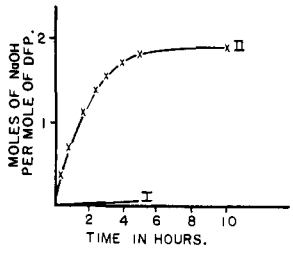


Fig. 1.—Hydrolysis of DFP in the presence and absence of sodium molybdate. Titration to constant *p*H with *N*/100 NaOH in the Beckman autotitrator: I, 57.5 μmoles of DFP in an initial volume of 25 ml water, *p*H 7.4, 25°, II. 57.5 μmoles of DFP + 287.5 μmoles of sodium molybdate in an initial volume of 25 ml., *p*H 7.4, 29°.

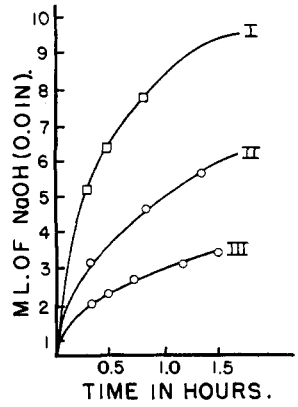


Fig. 3.—Hydrolysis of an aqueous mixture of 57.5 μmoles of DFP in the presence of an equimolar mixture of 287.5 μmoles of (I) ethylenediamine + $CuSO_4$, (II) diethylenetriamine + $CuSO_4$, (III) triethylenetetramine + $CuSO_4$. Initial volume = 25 ml., T = 27°, titration with 0.01 N NaOH to constant *p*H (7.4).

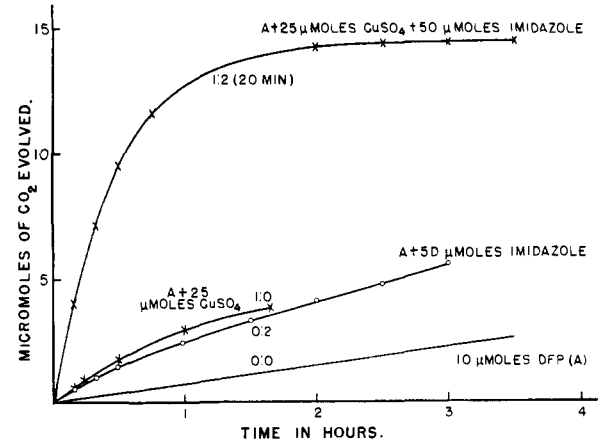


Fig. 2.—Hydrolysis of DFP (A) in the presence of copper sulfate, imidazole and a mixture of both substances in 2.2 ml. of sodium bicarbonate–CO_2 buffer at *p*H 7.6 and 38°; figures on curves = molar ratio of copper sulfate to imidazole; value in parentheses = "half-life."

By combination of the two catalysts a very strong enhancement of the rate of hydrolysis of DFP is obtained which exceeds, by far, the simple additive effect. The half-life of DFP under the conditions of the experiments whose results are shown in Fig. 2 is approximately 2 days. It was reduced to 20 minutes in the presence of the soluble, deeply blue-colored $CuSO_4$–imidazole complex of molar ratio 1:2.

Increase in the concentration of imidazole from a 1:2 ratio to a 1:4 ratio caused no further enhancement of the rate of hydrolysis. At a ratio of 1:1 the rate of hydrolysis was much less ($L_{1/2}$ = approx. 50 min.) than in the presence of the 1:2 or 1:4 mixture. From an aqueous mixture of copper sulfate and imidazole a deeply blue-colored complex, containing the components in a ratio of 1:3, could be isolated by precipitation with dioxane. Its activity as a catalyst for the hydrolysis of DFP, based on its copper content, was approximately the same ($L_{1/2}$ = 19 min.) as that of the 1:2 or the 1:4 mixture.

Almost the same half-life (20 to 22 minutes) as that shown above was obtained when 1-methylimidazole or 4-hydroxymethylimidazole was substituted for imidazole in the mixture of 1 mole of $CuSO_4$ with 2 moles of complexing agent.

A strong increase in the hydrolysis rate of 10 μmoles of DFP was also produced by the mixtures of 25 μmoles of $CuSO_4$ and 50 μmoles of various amino acids (see the Experimental part, Table III), which, by themselves, are without effect. No direct relationship between the catalytic activity and stability of copper–amino acid complex was found under the conditions of this study. Thus, catalytic activity falls in the sequence: sarcosine, arginine, β-alanine, glycine, glutamic acid, aspartic acid, lysine, alanine, serine, methionine, threonine and phenylalanine whereas the stability of 1:2 Cu(II)–amino acid complexes decreases in the order: glutamate, phenylalanine, threonine, serine, arginine and lysine.[9]

By raising the ratio of cupric ion to amino acid from 0.5:1 to 1:1 the catalytic activities of the less effective amino acid chelates, with the exception of that of methionine, were strongly increased. Threonine–$CuSO_4$ actually became the most potent catalyst in this series, with an activity that was almost as high as that of the corresponding imidazole–$CuSO_4$ mixture.

Inasmuch as the copper–imidazole complex is such an effective catalyst of the DFP-hydrolysis, it was of particular interest to study the behavior of the imidazole-containing amino acid histidine. A 1:2 molar mixture of copper(II) sulfate and histidine in bicarbonate did not accelerate the hydrolysis of DFP at *p*H 7.6 beyond the rate predicted from the sum of the individual effects of the metal salt and the amino acid. In this case, chelation of the metal obviously is divided unfavorably between the carboxyl, the amino and the imidazole groups. This assumption is supported by the fact that the catalytic effect of an equimolar mixture of copper sulfate, alanine and imidazole is much smaller than that of imidazole + $CuSO_4$ (1:1), or alanine + $CuSO_4$ (1:1) alone. Further, when the carboxy group is removed, as in histidinol, the 1:2 copper complex is an effective catalyst for DFP hydrolysis ($L_{1/2}$ = 22 min.).

With the addition of a second half equivalent of $CuSO_4$ to histidine, the catalytic activity increased tremendously and became even more effective than the equivalent $CuSO_4$–imidazole combination (see the Experimental part, Table IV).

Ethylenediamine (en) accelerates the hydrolysis of DFP at *p*H 7.6 to a much smaller extent than does imidazole.[10] However, by the addition of

(9) N. C. Li and E. Doody, THIS JOURNAL, **74**, 4184 (1952). The formation of 1:1 Cu(II)–amino acid chelates has been described recently by the same authors; *ibid.*, **75**, 221 (1953).

