immediately precipitated by the addition of water. (Longer standing in solution caused hydrolysis.) The white precipitate was washed until neutral and recrystallized twice from ethanol to give very pale yellow needles (pale yellow fluorescence) of N-hydroxyphthalimide.

C.—While chromatographic purification of the yellow form of N-hydroxyphthalimide was unsuccessful, this method was effective with the less polar N-acetoxyphthalimide. The acetates from both forms are colorless,[12] but the acetate from yellow N-hydroxyphthalimide has a strong bluish-white ultraviolet-excited fluorescence, while that from the colorless form does not fluoresce. When the acetate from the yellow form was chromatographed on silicic acid–Celite (3:1), it became non-fluorescent; a bluish-white fluorescent band remained on the column, but nothing could be isolated from this band.

D.—Aqueous ammonia was added to a solution of yellow N-hydroxyphthalimide in hot 95% ethanol. The precipitated ammonium salt was separated, dissolved in a minimum amount of water, and ethanol was added. On cooling the solution, a crystalline salt formed; it was separated and the recrystallization repeated. The orange crystals were then dissolved in a minimum amount of cold water and the solution acidified with dilute hydrochloric acid. The precipitate was washed to remove acid and recrystallized from ethanol to give colorless, non-fluorescent needles.

N-Benzyloxyphthalimide.—This derivative, hitherto incompletely characterized,[5] was prepared from both the colorless and yellow forms of N-hydroxyphthalimide by treatment of the silver salts with benzyl chloride in dilute ether solution at room temperature for one week.[2] The products which separated upon concentration of the ether filtrate were fractionally crystallized to separate the mixture of regenerated N-hydroxyphthalimide and the desired benzyl derivative. The N-hydroxyphthalimide samples recovered from the two reactions were colorless and yellow, respectively. The yield of benzyl derivative in each case was 12%; the melting points separately were 143.0–144.5° in each case, on mixing, 143.0–144.0°.

Anal. Calcd. for $C_{15}H_{11}O_3N$: C, 71.13; H, 4.38; N, 5.53. Found: C, 71.03, 70.88; H, 4.51, 4.45; N, 5.15, 5.09. (The two figures in each instance refer to the product derived from colorless and from yellow N-hydroxyphthalimide, respectively.)

Preparation of Model Compounds.—Phthalanil was prepared by heating phthalanilic acid above its melting point. Isophthalanil was prepared by acetyl chloride dehydration of phthalanilic acid.[13] We were unable, following the procedure of Hoogewerff and van Dorp,[14] to prepare N-methylisophthalimide, desired as a second reference compound.

The infrared spectra of N-hydroxy-, N-methoxy- and N-benzyloxyphthalimide closely resemble the spectra of the normal phthalimides and are characterized by a broad and very intense absorption at about 1735 cm.$^{-1}$ and a sharp band of moderate intensity in the vicinity of 1790 cm.$^{-1}$. Isophthalanil has similar absorptions in this region, but they appear with approximately equal intensity and width.

(12) The pale yellow acetate from the yellow form, described by Orndorff and Pratt,[3] could not be obtained; presumably the yellow color was due to incomplete acetylation.

(13) M. L. Sherrill, F. L. Schaeffer and E. P. Shoyer, THIS JOURNAL, **50**, 474 (1928).

(14) S. Hoogewerff and W. A. van Dorp, *Rec. trav. chim.*, **13**, 98 (1895).

CHICAGO, ILLINOIS

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, THE FLORIDA STATE UNIVERSITY]

Cuprous Ion Formation in Cupric Ion Catalyzed Oxidations[1,2]

BY ROBERT FLITMAN AND EARL FRIEDEN

RECEIVED MARCH 28, 1957

The specific cuprous ion complexing reagent, cuproin, was used to explore the extent and mechanism of some cupric ion catalyzed oxidations. The cupric ion catalyzed oxidation of ascorbic acid is inhibited by cuproin (2,2′-biquinoline). Sulfhydryl compounds, some phenols and certain reducing agents produced cuprous ions under the described experimental conditions. Kinetic studies of the reduction of cupric ion by hydroquinone reveal that the reaction is bimolecular over-all and monomolecular with respect to cupric ion. This suggests a free radical intermediate for the oxidized compound. No simple order is observed for the interaction between cupric ion and *p*-hydroxyanisole or cysteine.

