is affected by substitution: uracil, 4; thymine, 1.6; 1-methyluracil, 3.2. Substitution at the C5 position has a more pronounced effect than at the N1 position. (4) The oxidation of the 5-OH-6-yl-Py radical leads to the formation of pyrimidine glycols, 5,6-Py(OH)$_2$. (5) The yield of pyrimidine glycol in the presence of oxidizing agents at pH 7, as measured by HPLC, and the yield of 5-OH-6-yl-Py radical, as measured by pulse radiolysis, are identical for the pyrimidines studied. (6) In the absence of oxidizing agents, the much lower yields of 5,6-Py(OH)$_2$ (Table III) indicate oxidation of reducing radical (6-yl) by the oxidizing radical (5-yl) only and the absence of disproportionation reaction between identical radicals. (7) On the basis of presented evidence it is concluded that pyrimidine glycols are formed exclusively on oxidation of 5-OH-6-yl-Py radicals.

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Registry No. T, 65-71-4; T (radical A), 23402-95-1; T (radical B), 23402-94-0; T (radical C), 80857-92-7; U, 66-22-8; U (radical A), 23402-99-5; U (radical B), 14836-21-6; 1-MeU, 615-77-0; 1-MeU (radical A), 103478-73-5; 1-MeU (radical B), 103478-74-6; 1-MeU (radical D), 50656-54-7; *cis*-T(OH)$_2$, 1124-84-1; *trans*-T(OH)$_2$, 1431-06-7; *cis*-U(OH)$_2$, 3683-34-9; *trans*-U(OH)$_2$, 3952-56-5; *cis*-1-MeU-(OH)$_2$, 64629-87-4; OH$^\bullet$, 3352-57-6.

Possible Model Reaction for Some Amine Oxidases. Kinetics and Mechanism of the Copper(II)-Catalyzed Autoxidation of Some Diaminouracils[1a,b]

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Abstract: It was found that the autoxidation of various 6-N-substituted 5,6-diaminouracils is strongly catalyzed by Cu(II). Since this reaction is believed to be similar to one of the steps in the reaction catalyzed by the copper-containing amine oxidases, it has been studied in some detail. Evidence is presented that the catalyzed reaction involves the reduction of O_2 to H_2O_2 and the oxidation of the diaminouracil by two electrons to 5,6-diiminouracil or its tautomer. However, this initial product is unstable and hydrolyzes eventually to alloxan and NH_3 (an amine is also formed when a 6-N-substituted derivative is autoxidized). Under all conditions examined, the reaction is first order in O_2. With diaminouracil in excess the reaction is first order in Cu(II), and with Cu(II) in excess it is first order in diaminouracil. However, at higher concentrations the rate approaches zero order in each reactant at a constant concentration of the other. Among other observations are the following: the reaction proceeds more rapidly in H_2O than in D_2O by a factor of 2.2–4.7, superoxide dismutase has no effect on the rate, and Cu(II) does not oxidize the diaminouracil in the absence of O_2. The evidence is most consistent with a mechanism involving a ternary complex of the diaminouracil—Cu(II) and O_2 reacting by an ionic two-electron mechanism to give products. In such a mechanism the Cu(II) is believed to have two important functions: by binding to both reactants it provides a pathway for transferring electrons from one to the other, and by having an unpaired electron it allows the two-electron reduction of O_2 to occur in one step.

The Cu(II)-containing amine oxidases[2a] catalyze the reaction shown in eq 1, where the reactant can be a mono-, di-, or polyamine depending on the specificity of the individual enzymes.

$$RCH_2NH_2 + O_2 + H_2O \rightarrow RCHO + H_2O_2 + NH_3 \quad (1)$$

These enzymes as a class are of considerable biological interest because they are involved in several important metabolic pathways in animals. As examples, some are responsible for the degradation of the biogenic amines (histamine, tyramine, norepinephrine, putrescine, spermine, etc.) while others participate in the biosynthesis of collagen.