(10) Similarly 1,2-diaminopropane was found to enhance the hydrolysis of diethyl fluorophosphate only very slightly. This base has a much smaller effect than imidazole and was even less active than pyridine (*p*H 7.6).

copper sulfate, a catalytic action of comparable vigor to that of the copper–imidazole complex is obtained (Table VI). Mixtures of $CuSO_4$ and ethylenediamine with a molar ratio 1:1 and 2:1 are more effective catalysts for the hydrolysis of DFP than the 1:2 combination.[11]

The catalytic effect on the hydrolysis of DFP at a *p*H of 7.4 by the $CuSO_4$ chelates of ethylenediamine, diethylenetriamine and triethylenetetramine diminished in that order when the components were used in 1:1 molar ratios (Fig. 3). It has been reported that the stability of the Cu chelates increases in the same order.

Ethylenimine enhances the rate of hydrolysis of DFP to about the same slight extent as did imidazole. However, its combination with cupric sulfate did not give the same high catalytic activity that was obtained with the imidazole or the ethylenediamine–$CuSO_4$ chelates at the same molar concentrations. In the case of ethylenimine–$CuSO_4$ mixtures, the catalytic activity increased strongly with increasing amounts of the nitrogen base (Table VI) which probably indicates that the reactive complex is rather readily dissociable.

Mixtures of one mole of copper sulfate with 0.5 or one mole of ethylenediamine-tetracarboxylic acid (Versene) had a relatively small effect on the hydrolysis of DFP under standard conditions.[12] Since in the Cu(II)–Versene chelate the metal is very firmly bound to the sequestering agent, the factor of stability of the complex does not seem to be the decisive one in determining its catalytic activity.

A particularly effective catalyst for DFP hydrolysis is the combination of $CuSO_4$ and α,α'-dipyridyl-(2,2'-bipyridyl) in a 1:1 molar ratio. Increasing the molar ratio base/$CuSO_4$ causes a strong reduction in the catalytic activity of the complex. The copper complexes of phenanthroline, 4,4'-dimethyl-2,2'-bipyridyl and α,α',α''-tripyridyl were somewhat less effective in our experiments than the corresponding dipyridyl chelates (Table VII).

The most effective chelate catalysts that were found for the hydrolysis of DFP at *p*H 7.6 are listed in Table I, in increasing order of activity.[13] For comparison, it might be mentioned that the corresponding half-times for the phosphorylation of catechol and gallic acid with DFP under identical conditions are 29 and 7 minutes, respectively.

(11) The isolation of $Cu(en)SO_4$ and $Cu(en)_2SO_4$ in crystalline form is described in the Experimental part. Corresponding chlorides, $Cu(en)Cl_2$ and $Cu(en)_2Cl_2 \cdot H_2O$ have been reported by H. B. Jonassen and T. H. Dexter, THIS JOURNAL, **71**, 1553 (1949).

(12) The 1:1 copper sulfate–N,N-di-(β-hydroxyethyl)-glycine mixture was somewhat more active (half-life = 125 min. for 10 μmoles of DFP + 50 μmoles chelate in 2.2 ml. of bicarbonate–CO_2 buffer at *p*H 7.6 and 38°). The corresponding DFP half-lives in the presence of β-hydroxethyliminodiacetic acid + $CuSO_4$ (1:1) and tris-(hydroxymethyl)-aminomethane + $CuSO_4$ (1:1) were 57 and 35 minutes, respectively.

(13) Since not all of the complexes listed in Table VII were entirely soluble at the concentration used, and several had to be tested in the form of their suspensions, the order of their activity might change when tested at lower concentrations. It is further understood that the order given may only be true at *p*H 7.6 in bicarbonate buffer, which inhibits the chelate catalyzed hydrolysis of DFP (see Table X). The discovery of the inhibition by bicarbonate was made after a considerable part of the study had been completed. Thus, all half-lives which were reported earlier in the paper would probably be smaller in bicarbonate free solutions.

TABLE I

EFFICIENT COPPER(II) CHELATE CATALYSTS FOR THE DFP HYDROLYSIS AT 38° IN BICARBONATE–CO_2 BUFFER, *p*H 7.6 (4.5×10^{-3} molar DFP; 22.8×10^{-3} molar complexing agent + $CuSO_4$ (1:1))

Complexing agent	Half-life, min.	Complexing agent	Half-life, min.
(No catalyst)	(>2500)	Threonine	18
β-Alanine	29	Ethylenediamine	16
Glycine	27	Imidazole	14
Aspartic acid	26	*o*-Phenanthroline	14
Glutamic acid	25	4,4'-Dimethyl-2,2'-bipyridyl	9
Arginine	23	L-Histidine	8
Lysine	23	α,α'-Dipyridyl	4.5

α,α'-Dipyridyl + $CuSO_4$, the most effective chelate found, was compared with naturally occurring fluorophosphatase. Under the same experimental conditions as in Table VII, 50% of the DFP was hydrolyzed after 23 minutes in the presence of 1 ml. of horse serum, and after 28 minutes in the presence of 1 ml. of human plasma. One ml. of horse serum (approx. 90 mg. dry weight) had the same "fluorophosphatase" activity as 10 μmoles (3.16 mg.) of α,α'-dipyridyl–$CuSO_4$ (1:1). At *p*H 8, in the absence of $NaHCO_3$, 2–2.5 mg. of the chelate was necessary to correspond to the fluorophosphatic action of 1 ml. of fresh rabbit serum.

The α,α'-dipyridyl-$CuSO_4$ (1:1) chelate also accelerates the hydrolysis of tetraethyl pyrophosphate (TEPP). At 30°, the half-life of 10 μmoles of TEPP in 2.2 ml. of bicarbonate–CO_2 buffer, *p*H 7.6, was reduced from approximately 3 hours to 4 minutes in the presence of 50 μmoles of the chelate.

(b) **Chelates of Other Metals.**—Chelates of nickel(II) and cobalt(II) were very much less effective catalysts of the hydrolysis of DFP than those of copper(II). Iron(II) and manganous(II) chelates seem to be inactive.

3. Factors Influencing the Catalytic Activity of Copper Chelates and Possible Mechanism of Reaction.—With bidentate donor groups such as dipyridyl and ethylenediamine, we find that Cu(II)–dipyridyl and Cu(II)–en are more effective catalysts for the hydrolysis of DFP than Cu(II)–$dipyridyl_2$ and Cu(II)–en_2, respectively. The product of Cu(II) and a tetradentate ion such as ethylenediaminetetraacetic acid, in which the metal is completely surrounded by the chelating agent, is a very poor catalyst for the hydrolysis of DFP. Thus, it seems that maximum activity is found in those copper(II) chelates in which the chelating agent does not completely fill the sphere of the metal atom. In the case of unidentate donors such as imidazole, maximum activity is reached at the Cu(II):imidazole ratio of 1:2, and remains at that level with further addition of the heterocyclic base. This might be explained by the considerably decreased stability of the coördinate complexes having unidentate donors, so that the concentration of the incompletely saturated complex Cu(II)–$imidazole_2$ remains high even in the presence of 3 or 4 moles of imidazole per one mole of Cu(II).