Copper ion catalyzed oxidation reactions afford an outstanding example of a model system for studying enzymatic oxidations. Copper enzymes have been considered in several recent reviews[3,4] and included in a recent American Chemical Society Symposium.[5]

It has been suggested that cupric ion catalyzed oxidations proceed through a generalized two-step mechanism. For the extensively studied oxidation of ascorbic acid, the following steps have been inferred[6–8]

(1) ascorbate + $2Cu^{++} \longrightarrow$ dehydroascorbate + $2Cu^{+} + 2H^{+}$

(2) (a) $2Cu^{+} + 2H^{+} + {}^1/_2O_2 \longrightarrow 2Cu^{++} + H_2O$

(b) $2Cu^{+} + 2H^{+} + O_2 \longrightarrow 2Cu^{++} + H_2O_2$

In this system copper functions catalytically by transporting electrons, in the Cu^{I} state from the oxidizable substrate to oxygen. Both alternatives (a) and (b), are observed for reaction (2); reaction type (a) is observed in the enzymatic reaction involving ascorbic acid oxidase.[3] Reaction type (b) is observed for the cupric ion catalyzed oxidation.[3,4]

Evidence for the existence of the Cu^{I} intermediate was first presented by Barron, *et al.*,[9] who demonstrated the inhibition of ascorbic acid oxidation

(1) Presented in part at the Meeting-in-miniature of the Florida Section of the American Chemical Society, Tallahassee, May, 1956, and in part at the Miami Meeting of the American Chemical Society, April 10, 1957.

(2) This work was supported by a research grant A-146 to E. Frieden from the National Institute of Arthritis and Metabolic Diseases, Public Health Service. The authors are grateful to G. Edwin Lewis and Dr. Ernest Grunwald for many interesting discussions of the problem.

(3) W. McElroy and B. Glass, "Copper Metabolism," The Johns Hopkins Press, Baltimore, Md., 1950.

(4) T. P. Singer and E. B. Kearney, "The Proteins," Vol. IIA, 1954, pp. 135–159.

(5) O. Hayaishi, *et al.*, Symposium on Enzymatic Activation of Molecular Oxygen, 53C–58C, abstracts of papers presented at Atlantic City, N. J., September 16–21, 1956.

(6) A. O. Dekker and R. G. Dickinson, THIS JOURNAL, **62**, 2165 (1940).

(7) A. Weissburger and J. LuValle, *ibid.*, **65**, 1934 (1943).

(8) H. Nord, *Acta Chem. Scand.*, **2**, 442 (1955).

(9) E. S. G. Barron, R. H. DeMeio and F. Klemperer, *J. Biol. Chem.*, **112**, 625 (1935).

by CO, since CO forms a stable carbonyl with Cu^{I} but not with Cu^{II}. The copper oxidases may function in an analogous manner.[10] The CO inhibition of tyrosinase has been demonstrated[11] and the admonition of Warburg that "a respiration partly non-light sensitive (to CO) would indicate that copper proteids are involved,"[12] carries with it the implication of a Cu^{I}–protein intermediate. The likelihood of the $Cu^{II} \rightarrow Cu^{I}$ cycle is also indicated by recent kinetic studies on the effect of specific cuprous and cupric reagents on copper enzymes.[13] For tyrosinase, the specific cupric ion reagent, biscyclohexanoneoxalyldihydrazone, competed directly with the substrate catechol, while the specific cuprous ion reagent, 2,9-dimethyl-1,10-phenanthroline, inhibited the enzyme uncompetitively. Klotz and Klotz[14] found evidence for analogous changes in state of the oxygen-carrying copper protein, hemocyanin.

The ability of cuproin (2,2′-biquinoline) and other cuprous ion reagents to form a colored complex specifically with Cu^{I}[15] provides a fresh experimental approach to the study of these reactions from two points of view. The rate of Cu^{I} formation can be followed spectrophotometrically by the estimation of the concentration of the Cu^{I}–cuproin complex as a function of time. Also cuproin can be used to inhibit Cu^{II}-catalyzed autoöxidations, thus providing further evidence for the Cu^{I} intermediate.

Experimental

The insolubility of cuproin in water necessitated the use of aqueous organic solvents. Ethanol–water and acetone–water mixtures provided an effective compromise between the desire for cuproin solubility and for a substantially polar medium as highly aqueous as possible. For most of these studies, a 70% aqueous acetone solvent was chosen. No reaction between Cu^{II} and acetone or alcohol to form Cu^{I} was detected under our experimental conditions.

The cuproin inhibition of ascorbate autooxidation was demonstrated in two ways. First, by following the disappearance of ascorbic acid in the Beckman DU spectrophotometer similar to the method of Racker,[16] and also by measuring the rate of oxygen uptake using conventional Warburg techniques. In both cases the temperature was held at 30.0 ± 0° and 0.0100 molar phosphate or tris buffer (trishydroxymethylaminomethane), *p*H 7.2, was used as buffer. Other details of this procedure recently have been described.[17]

A spectrophotometric method was used to determine the rate at which Cu^{I} was generated *in situ*. The Cu^{I}–cuproin colored complex rigorously follows the Beer–Lambert law at 545 mμ at the concentrations used. The absorbancy of the Cu^{I}–cuproin complex was determined at various times during the reaction and the value obtained related to the Cu^{I} concentration.[18]

No indication of the air reoxidation of Cu^{I} to Cu^{II} prior to formation of the Cu^{I}-cuproin complex was noted. Quantitative conversion of Cu^{II} to Cu^{I} was observed for reductants such as ascorbate and catechol. In most instances the reaction was started by adding 1 ml. of the substrate in 70% aqueous acetone to 2 ml. of the same solvent containing buffer, cuproin and Cu^{II} in the cell. Other details are included in the figure legends.