In addition to the metal ion, these enzymes possess a covalently bound organic prosthetic group that contains a reactive carbonyl. Although it was originally thought that this group is a pyridoxal derivative, that now seems unlikely. Recently, G.A.H. proposed[2a] that the reactive carbonyl compound is an oxidized amino-hydroxyuracil derivative (**1**), formed by hydrolytic cleavage of a flavin covalently bound to the enzyme.[2b] Furthermore, an overall mechanism for the reaction was proposed, and this is illustrated in eq 2.

More recently, Duine and his co-workers[3] have reported evidence that the cofactor is pyrroloquinoline quinone (**6**), presumably covalently bound to the enzyme through one of its carboxyl groups. Because **6** is expected to have reactivity characteristics similar to those of **1**, a mechanism related to that in eq 2, but which involves **6** as the reactant, can be written,[4] and that is illustrated in eq 3. In either case **1** or **6** may not be a true catalytic cycle intermediate because a simple trans-Schiffization of **5** or **10** would lead directly to **2** or **7**.

(1) (a) This work was supported by a research grant (AM 13448) from the National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases, Public Health Science. (b) Taken from: Al-Arab, M. M. Ph.D. Thesis, The Pennsylvania State University, 1983. (c) Present address: Department of Chemistry, Yarmouk University, Irbid, Jordan.

(2) (a) Hamilton, G. A. In *Cooper Proteins*; Spiro, T. G., Ed.; Wiley-Interscience: New York, 1981; pp 193–218. (b) Wessiak and Bruice have recently synthesized and studied the characteristics of a model for **1**: Wessiak, A.; Bruice, T. C. *J. Am. Chem. Soc.* **1983**, *105*, 4809–4825. They also briefly investigated the autoxidation of reduced **1** but did not report any metal ion catalysis of the reaction.

(3) Lobenstein-Verbeek, C. L.; Jongejan, J. A.; Frank, J.; Duine, J. A. *FEBS Lett.* **1984**, *170*, 305–309.

(4) Eckert, T. S.; Bruice, T. C. *J. Am. Chem. Soc.* **1983**, *105*, 4431–4441. Sleath, P. R.; Noar, J. B.; Eberlein, G. A.; Bruice, T. C. *J. Am. Chem. Soc.* **1985**, *107*, 3328–3338. Noar, J. B.; Rodriguez, E. J.; Bruice, T. C. *J. Am. Chem. Soc.* **1985**, *107*, 7197–7199.

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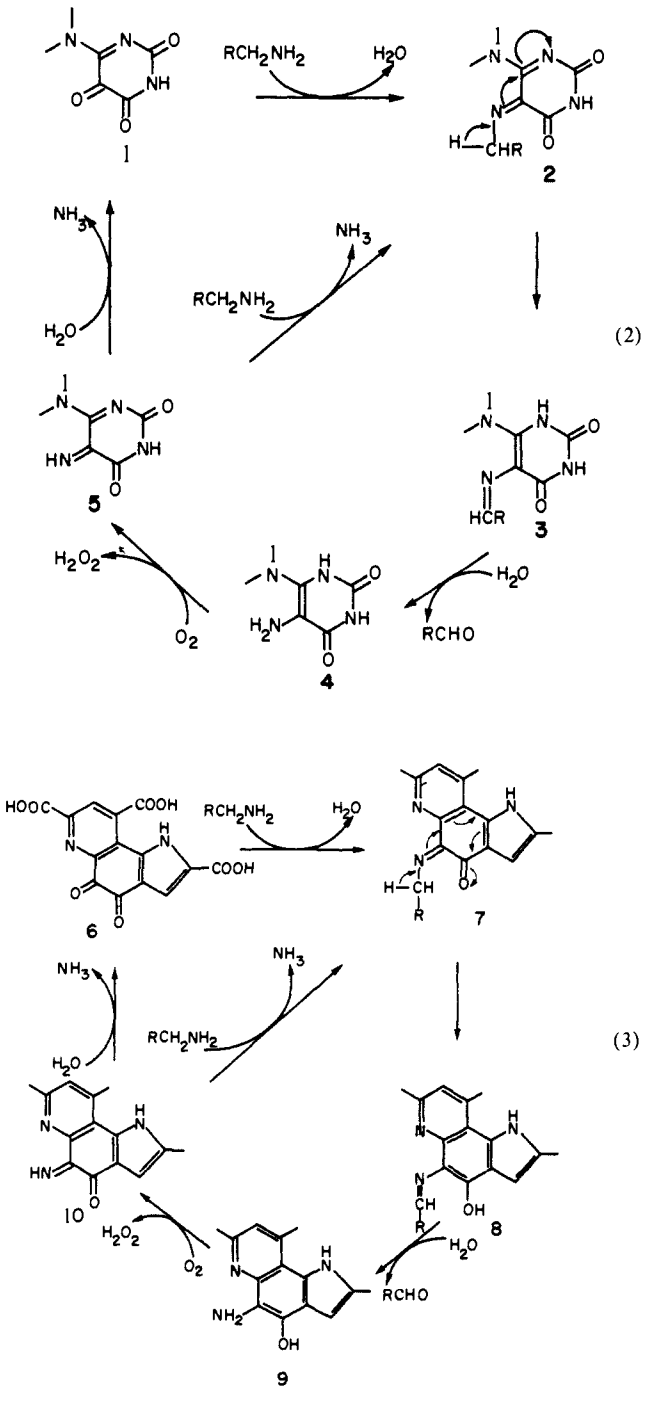
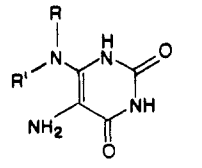