In order to obtain some insight into the mechanism of catalytic action of the chelates of copper(II) on the hydrolysis of DFP, a limited study was made

on the properties of the 1:1 copper(II)–α,α'-dipyridyl chelate. It can be assumed that in 1:1 Cu(II) chelates, where only two of the four coördination positions are occupied by a bidentate chelating agent, the other two positions are filled by water molecules, *e.g.*, formula A. It was suggested to us by Messrs. F. M. Fowkes, L. B. Ryland and G. S. Ronay of the Shell Development Company, Emeryville, Cal., that the hydroxy aquo chelate (formula B) and the dihydroxy chelate (formula C) which are formed upon addition of alkali to (A) may also be involved as catalysts in the hydrolysis of DFP

$$\text{dipy Cu}^{++}(H_2O)_2 \underset{+H^+}{\overset{+OH^-}{\rightleftharpoons}}$$

A

$$\text{dipy Cu}^{+}(OH)(H_2O) \underset{+H^+}{\overset{+OH^-}{\rightleftharpoons}} \text{dipy Cu}(OH)_2$$

B C

The formation of B in alkaline solution seems to be indicated by a distinct inflection point in the titration curve of A when one equivalent of base has been added (Fig. 4).[14, 15] It appears possible that with increasing alkalinity a second hydroxyl may coördinate with the copper chelate to give C.

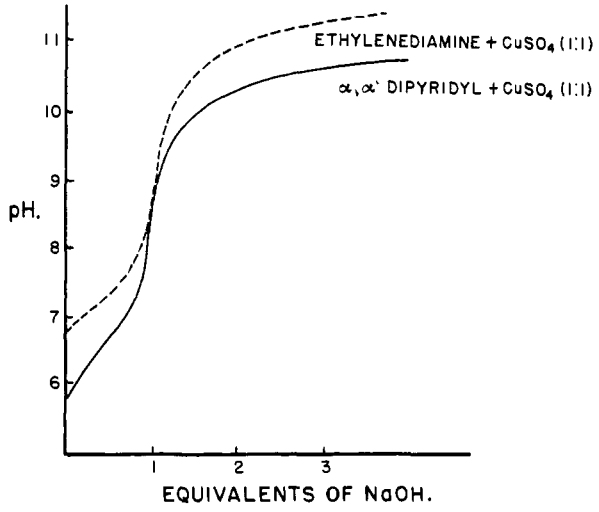


Fig. 4.—Titration curves of 1:1 molar mixtures of copper(II) sulfate/α,α'-dipyridyl and /ethylenediamine: 28.75 μmoles of the chelate in an initial volume of 25 ml. of 0.05 *M* KNO_3. Titration with 0.01 *N* NaOH at 27°. The $CuSO_4$–ethylenediamine mixture was turbid above *p*H 7.6.

We isolated species A and B in the form of their sulfates and acetates and compared their properties. The sulfate of A is of a lighter blue color than that of B, and in 0.1% aqueous solution gave a *p*H of 5.5, whereas under similar conditions the latter gave a *p*H of 8.0. The corresponding *p*H values for the acetates are 6.1 and 8.6. The two forms can be interconverted by suitable addition of acid and base.

(14) The titration curves for the 1:1 mixtures of $CuSO_4$ with *o*-phenanthroline and with 4,4'-dimethyl-2,2,-bi-pyridyl are of a similar type.

(15) Since the bicarbonate anion strongly inhibits the catalytic activity of copper-dipyridyl (see Table X) one might assume that in an $NaHCO_3$ solution the active chelate is converted in part to a catalytically less active or inactive form, *e.g.*, (dipyridyl) Cu+$(HCO_3)(H_2O)$.

The titration curve of a 1:1 $CuSO_4$–ethylenediamine mixture, which is included in Fig. 4, also indicates the formation of an hydroxy aquo chelate, which however is not stable. An attempt to isolate this species yielded copper(II) hydroxide and $Cu(en)_2SO_4$, evidently formed by disproportionation of the hydroxy aquo complex: $2(en)Cu^{+}(OH)(H_2O) = Cu(OH)_2 + (en)_2Cu^{++} + 2\,H_2O$.

The rate of DFP hydrolysis in the presence of the 1:1 copper–dipyridyl chelate was studied at various *p*H levels. In Fig. 5 the monomolecular hydrolysis constants are plotted against *p*H. It can be seen that the rate of hydrolysis of DFP in the presence of copper–dipyridyl (1:1) increases considerably between *p*H 6.5 and 7.5, very slightly up to *p*H 10; above *p*H 10 a very marked increase occurs.[16]

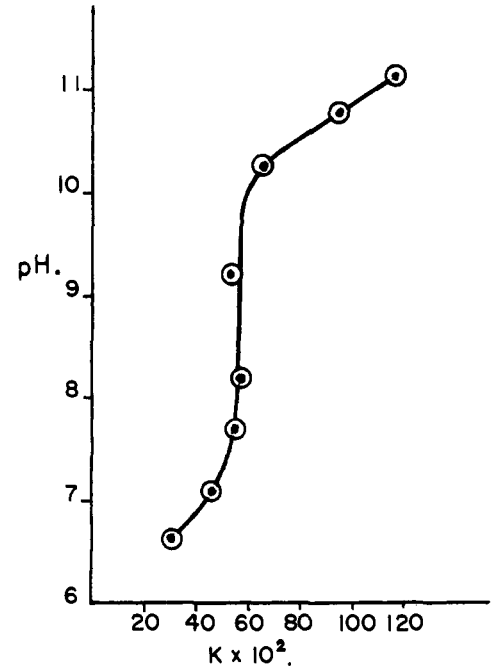


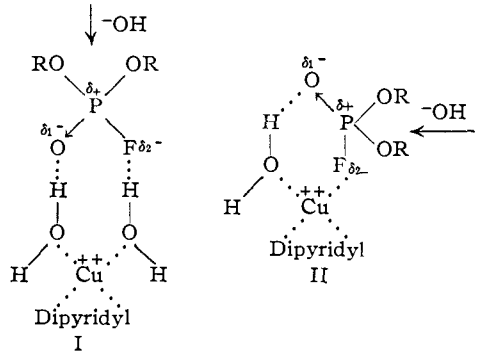
Fig. 5.—*p*H dependency of the monomolecular reaction rate constants for the hydrolysis of DFP in the presence of $CuSO_4$/α,α'-dipyridyl (1:1) at 30°. Figures are taken from Table II.