Twice-distilled water, acetone and ethanol were used throughout. The cuproin was purchased from the Eastman Kodak Co. Standard cupric chloride solutions were prepared by dissolving cupric oxide (analytical reagent grade, Mallinckrodt Chemical Works) in concentrated hydrochloric acid and fuming to remove excess acid. All other chemicals employed were either C.P. or the best available grade from the Nutritional Biochemical Company, Cleveland, Ohio, or the Eastman Kodak Co.

Results and Discussion

Cuproin Inhibition of Copper Catalysis.—The inhibitory effect of cuproin on the copper catalysis of ascorbate autoöxidation is compared in Fig. 1

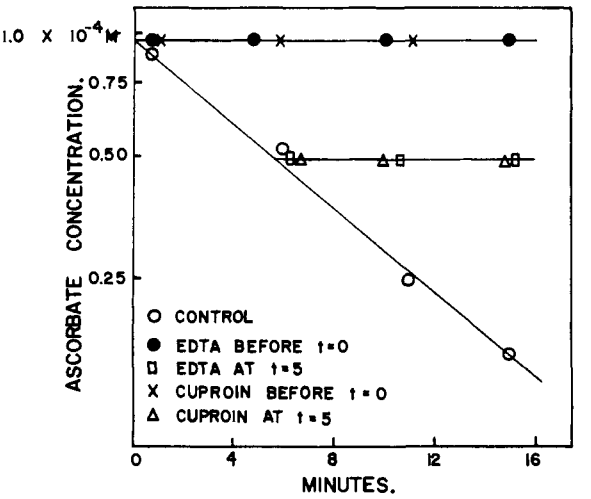


Fig. 1.—Effect of cuproin (1.00×10^{-6} *M*) and EDTA (1.00×10^{-5} *M*) added at the indicated times. The reaction mixture consisted of a 50% ethanol–water solution of 5.00×10^{-6} *M* Cu^{II}, 0.010 *M* tris buffer, *p*H 7.2, and 1.00×10^{-4} *M* ascorbate at 30.0°. Ascorbate concentration was determined in a Beckman spectrophotometer at 265 mμ.

with the inhibitory effect of ethylenediaminetetraacetate (EDTA). The latter substance is considered to be a general chelator of copper ions and other metal ions, whereas the former inhibits by a specific chelation of the cuprous ion intermediate. Under the conditions of the experiment shown in Fig. 1 the rate at which ascorbic acid disappears is pseudo-first order; hence the linearity of the logarithmic plot. The effect of adding the inhibitors after the reaction was started is the same in either case and indicates the ability of the complexer to disrupt any copper ion–ascorbate complex. From the data in Fig. 1, the amount of cuprous ion formed in the presence of cuproin was insignificant compared to the total amount of ascorbate oxidized.

Cuproin inhibition of the cupric ion catalyzed oxidation of ascorbate also was found using the Warburg technique. In a solvent containing 50% alcohol, equimolar concentrations of cuproin appeared to afford complete inhibition of the cupric ion catalyzed oxidation of ascorbate. The effect of varying cuproin concentration was studied in this system and unexpected concentration effects were obtained. Similar unusual results were observed in aqueous solutions using the water-soluble Cu^{I} reagent, 2,9-dimethyl-1,10-phenanthroline.

(10) M. Joselow and C. R. Dawson, *J. Biol. Chem.*, **191**, 11 (1951).
(11) F. Kubowitz, *Biochem. Z.*, **292**, 221 (1937).
(12) O. Warburg, "Heavy Metal Prosthetic Groups and Enzyme Action," Oxford University Press, London, 1949, p. 181.
(13) E. Frieden, G. Ezell and Y. Karkhanis, *Fed. Proc.*, **16**, 183 (1957).
(14) I. M. Klotz and T. A. Klotz, *Science*, **121**, 477 (1955).
(15) J. Hoste, *Anal. Chem. Acta*, **4**, 23 (1950).
(16) E. Racker, *Biochem. Biophys. Acta*, **9**, 577 (1952).
(17) E. Frieden and B. Naile, *Arch. Bioch. Biophys.*, **48**, 448 (1954).
(18) R. A. Guest, *Anal. Chem.*, **25**, 1484 (1953).

Because of their bizarre nature, the effects of these Cu^{I} reagents are being explored in some detail and will be reported on separately.[19] The interesting possibility of the use of cuproin and similar reagents as a specific Cu^{I} inhibitor in systems in which catalytic or autoöxidations are proceeding in the presence of a variety of metal ions needs only to be mentioned at this point.

Specificity of Cupric Ion Reduction.—It was possible to devise a convenient test method for a variety of organic compounds as to their ability to reduce Cu^{II}. An excess of the test compound was dissolved in 5 ml. of 70% aqueous acetone and the reaction was started by adding 10 ml. of all the other reactants. The cuprous–cuproin complex was then measured at the indicated times after the reaction was started. These data are summarized in Table I. It is emphasized that the quantitative aspects of these data are significant only as far as comparative rates of cuprous–cuproin complex formation under these precise conditions are concerned.

The fact that many organic compounds possess the ability to complex one or both of the ionic species of copper must also be taken into account in assessing the data in Table I. Theoretically, it should be possible to achieve a ratio of Cu^{I} to total Cu ion of unity. However, several sulfhydryl compounds such as glutathione, 5-iodo-2-thiouracil and cysteine do not yield apparent ratios of one. This might be due to copper ion complexing or the fact that some of these compounds are already partially oxidized even in the solid state.

As indicated in Table I and Fig. 2, numerous sulfhydryl compounds, phenols and polyphenols and

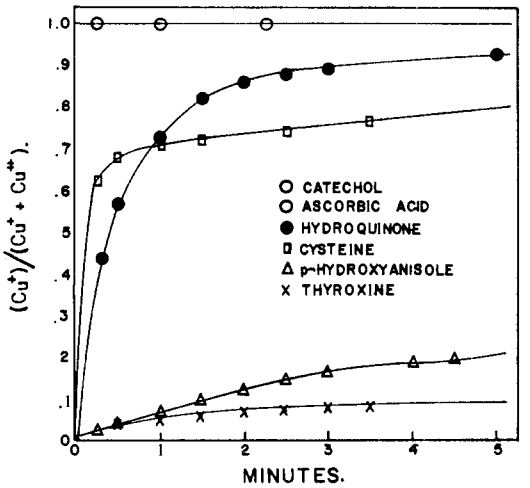


Fig. 2.—Relative rates of cuprous ion formation for several different organic compounds. The formation of the cuprous cuproin complex was measured at 545 mμ. The reaction was initiated by the addition of the test compound (final concentration 1.00×10^{-4} *M*). The reaction mixture contained 0.010 *M* tris buffer, *p*H 7.2, 5.00×10^{-5} *M* Cu^{II}, 1.00×10^{-4} *M* cuproin at 30.0° and 70% acetone.

other oxidizable compounds readily form Cu^{I} under the described test conditions. Several other compounds are included in subsequent parts of this paper. Negative results were obtained with the compounds listed at the bottom of Table I. Obviously, not all phenols are readily sensitive to copper-catalyzed oxidations and many common so-called "reducing" agents do not produce detectable cuprous ion. It is conceivable that this simple test permitting the convenient detection of a certain category of organic and inorganic reducing agents might be useful in qualitative analytical schemes.

TABLE I

FORMATION OF CUPROUS IONS BY CERTAIN ORGANIC COMPOUNDS

The solvent system used was 70% aqueous acetone by volume. Initial concentrations of components were: cupric chloride 1.0×10^{-4} *M*; 0.01 *M* tris buffer at *p*H 7.2; cuproin 2.5×10^{-4}. The concentrations of the substrates were approximately ten times the $CuCl_2$ concentration. The temperature was 25 ± 1°.

	$Cu^{I}/(Cu^{I} + Cu^{II})$ 45 min.	12 hr.
1. Sulfhydryl compounds		
Glutathione	0.68	0.82
Thiouracil	.79	1.00
Thiocytosine	.15	0.33
5-Iodo-2-thiouracil	.42	0.63
2. Phenols		
Phenol	0.09	0.41
Catechol	1.00	1.00
Resorcinol	0.23	0.73
p-Hydroxybenzoic acid	.01	.05
p-Hydroxyphenylacetic acid	.11	.29
p-Hydroxyphenylglycine	.89	1.00
Syringic acid	.85	1.00
Nordihydroguaiuretic acid	.88	1.00
Gallic acid	.91	1.00
p-Aminophenol	.93	1.00
4,4′-Dihydroxybiphenyl	.50	0.90
3. Miscellaneous		
Uric acid	0.85	1.00
Benzyl alcohol	.01	0.04
Hydroxylamine	.10	.72
Pyridoxal	.11	.21
Penicillin G, K salt	.25	.43

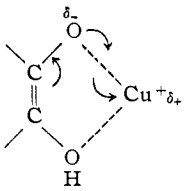
The following compounds showed negligible reducing ability under the conditions of this test: tyrosine, alloxan, allantoic acid, allantoin, urea, glucose, lactose, maltose, formaldehyde, menadione, niacinamide, 2-amino-3,5-diiodobenzene, *o*-nitrophenol, 2,4-dinitrophenol, starch, *o*-chlorobenzoic acid.