This behavior seemed to indicate that the acid form of the chelate (A) has a smaller activity than both the hydroxy aquo chelate (B), and the dihydroxy chelate (C). However, a quantitative study of the relations involved has shown that the true picture of the mechanism is probably more complex, due to a strong tendency of B to dimerize, yielding an inactive form. Thus, in the *p*H range below which the dihydroxy form (C) is present, catalytic activity appears to be proportional to the concentration of both the diaquo species (A) and the hydroxyl ion.[17]

(16) Hydrolysis of DFP in 10^{-3} *M* NaOH (*p*H 11), under the experimental conditions as in Table II, h, gave an $L1/2$ of 17 minutes. In the presence of 1.15×10^{-3} *M* dipyridyl–$CuSO_4$ (1:1), at a *p*H of 9, the $L_1/_2$ was 14 minutes. This demonstrates very clearly that in the presence of the chelate the same hydrolytic effect is produced at slightly alkaline *p*H that is found in strong alkali in the absence of chelate.

(17) Dr. Fowkes intends to report his study on the equilibria present in the chelate solution and the mechanism of reaction with DFP in a separate paper.

Intermediary addition complexes, *e.g.*, formulas I or II, etc., are suggested to explain the mechanism of the hydrolysis catalyzed by the chelate. The electrophilic Cu^{++} probably increases the polarization of the P=O and the P–F bonds and thereby facilitates the approach of a hydroxyl ion to the phosphorus atom, followed by the expulsion of the fluorine anion.[18] Since cupric salts themselves are catalytically active (Fig. 2), it is possible that the main action of the chelating agent is to keep the metal ion in solution at a *p*H above which it normally precipitates.



Experimental

A. **Substances Employed.**—The amino acids, imidazole, α,α'-dipyridyl and α,α',α''-tripyridyl were ordinary commercial preparations. 4,4'-Dimethyl-2,2'-bipyridyl was prepared according to the method of Case.[19] Purified globin and D-leucyl-L-tyrosine were purchased from Nutritional Biochemicals Corporation. The globin was dissolved in water, with the addition of acetic acid. Upon addition of alkali to *p*H 7.6, the protein gradually flocculated; this suspension was used for the manometric experiments.

Samples of histidinol, L-tyrosyl-L-tyrosine, L-prolyl-L-tyrosine, 73% β-metal-combining globulin (Squibb Fraction IV-7), vitamin B_{12} and vitamin B_{12a} were obtained through the courtesy of Dr. N. J. Steinman of the National Institutes of Health, Bethesda, Md., Dr. A. B. Barkdoll, Hockessin, Delaware, Dr. K. Pfister of Merck and Co., Inc., Rahway, N. J., Dr. D. M. Surgenor, Laboratory of Physical Chemistry of Harvard University, and Dr. K. Folkers of Merck and Co., Rahway, N. J., respectively.

B. **Measurement of the Rate of Hydrolysis of DFP.** (1) **Titration at Constant *p*H in the Absence of Buffers.**—A Beckman Model K Automatic Titrator which delivered CO_2-free NaOH solution was used for this purpose. In the majority of the experiments the anticipation was set at 1 or at 3. The reactions were run in a jacketed beaker controlling the temperature by means of a Bronwill Constant Temperature Circulator. To avoid uptake of CO_2 from the atmosphere, the reacting vessel was covered with cardboard and a slow stream of nitrogen passed over the solution. Passage of the inert gas may cause a slight loss of DFP by evaporation, so that slightly less than the theoretical quantity of sodium hydroxide sometimes was required for complete reaction (approx. 5–10%). Since comparison of the rates of reaction is based upon the time required for 50% hydrolysis, this error is of minor importance.

However in the determination of the half-life figures presented in Table II, at higher acidity and alkalinity considerable deviations from the amount of NaOH calculated for complete reaction were observed. At *p*H 6.5 with the low concentration (*l*) of DFP, and at *p*H 9 and 10 with the high concentration (*h*) of DFP, the deficit was 22, 27 and 21%, respectively. The reason for this phenomenon could not be ascertained. Since the plots of $\log a/(a-x)$ *vs.* time gave straight lines to about 80–90% hydrolysis the reaction appeared to be essential monomolecular. We believe that the $L_{1/2}$ values obtained give a fair picture of the *p*H-dependence of the reaction. However, a more refined study of the kinetics would have to be made to learn the details of the reaction mechanism.

TABLE II

DETERMINATION OF THE RATE OF HYDROLYSIS OF DFP AT VARIOUS *p*H IN THE PRESENCE OF $CuSO_4$–DIPYRIDYL (1:1)

	Monomolecular rate constants/min.[a]		Half-life, min. Calcd.[b]		Half-life, min. Obsd.[d]	
	l[c]	h[e]	l	h	l	h
6.5	0.0277		25		27	
7.0	.0443		15.7		16	
7.6	.0544		12.7		12.5	
8.0	.0558		12.4		12	
9.0	.0512	0.0514	13.6	13.5	12.5	14
10.0		.0598		11.6		12
10.5		.0895		7.8		7
10.9		.109		6.4		6

[a] Determined from hydrolysis curves. [b] Calculated from rate constants. [c] 28.7 μmoles of $CuSO_4$ and of dipyridyl + 57.5 μmoles of DFP in 25 ml. of 0.05 *M* KNO_3. Titration with 0.01 *N* NaOH at 30°. [d] In 0.05 *M* KCl solution identical half-life values (covering the *p*H range 6.5 to 8.0) were obtained. In pure water, the reaction rates were slightly lower. The latter difference is probably only an apparent one, and is most likely due to the slower response of the instrument (Beckman automatic titrator) in solutions of low ionic strength. [e] As under *c*, but 575 μmoles of DFP; titration with 0.1 *N* NaOH.

(2) **Manometric Measurements in Bicarbonate-CO_2 Buffer, and Preparation of Reaction Mixtures.**—Most of the experiments were performed at 38° and at *p*H 7.6, in a total volume of 2.2 ml. In general the concentration of DFP was 4.6×10^{-3} molar, that of the catalyst varied between 2.3×10^{-3} and 2.3×10^{-2} molar. Controls were run with the catalysts, in the absence of DFP. In general, they gave very small readings, which were subtracted from those of the corresponding experiments with DFP. The reproducibility of the half-life values reported was of the order of ±10%.

(a) **Cupric Sulfate.**—When the suspension obtained by addition of $CuSO_4$ to bicarbonate-CO_2 buffer, in the absence of complexing agent, was used as a catalyst for the hydrolysis of DFP, a steady evolution of CO_2 was observed for only the first 2 hours. After that, gas evolution generally stopped, and CO_2 absorption took place. Several hours later CO_2 evolution started again, but at a very reduced rate. The observed irregularities are not unexpected since it is known that precipitates of Cu(II) carbonate change their structure upon aging. When the mixture of $CuSO_4$ + bicarbonate-CO_2 buffer, was maintained at 38° for a period of 16 hours, the supernatant liquid became colorless, a white precipitate fell, and CO_2 absorption took place. The aged $CuSO_4$-bicarbonate-CO_2 mixture exerted no catalytic effect upon the hydrolysis of DFP. In order to explain this phenomenon we may assume that basic Cu(II)-bicarbonates or -carbonates are formed first which are slightly soluble in water and that only this soluble portion is responsible for the catalytic effect observed. On aging, the copper salts obtained are transformed into less soluble products which have no catalytic activity.