Relative Rates of Cupric Ion Reduction.—A more detailed study of the rates at which several organic compounds will reduce cupric ion (Fig. 2) reveals that the enediols, ascorbic acid and catechol, gave the most rapid rate. Under normal conditions, these substances react so rapidly that by 15 seconds after zero time the reaction is completed. In view of this fact, and the fact that hydroquinone gave a significantly slower rate, it seems reasonable to postulate a more stable bimolecular transition state for the enediol cupric reduction. A similar activated complex stabilized by resonance is less likely for a hydroquinone–cupric ion complex.

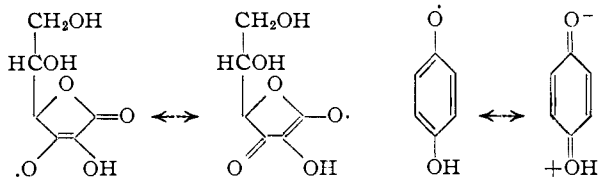
The monomethyl ether of hydroquinone, *p*-hydroxyanisole, reduced Cu^{II} at a slower rate than did hydroquinone itself under identical experi-

(19) G. Ezell and E. Frieden, unpublished data.

mental conditions. If the site of attack of Cu^{II} is presumed to be the dissociated hydroxyl group of the substrate, then this lowered rate can be accounted for, at least in part, by the decreased number of such sites in the *p*-hydroxyanisole molecule as compared to hydroquinone. However, since the two reactions may proceed by a different mechanism, this must be considered as one of several possible explanations. Subsequently it was found possible to increase the rate of Cu^{I} formation by *p*-hydroxyanisole by increasing the cuproin concentration.



Since a single cupric ion is capable of accepting only one electron, postulation of a bimolecular reaction leads to the assumption of a substrate free radical intermediate. The reduced quinones or quinone-ethers studied are all theoretically able to form reasonance-stabilized free radicals as illustrated for ascorbic acid and hydroquinone.



Ascorbic acid Hydroquinone

Considerable additional evidence is available for the existence of such free radicals.[20-23]

For ascorbic acid, catechol and hydroquinone, the end products of oxidation are dehydroascorbic acid, *o*- and *p*-benzoquinone, respectively. The reaction amounts to the removal of a pair of protons and a pair of electrons. However, because of the negligible energy of methyl carbonium ion hydration compared to the energy of proton hydration, *p*-hydroxyanisole would not likely give an analogous quinone product. Additional hydroxylation in the *o*-positions is likely to occur.

Comparative Cupric Ion Reduction by the Thyroxin and Nitrophenol Series.—An important group of substituted hydroquinone derivatives consists of compounds related to the thyroid hormones including thyroxin and triiodothyronine. We have reported earlier[24] that these hormones also reduce Cu^{II} as measured by the formation of the Cu^{I}–cuproin complex. The rates of Cu^{I} production are less than those reported here for hydroquinone or its monomethyl ether. Further studies have also shown that increasing the cuproin concentration results in a rapid, quantitative reduction of Cu^{II} by thyroxin, triiodothyronine, and other related phenols. Of additional interest is the absence of measurable Cu^{II}-reducing ability by several nitrophenols in contrast to the substituted *o*-diiodophenols of the thyroxin series. This inability of the nitrophenols to reduce Cu^{II} may arise from their superiority as oxidizing agents or be due to their inability readily to form free radical intermediates.

These two series of compounds produce many biological effects in common, *e.g.*, increases in oxygen consumption, uncoupling of oxidative phosphorylation, activation of ATPases, etc.[25] This may be due to the exaggerated phenolic character of both the nitrophenols and the iodinated thyronines. However, recently[26] subtle but important differences havd been noted between the effects of these compounds on oxidative phosphorylation in rat liver mitochondria which remain to be explained. The difference in chemical behavior cited here might account for these divergences in biological effects on the thyroxin series and the nitrophenols series. Park, *et al.*,[27] reported that EDTA and glutathione prevented the uncoupling effect of thyroxin but did not alter the uncoupling action of 2,4-dinitrophenol. According to the present view, the uncoupling action of thyroxin may arise from its free radical formation. Glutathione might block this action by serving as a free radical trap. EDTA would operate by reducing any interactions between Cu^{II} (or other oxidizing agent) and thyroxin by preferential chelation of Cu^{II}. If the uncoupling action of 2,4-dinitrophenol is not dependent on a free radical intermediate or is not dependent on its ability to be oxidized, it would not be expected to show sensitivity to such reagents as EDTA or glutathione. It is also possible that in these reactions the iodine atom of the iodinated phenols may play a significant role.

Kinetics of Cupric Ion Reduction by Hydroquinone, *p*-Hydroxyanisole and Cysteine.—If the first step of any cupric ion reduction by a reduced quinone is bimolecular, then the production of a free radical along with cuprous ion is expected. Accordingly, if no subsequent step, significant in the rate-determining reaction, involves copper ion in any of its oxidation states, simple second-order kinetics should be observed. The data in Fig. 3 indicate that reaction between hydroquinone and cupric ion is first order with respect to each component over almost the entire course of the reaction. The calculated second-order rate constant is 9.4×10^3 min.$^{-1}$ (moles/l.)$^{-1}$.

From these data and previous suggestions,[20] the mechanism shown by (3), (4)(5a,b) for the oxidation of hydroquinone by cupric ion seems reasonable. The *p*H dependence of these reactions suggests that at least a monophenylate ion is involved as in reaction 3. Reaction 4 follows from the bimolecularity of the reaction as shown here. Two possibilities

(20) L. Michaelis in "The Enzymes," Vol. II, Part 1, Academic Press, Inc., New York, N. Y., 1951, pp. 1–54.

(21) M. B. Mathews, *J. Biol. Chem.*, **189**, 695 (1951).

(22) M. Kern and E. Racker, *Arch. Biochem. Biophys.*, **48**, 448 (1954).

(23) W. D. Woisloit, A. Nason and A. J. Terrell, *J. Biol. Chem.*, **206**, 277 (1954); *Arch. Biochem. Biophys.*, **48**, 233 (1954).

(24) E. Frieden and R. Flitman, *ibid.*, **64**, 513 (1956).

(25) See summary by H. A. Lardy and G. F. Maley in *Recent Progress in Hormone Research*, **10**, 229 (1951).

(26) A. L. Lehninger in "Enzymes: Units of Biological Structure and Function, edited by O. H. Gaebler, Academic Press, New York, N. Y., 1956, pp. 217–234.

(27) J. H. Park, B. P. Meriwether, C. R. Park, S. H. Mudd and F. Lipmann, *Biochem. Biophys. Acta*, **22**, 403 (1957).

are offered for the final reaction 5. Both result in identical products and stoichiometry. Although the rate of reaction with either catechol or ascorbic acid was extremely fast so that a second-order rate could not be verified with the available methods, it is possible that these substances are oxidized *via* the same mechanism.

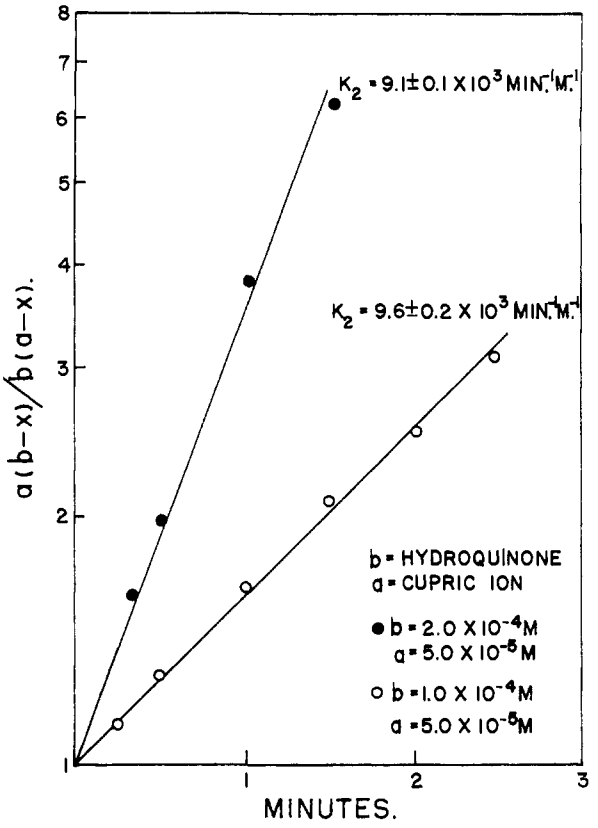
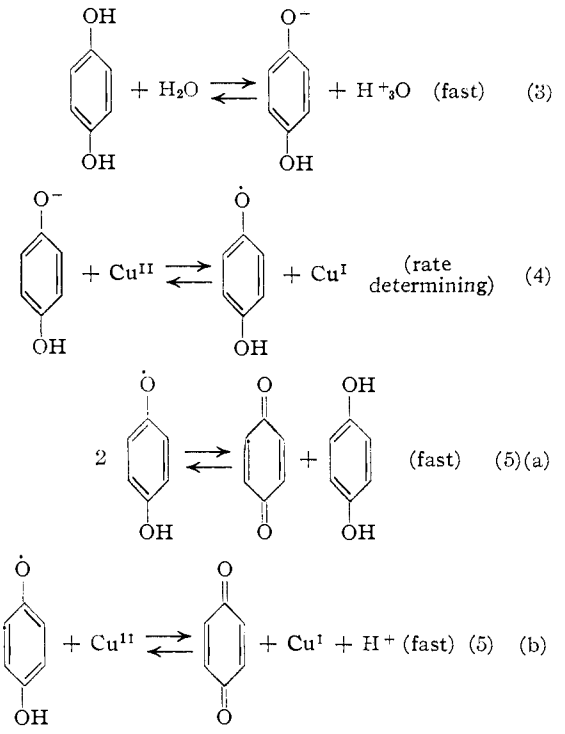
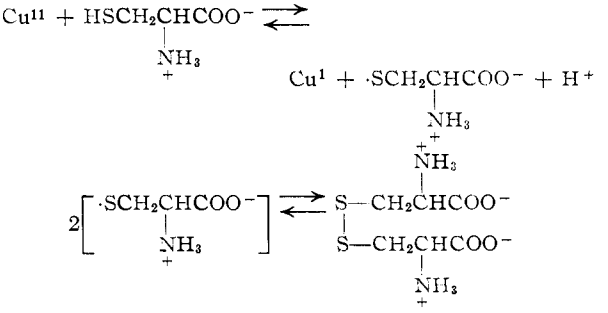