(b) **Imidazole + $CuSO_4$.**—One example is described in detail in order to demonstrate the manner in which the reaction mixtures were prepared. A 10.24-mg. sample (150 μmoles) of imidazole and 18 mg. (75 μmoles) of crystalline copper sulfate were dissolved in 3 ml. of distilled water. Sodium hydroxide solution (0.1 *N*) was added, with stirring, until a *p*H of 7.6 was reached. Sodium bicarbonate (0.75 ml. of a 0.2 *M* solution) was added, and the mixture diluted to 6 ml. with water. Two-ml. aliquots of the blue solution were pipetted into the main compartment of each of two Warburg flasks of 17–20 ml. capacity. The vessels were gassed on the manometers for 10 minutes with a stream of 5% carbon dioxide in nitrogen. At the end of this time, 0.2 ml. of a freshly prepared solution of 0.044 ml. DFP (10 μmoles) in 5 ml. of 0.025 *M* $NaHCO_3$ was added to the side-arm of the first vessel, and 0.2 ml. of 0.025 *M* $NaHCO_3$ was added to the side-arm of the second vessel which served as a control. The other operations were carried out in the usual manner.

(18) This formulation of the mechanism of reaction is similar to that given for the acceleration of the hydrolysis of amino acid esters by heavy metal ions, H. Kroll, THIS JOURNAL, **74**, 2036 (1952).

(19) F. H. Case, *ibid.*, **68**, 2574 (1946).

No visible change in solution color occurred when the bicarbonate buffered imidazole–copper sulfate mixture (2:1 molar) was kept at 38° for a period of 16 hours. Only a very small amount of a white precipitate formed during this period of incubation.

Figure 6 shows the influence of varying amounts of $CuSO_4$, with the concentration of imidazole maintained at 50 μmoles per 2.2 ml. The curves demonstrate that an increase in the quantity of copper sulfate strongly enhances the catalytic activity of imidazole until a molar ratio of 0.5:1 is reached. After that, little improvement is noted over the half-life at 0.5:1 of 18 min. The difference of 2 minutes between this value and that shown in Fig. 2 demonstrates the limits of accuracy of the method.

(c) **Amino Acids + $CuSO_4$.**—The influence of Cu(II)-amino acid chelates on the hydrolysis of DFP is demonstrated in Table III.

TABLE III

HALF-LIFE OF 10 μMOLES OF DFP IN THE PRESENCE OF MIXTURES OF AMINO ACIDS AND COPPER SULFATE

v = 2.2 ml.; t = 38°; sodium bicarbonate–CO_2 buffer, pH 7.6

Amino acid 50μ moles	Half-life, min. $CuSO_4$ 25 μmoles	50 μmoles
Sarcosine	21	21
L-Arginine	24	23
β-Alanine	26	29
Glycine	33	27
L-Glutamic acid	37	25
DL-Aspartic acid	41	26
L-Lysine	55	23
DL-Alanine	60	27
DL-Serine	70	37
DL-Methionine	103	100
DL-Threonine	115	18
DL-Phenylalanine	138	..

The effect of varying the concentration of the 1:1 histidine–$CuSO_4$ chelate can be seen in Table IV. By doubling the concentration of the catalyst from 12.5 μmoles to 50 μmoles, the rate of hydrolysis was doubled. Upon further increase, the linearity of the relationship fell off. This was not unexpected, since at the 50 μmole/2.2 ml. concentration the chelate was no longer completely soluble.

TABLE IV

HALF-LIFE OF 10 μMOLES DFP IN THE PRESENCE OF 1:1 MIXTURES OF $CuSO_4$ AND L-HISTIDINE AT pH 7.6 AND 38°

v = 2.2 ml.; bicarbonate–CO_2 buffer.

	Half-life, min.
12.5 μmoles $CuSO_4$-histidine (1:1)	22
25 μmoles $CuSO_4$-histidine (1:1)	11
50 μmoles $CuSO_4$-histidine (1:1)	8

(d) **Dipeptides + $CuSO_4$.**—As demonstrated in Table V, several dipeptide–$CuSO_4$ complexes were quite effective catalysts for the hydrolysis of DFP. However, none was found to approach the better amino acid–$CuSO_4$ complexes in efficiency.

TABLE V

HALF-LIFE OF 10 μMOLES OF DFP IN THE PRESENCE OF MIXTURES OF DIPEPTIDES AND COPPER SULFATE AT pH 7.6 AND 38°

v = 2.2 ml.; bicarbonate–CO_2 buffer

	Half-life, min. (approx. values)
25 μmoles D-leucyl-L-tyrosine + 25 μmoles $CuSO_4$	45
25 μmoles L-prolyl-L-tyrosine + 25 μmoles $CuSO_4$	50
25 μmoles N-glycyl-serine + 25 μmoles $CuSO_4$	50
10 μmoles L-tyrosyl-L-tyrosine + 10 μmoles $CuSO_4$	55

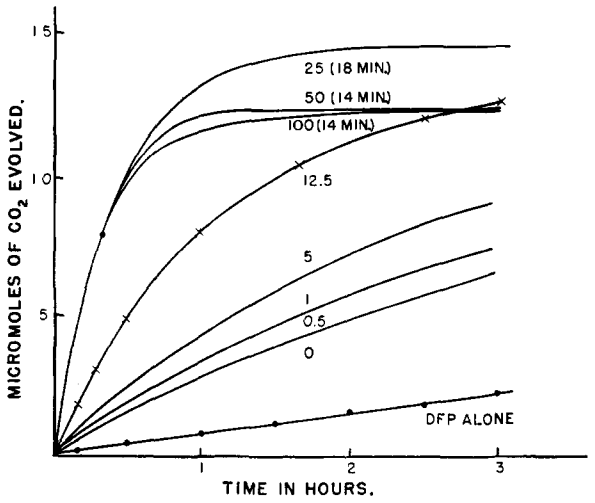


Fig. 6.—Hydrolysis of 10 μmoles of DFP in the presence of 50 μmoles imidazole and varying amounts of copper sulfate, in 2.2 ml. of bicarbonate–CO_2 buffer, at pH 7.6 and 38°; figures on curves = micromoles of $CuSO_4 \times 5H_2O$ contained in reaction mixture; values in parentheses are "half-lives."