Fig. 3.—Second order plot of the reaction between hydroquinone and cupric ion. Reaction conditions are 30.0°, 0.010 *M* tris buffer, *p*H 7.2, cuproin = 2×10^{-4} *M* in 70% acetone; *a* and *b* are the initial concentrations of cupric ion and hydroquinone, respectively, *x* is the amount of cuprous ion formed at the indicated times. The reaction is followed by measuring the Cu^I formed by its colored complex with the cuproin reagent at 545 mμ.

A second-order plot was also attempted for *p*-hydroxyanisole. Marked deviation from a second-order plot was observed for this order as well as for other orders attempted. The complexity of the reaction sequence for *p*-hydroxyanisole is understandable in terms of the mechanism proposed for hydroquinone. The first two steps in this mechanism might be expected to operate for *p*-hydroxyanisole. However, the disproportionation of two *p*-hydroxyanisole free radicals in a similar manner to (3) is clearly impossible. Because the product arising from *p*-hydroxyanisole is not known, it is difficult to surmise the subsequent fate of the free radical intermediate. However, since simple second-order kinetics were not obeyed, *i.e.*, the reaction is not first order with respect to *p*-hydroxyanisole and first order with respect to cupric ion, it appears likely that one or more latter stages of the reaction involves copper or the phenol in a step which contributes to the determining rate.



When the kinetics of the oxidation of cysteine were studied, a situation not unlike the Cu^{II}–ascorbate reaction was encountered. The rapidity of the reaction was noted in Fig. 2, more than 60% of the available Cu^{II} having been reduced to Cu^I at 15 seconds after zero time. However, unlike the ascorbate oxidation, the product of the cysteine oxidation, cystine, is a powerful chelator of copper ion.[17] Cystine can be shown to compete for copper ion when present in the reaction medium at zero time, although its effect is not appreciable during the early stages of the reaction when it arises as a product. The data for this reaction did not fit simple first- or second-order kinetics. However, the possibility of second-order competitive consecutive kinetics has not been eliminated. If a bimolecular reaction between cysteine and cupric ion occurred, recombination of two free radicals would produce cystine as a product according to the reaction sequence



NOTE ADDED IN PROOF.—Subsequently it has been found that Cu^{II} ($2 \times 10^{-4}M$) and tris buffer (0.010 *M*, *p*H 7.2) exhibits complex formation in aqueous solution at 25°. This

complex was detected by a considerable increase in ultraviolet absorption at 238 mu. Even if this finding can be extended to the mixed solvents used in this work, we need not necessarily modify the interpretation of the data presented here. For example, the presence of a more effective complexing substance such as EDTA or cysteine would eliminate any competitive effect of tris. The Cu^{II}-tris complex may also show comparable catalytic activity in oxidation reactions. The complex might also rapidly release its Cu^{II} by dissociation as the free Cu^{II} is reduced to Cu^{I}.

TALLAHASSEE, FLORIDA

[CONTRIBUTION FROM THE SCHOOL OF PHARMACY, UNIVERSITY OF NORTH CAROLINA]

Amino Acids. XV. Michael Addition Reactions of Diethyl Acetamidomalonate[1]

BY GEORGE H. COCOLAS[2] AND WALTER H. HARTUNG[3]

RECEIVED APRIL 29, 1957

The Michael addition between diethyl acetamidomalonate and ethyl acrylate or ethyl crotonate may give one of two products, depending on the reaction conditions. Mild conditions yield the normal product, whereas reflux temperatures favor the formation of a pyrrolidinone. Pyrrolidinones were also prepared from the normal addition products of diethyl acetamidomalonate or diethyl carbobenzyloxyaminomalonate and the α,β-unsaturated ester. The pyrrolidinones were converted to their corresponding di- and monocarboxylic acids.

The Michael addition of diethyl acetamidomalonate to an appropriate derivative of acrylic acid has become a convenient route to the synthesis of glutamic acid or a suitably substituted glutamic acid.[4-10] However, except for Fillman and Albertson,[6] who noted the loss of a $C_3H_5O_2$ fragment, the intermediates of the reaction or the full path have been ignored. A program calling for the synthesis of analogs and homologs of glutamic acid afforded an opportunity to study both the character of the intermediates and the likely mechanism of the condensation. It soon was apparent that the identity of the adducts depends on the conditions of the reaction. This is true when acrylic and crotonic esters are employed, and it may also apply to esters of other α,β-unsaturated acids.

When the reaction is carried out in the presence of $^1/_{10}$ to $^1/_5$ equivalent of sodium ethoxide under mild conditions, *i.e.*, at either room temperature or in the cold, the normally expected product is formed; but under more strenuous conditions, *i.e.*, at reflux temperatures, a pyrrolidinone derivative forms. These differences may be indicated in Fig. 1.

Support for structure III is afforded by Talbot, Gaudry and Berlinguet,[11] who synthesized IIIa by the reaction between diethyl acetamidomalonate and β-propiolactone and by Kato and his co-workers,[12] who prepared IIIa by the conventional alkylation method from diethyl acetamidomalonate and ethyl β-iodopropionate.

Proof for the pyrrolidinone structures IV was obtained from their preparation by two other methods, namely, (i) formation of IVa and IVb by the

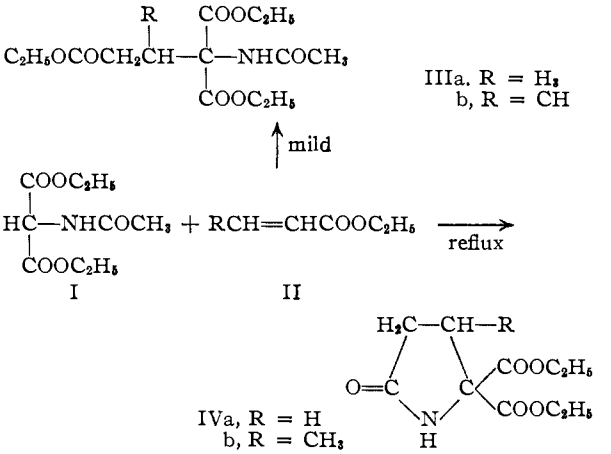
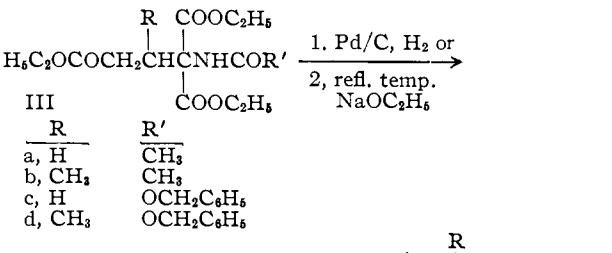


Fig. 1.



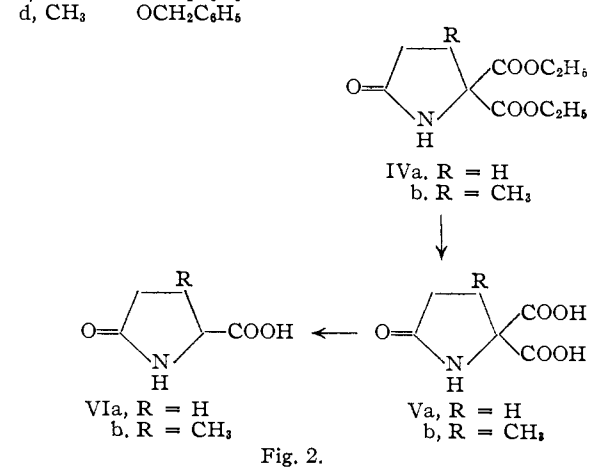


Fig. 2.

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(2) Research Laboratories, National Drug Co., Haines and McCallum Streets, Phila. 44, Pa.

(3) School of Pharmacy, Medical College of Virginia, Richmond, Va.

(4) J. Andrako, Ph.D. Thesis, U. of North Carolina, 1953.

(5) N. Albertson and S. Archer, THIS JOURNAL, **67**, 2043 (1945).

(6) J. Fillman and N. Albertson, *ibid.*, **74**, 2969 (1952).

(7) D. Morrison, *ibid.*, **77**, 6072 (1955).

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