(e) **Proteins + $CuSO_4$.**—It is known that the imidazole group of the histidine residues in proteins is important for the binding of metal ions.[20] Since globin is very rich in histidine, an appreciable catalytic effect by this protein in combination with cupric ions on the hydrolysis of DFP was to be expected and, indeed, was found. The half-life of DFP was 65 minutes in the presence of 20 mg. globin + 25 mg. $CuSO_4$, under those conditions listed in Table III. A much smaller effect was obtained with the combination of $CuSO_4$ and the β-metal-combining globulin.

(f) **Ethylenediamine + $CuSO_4$, Ethylenimine + $CuSO_4$.**—Where ethylenediamine and $CuSO_4$ were mixed in 1/1 molar ratio in the bicarbonate–CO_2 buffer, there were obtained a blue supernatant liquid and a considerable precipitate. After incubation at 38° for 72 hours, the supernatant had changed to violet color. The $L_{1/2}$ for DFP fell from 24 min. for the freshly prepared mixture to 55 min. for the incubated sample. It would appear probably that aging caused disproportionation of the 1/1 complex to copper hydroxide and the catalytically less active 1/2 complex. This assumption is supported by the analogous decomposition of the ethylenediamine hydroxy aquo Cu(II) chelate which has been reported in a preceding chapter. The clear, deeply violet-colored solution of 25 μmoles of $CuSO_4$ and 50 μmoles of ethylenediamine (anhydrous) in 2.2 ml. of bicarbonate–CO_2 buffer (pH 7.6) did not display the aging phenomenon.

TABLE VI

HYDROLYSIS OF DFP IN THE PRESENCE OF MIXTURES OF $CuSO_4$ WITH ETHYLENEDIAMINE AND WITH ETHYLENIMINE AT pH 7.6 AND 38°

v = 2.2 ml.; bicarbonate–CO_2 buffer

	Half-life, min.
A = 10 μmoles DFP	2500–3000
A + 25 μmoles $CuSO_4$ + 12.5 μmoles ethylenediamine	24
+ 25 μmoles $CuSO_4$ + 25 μmoles ethylenediamine	24
+ 25 μmoles $CuSO_4$ + 50 μmoles ethylenediamine	80
+ 50 μmoles $CuSO_4$ + 50 μmoles ethylenediamine	16
+ 25 μmoles $CuSO_4$ + 12.5 μmoles ethylenimine	120
+ 25 μmoles $CuSO_4$ + 25 μmoles ethylenimine	60
+ 25 μmoles $CuSO_4$ + 50 μmoles ethylenimine	33

(g) **Polypyridyls + $CuSO_4$.**—Aqueous solutions of the complex salts of α,α′-dipyridyl, α,α′,α″-tripyridyl and o-phenanthroline were obtained by dissolving the complexing agents in copper sulfate solution of the proper concentra-

(20) F. R. N. Gurd, J. T. Edsall, *et al.*, *Federation Proc.*, **11**, 224 (1952).

tion, with warming when necessary. Insoluble products did not form at the usual concentration in the presence of the bicarbonate–CO_2 buffer at *p*H 7.6. For the preparation of more concentrated chelate solutions, the suspension of the complexing agent in aqueous $CuSO_4$ had to be made alkaline by addition of 0.01 *N* NaOH. Before use, the *p*H was adjusted to 7.6 by addition of dilute acid, generally without precipitation of the chelate. A solution of 25 μmoles of α,α′-dipyridyl $CuSO_4$ (1:1) in 2.2 ml. of bicarbonate–CO_2 buffer (*p*H 7.6), kept at 38° for 16 hours, had substantially the same activity as unaged material (half-life of DFP, 7 and 8 minutes, respectively).

TABLE VII

HYDROLYSIS OF 10 μMOLES DFP IN THE PRESENCE OF THE COPPER COMPLEXES OF POLYPYRIDYLS AND *o*-PHENANTHROLINE

v = 2.2 ml.; *t* = 38°; $NaHCO_3$–CO_2 buffer, *p*H 7.6

Chelate	$L_{1/2}$, min.	$L_{1/2} \times C$
None	>2500	
10 μmoles $CuSO_4$ + 30 μmoles 2,2′-bipyridyl, (1:3)	95	
10 μmoles $CuSO_4$ + 20 μmoles 2,2′-bipyridyl, (1:2)	70	
5 μmoles $CuSO_4$ + 2,2′-bipyridyl, (1:1)	51	255
10 μmoles $CuSO_4$ + 2,2′-bipyridyl, (1:1)	23	230
25 μmoles $CuSO_4$ + 2,2′-bipyridyl, (1:1)	8	200
50 μmoles $CuSO_4$ + 2,2′-bipyridyl, (1:1)	4.5	225
50 μmoles $CuSO_4$ + 4,4′-dimethyl-2,2′-bipyridyl, (1:1)	9	
50 μmoles $CuSO_4$ + *o*-phenanthroline, (1:1)	14	
20 μmoles $CuSO_4$ + 10 μmoles α,α′,α″-tripyridyl, (2:1)	33	
30 μmoles $CuSO_4$ + 10 μmoles α,α′,α″-tripyridyl, (3:1)	38	

It may be noted that the product of the half-life ($L_{1/2}$) and the catalyst concentration (C) remains relatively constant over a moderately wide range of concentrations (average value = 227.5; ±10% deviations). This indicates that the rate of reaction under the conditions given is approximately directly proportional to the concentration of the catalyst. Variations in DFP concentration, with concentration of catalyst held constant, gave a slight increase in hydrolysis rate with higher DFP concentration (Table VIII).

TABLE VIII

HYDROLYSIS OF DFP AT VARIOUS CONCENTRATIONS IN THE PRESENCE OF 5 μMOLES $CuSO_4$ + α,α′-DIPYRIDYL (1:1)

v = 2.2 ml.; $NaHCO_3$–CO_2 buffer, *p*H 7.6; *t* = 38.5°

	$L_{1/2}$, min.
5 μmoles DFP	42
10 μmoles DFP	39
15 μmoles DFP	36
20 μmoles DFP	34

C. **CO_2-Retention Controls.**—It may be noted from the Figs. 2 and 6 that in the presence of imidazole somewhat less than the calculated final yields of 20 μmoles of CO_2 per 10 μmoles of hydrolyzed DFP were obtained. This is due to retention of CO_2 caused by the buffering capacity of the base. The effects of the concentration of imidazole and of ethylenediamine, upon the magnitude of CO_2 retained, are given in Table IX. The experiments were run in the same manner as described in an earlier paper.[1a]

TABLE IX

CO_2-RETENTION

A. CO_2 obtained from 10 μmoles of hydrolyzed DFP in 2.2 ml. of bicarbonate–CO_2 buffer at *p*H 7.6 and 38° in presence of listed chelate.

B. CO_2 obtained by the hydrolysis of DFP in the presence of listed chelate.

Chelate	CO_2, % A	CO_2, % B
Blank (no chelate)	100	..
50×10^{-6} mole imidazole + 25×10^{-6} mole $CuSO_4$	80	80[a]
50×10^{-6} mole imidazole + 100×10^{-6} mole $CuSO_4$	69	69[a]
100×10^{-6} mole imidazole + 25×10^{-4} mole $CuSO_4$	58	58[a]
25×10^{-6} mole ethylenediamine + 25×10^{-4} mole $CuSO_4$	92	90[b]
50×10^{-6} mole ethylenediamine + 25×10^{-6} mole $CuSO_4$	99	93

[a] These figures are calculated from the final volumes of the experiments in Figs. 2 and 6, and similar experiments, with consideration of the fact that the sample of DFP used was 91% pure. [b] These figures correspond to experiments reported in Table VI.

D. ***p*H-Dependency of the Hydrolysis of DFP in the Presence of $CuSO_4$-α,α′-Dipyridyl (1:1), in Bicarbonate–CO_2 Buffer.**[21]—In the hydrolysis experiments reported in Table X two moles of CO_2 was obtained per mole of DFP in bicarbonate buffer in the presence of $CuSO_4$–dipyridyl (1:1) at *p*H 7.2 and 8.0, however only 1.5 moles at *p*H 6.6. The same phenomenon was observed when the DFP was replaced by previously hydrolyzed DFP.

TABLE X

Concn. of $NaHCO_3$, *M*	2.6×10^{-3}	11×10^{-3}	66×10^{-3}
*p*H	6.6	7.2	8.0
$L_{1/2}$	....	87 min.[a]	5 hr.[a]
	27 min.[b]	80 min.[b]	5 hr.[b]
	....	21 min.[c]	87 min.[c]

[a] 2.5 μmoles of $CuSO_4$–dipyridyl (1:1) + 5 μmoles DFP in 2.2 ml. $NaHCO_3$–CO_2 buffer. [b] 10 μmoles of $CuSO_4$–dipyridyl (1:1) + 57 μmoles DFP in 6.6 ml. $NaHCO_3$–CO_2 buffer. [c] 10 μmoles of $CuSO_4$–dipyridyl (1:1) + 5 μmoles DFP in 2.2 ml. $NaHCO_3$–CO_2 buffer.

E. **Experiments with Chelates of Metals Other than Copper.**—Under the conditions stated below Fig. 2, the catalytic activity of cobaltous chloride was only doubled by the addition of imidazole. Inasmuch as the activity of the cobalt salt itself is lower than that of the copper salts, its complex is of little interest as a catalyst for the hydrolysis of DFP. A combination of manganous sulfate and imidazole in 1:2 molar ratio was no more effective than the base alone.

The amino acids failed to increase the activity of cobaltous or manganous ions. Thus, an equimolar mixture of cobaltous chloride and serine (25 μmoles in 2.2 ml.) was about as active as $CoCl_2$ alone, and a mixture of manganous sulfate with glutamic acid or lysine (50 μmoles each in 2.2 ml.) was entirely without effect. Manganous chloride, similarly failed to accelerate the hydrolysis of DFP in the presence of histidine or globin.

The Ni(II)- or Co(II)-α,α′-dipyridyl chelates were very much less effective catalysts than the Cu(II) chelate. The complexes of *o*-phenanthroline with $CoCl_2$ or $NiSO_4$ were no more active than the metal salts themselves. The α,α′-dipyridyl-Fe(II), complex had no catalytic effect whatsoever on the hydrolysis of DFP. Vitamin B_{12} and B_{12a} (cyano and hydroxo cobalamin) also were ineffective.

All the experiments reported in this section were made in bicarbonate–CO_2 buffer at *p*H 7.6.

F. **Isolation of Cu(II) Complex Salts in Solid Form.** 1. **Copper Salt of DL-Serine.**—This was prepared from copper oxide and DL-serine.[22] The blue crystals obtained decomposed at 205° (uncor.), with formation of red brown Cu_2O. *Anal.* Calcd. for $Cu(C_3H_6O_3N)_2$ (271.8): N, 10.4; Cu, 23.5. Found: N, 10.5; Cu, 22.9.

The isolated complex salt had the same catalytic activity for the hydrolysis of DFP as the corresponding mixture of copper sulfate and serine.

2. **Imidazole-Copper Sulfate (3:1).**—Acetone (5 ml.) was added to a solution of 192 mg. (2.8 mmoles) of imidazole and 350 mg. (1.4 mmoles) of $CuSO_4 \cdot 5H_2O$ in 3 ml. of H_2O. The dark blue oil which separated on storage of the solution in a refrigerator was mixed with 4 ml. of ethanol and 3 ml. of water. After centrifugation of the small quantity of solid which had formed, the clear supernatant liquid was precipitated with excess acetone yielding a dark blue solid. The solid was triturated with 3 ml. of water and the mixture centrifuged. The solid fraction which separated was blue but of lighter color. The water layer was precipitated with excess acetone as before and the trituration and precipitation process repeated once more. Finally, the substance was dissolved in 2 ml. of water, and 2 ml. of dioxane was added. Upon storage in the refrigerator, large dark blue crystals

(21) Manometric determination of CO_2 evolved in the Warburg apparatus.

(22) E. Fischer and W. A. Jacobs, *Ber.*, **39**, 2947 (1906); E. Abderhalden and E. E. Schnitzler, *Z. physiol. Chem.*, **163**, 99 (1927).

separated, which had the appearance of crystallized copper sulfate. The substance melted at 280°, with formation of a dark olive green melt. For analysis, the crystals were dried over P_2O_5 in a vacuum.

Anal. Calcd. for $CuSO_4 \cdot 3C_3H_4N_2 \cdot H_2O$ (381.8): N, 22.0; S, 8.4. Found: N, 21.7; S, 8.5.

3. **Ethylenediamine-Copper Sulfates.** (a) 1:1 **Chelate.**—Anhydrous ethylenediamine (3.33 ml. = 0.05 mole) and 6.25 g. of $CuSO_4 \cdot 5H_2O$ (0.025 mole) were dissolved in a small amount of water. A few ml. of alcohol and dioxane were added, and the solution was kept in a refrigerator. Deeply violet-colored crystals appeared and were separated by decantation. Treatment of these crystals with a small amount of water, followed by filtration, gave a solution which yielded dark violet crystals on addition of dioxane and cooling in the refrigerator. The product was recrystallized from water-dioxane. The crystals obtained became light blue after drying. They did not melt when heated to 295°. Analysis showed that the product obtained was a mixture of approximately 9 parts of the 1:1 chelate and 1 part of the 1:2 chelate.

Anal. Calcd. for: $CuSO_4 \cdot (H_2NCH_2CH_2NH_2)_{1.1} \cdot 1.5H_2O$ (252.6): N, 12.7; S, 12.2; Cu, 25.2; C, 10.5; H, 4.7. Found: N, 12.6; S, 12.3; Cu, 25.2; C, 11.2; H, 4.6.

(b) 1:2 **Chelate.**—From the mother liquor of the 1:1 chelate the 1:2 chelate could be isolated as dark violet colored crystals which also failed to melt when heated up to 295°.

Anal. Calcd. for $CuSO_4 \cdot (H_2NCH_2CH_2NH_2)_2$ (279.7): C, 17.2; H, 5.8; N, 20.5; Cu, 22.8; S, 11.5. Found: C, 17.2; H, 6.0; N, 19.4; Cu, 23.15; S, 11.5.

4. **α,α′-Dipyridyl-Copper(II) 1:1 Chelates. I. Sulfates.** (A) **Acid Form (Diaqua Chelate).**[23]—Three and a half grams (0.014 mole) of $CuSO_4 \cdot 5H_2O$ in 25 ml. of water was added to 2.0 g. (0.013 mole) of dipyridyl and the flask was shaken vigorously. The resulting light blue precipitate was filtered off and washed with water. When recrystallized from boiling water needle-like prisms were obtained. When examined with the polarized light microscope they showed parallel extinction. The crystals did not melt but charred when heated to temperatures of between 250 and 300°. A solution of 22 mg. in 20 ml. of water had a *p*H of 5.4. For analysis the crystals were dried over P_2O_5 *in vacuo*.

Anal. Calcd. for $C_{10}H_8N_2 \cdot CuSO_4 \cdot 2H_2O$ (351.8): Cu, 18.1; S, 9.1; C, 34.3; H, 3.43; N, 8.1. Found: Cu, 18.1; S, 9.4; C, 34.1; H, 3.3; N, 7.8.

(B) **Alkaline Form (Hydroxo Aqua Chelate).**—To an aqueous suspension of 1 g. (0.0028 mole) of the complex prepared above there was added 27.3 ml. of 0.0104 *N* NaOH. The solid dissolved and a dark blue solution resulted. The filtered solution was concentrated by vacuum evaporation. Recrystallization of the blue solid from boiling water gave large, well-formed, blue, rhombic crystals. They were dark between crossed prisms of the polarizing microscope when their diagonals were parallel to the crosshairs of the eyepiece (symmetrical extinction). A solution of 22 mg. in 20 ml. of water had a *p*H of 8.0. The crystals lost water when heated in a sealed capillary, decomposing at 225°, and were insoluble in organic solvents. For analysis they were dried over P_2O_5 *in vacuo*.

Anal. Calcd. for $(C_{10}H_8N_2)_2(CuOH)_2SO_4 \cdot 2H_2O$ (605.4): Cu, 21.0; S, 5.3; C, 39.6; H, 3.7; N, 9.3. Found: Cu, 21.2; S, 5.6; C, 39.9[24]; H, 3.8; N, 9.3.

Bubbling CO_2 into a warm saturated solution of the alkaline complex gave the blue needle-like prisms of the acid complex described in (A) above.

II. **Acetates.** (A) **Acid Form.**—A solution of 2.53 g. (0.0127 mole) of $Cu(C_2H_3O_2)_2 \cdot H_2O$ in 25 ml. of water was added to 2.0 g. (0.0127 mole) of dipyridyl. A violet solution resulted which was evaporated to dryness *in vacuo* and the solid recrystallized from water plus a large volume of acetone. The dark violet needle-like prisms which were obtained showed parallel extinction when examined between crossed prisms of a polarizing microscope. When dried in air the crystals shrunk and changed to light blue in color. They decomposed at 196–201°, were very soluble in water or ethanol and only slightly in acetone. The 0.1% aqueous solutions had a *p*H of 6.1.

For analysis the substance was dried at 100° *in vacuo*.

Anal. Calcd. for $C_{10}H_8N_2 \cdot Cu(C_2H_3O_2)_2$ (337.7): Cu, 18.9; C, 49.7; H, 4.2; N, 8.3. Found: Cu, 19.2; C, 49.3; H, 4.5; N, 7.9.

B. **Alkaline Form.**—To 2.0 g. (0.0056 mole) of the above complex there was added 54 ml. of 0.104 *N* NaOH. Acetone was added to the dark blue aqueous solution. Upon cooling a crop of small, poorly-formed blue crystals was obtained. The 0.1% aqueous solution had a *p*H of 8.6. When dried over P_2O_5 *in vacuo* the crystals became bluish green.

Anal. Calcd. for $C_{10}H_8N_2 \cdot Cu(C_2H_3O_2) \cdot OH \cdot H_2O$ (313.8): Cu, 20.3; C, 45.9; H, 4.5; N, 8.9. Found: Cu, 21.0; C, 46.2; H, 4.3; N, 8.6.

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(23) The isolation of this product had been described before by F. Blau, *Monatsh.*, **19**, 647 (1898).

(24) Combustion in the presence of V_2O_5.

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[CONTRIBUTION FROM THE RESEARCH LABORATORIES, VICTOR CHEMICAL WORKS]

A New Method of Preparation for Alkenylphosphonous Dichlorides and Alkenylthionophosphonic Dichlorides

BY E. N. WALSH, T. M. BECK AND W. H. WOODSTOCK

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The reaction products of phosphorus pentachloride with certain olefins are reduced by elemental phosphorus only in the presence of iodine or iodine-liberating compounds to form the corresponding alkenylphosphonous dichlorides. The latter add sulfur to form the alkenylthionophosphonic dichlorides. Iodine also will catalyze the known reaction of olefin–phosphorus pentachloride addition compounds with phosphorus pentasulfide with a fivefold increase in yield of alkenylthionophosphonic dichlorides.

Introduction

Phosphorus pentachloride is known to add to certain olefins,[1,2] to form the thermally unstable chloroalkylphosphorus tetrachlorides. Recently one of the authors[3] described the reaction of such products with phosphorus pentasulfide to form alkenylthionophosphonic dichlorides, but the yields were always below 20%. Subsequent research has been undertaken to find an improved synthesis for alkenylthionophosphonic dichlorides and has resulted in two new preparative methods.

The first method entails a catalyzed reduction of the olefin–phosphorus pentachloride reaction products with elemental phosphorus to form the

(1) J. E. Marsh and J. A. Gardner, *J. Chem. Soc.*, **59**, 648 (1891).

(2) E. Bergmann and A. Bondi, *Ber.*, **63**, 1158 (1930); **64**, 1455 (1931); **66**, 278, 286 (1933).

(3) W. H. Woodstock, U. S. Patent 2,471,472.