(2)

(3)

The focus of the present paper is on the possible role of the copper in the catalytic reaction. As discussed previously,[2a] the most likely step that would require the copper is the conversion of **4** to **5** if the mechanism of eq 2 obtains. Similar considerations would suggest that the **9** to **10** conversion is the one that would need the copper if the mechanism of eq 3 were correct. The present work was completed before the results of Duine and co-workers[3] were known, so the model reactions that were studied are closely related to the conversion of **4** to **5** rather than **9** to **10**. However, it is thought that the results are relevant to the probable mechanism of the putative **9** to **10** reaction as well. The autoxidation of four compounds, namely 5,6-diaminouracil (**4a**), 6-(*N*-methylamino)-5-aminouracil (**4b**), 6-(*N*-methylanilino)-5-aminouracil (**4c**), and 6-(*N*-ethylanilino)-5-aminouracil (**4d**), were studied. It was found that Cu(II) is a very effective catalyst for their autoxidation and that the model system has several characteristics similar to the enzymic reaction. Following a detailed study of this reaction, a mechanism consistent with both the model and enzymic reactions becomes apparent and is discussed.



4a: R = R' = H
4b: R = CH_3, R' = H
4c: R = CH_3, R' = C_6H_5
4d: R = C_2H_5, R' = C_6H_5

Results

Preliminary Observations. By following their UV absorptions, it was found that the various diaminouracils are stable for several hours in acid solution (pH 2) at room temperature in contact with air, either with or without EDTA being present. On the other hand, at a slightly basic pH (8.6) there is a decrease of up to 25% in the optical density after 15 min when EDTA is absent. This reaction is suppressed when EDTA (20 μM) is present; there is a negligible change in optical density after 15 min under otherwise similar conditions. These results suggest that trace metal ions are catalyzing the reaction of the diaminouracils. When various metal ions were added to reaction mixtures at pH 8.6, it was found that Cu(II) is a particularly effective catalyst; with 5 μM Cu(II) present the UV absorption due to the diaminouracils disappears in less than 5 min when the solutions are in contact with air. Of other metal ions tried [Zn(II), Mg(II), Ni(II), Mn(II), and Co(II)], only Mn(II) and Co(II) showed any catalytic activity, and these are less effective by approximately 1 and 2 orders of magnitude, respectively. Because it is so efficient as a catalyst and because it is the metal ion involved in the enzymic reaction, we have concentrated on studying the characteristics of the Cu(II)-catalyzed reaction.

Products and Stoichiometry. Considerable evidence indicates that the initial Cu(II)-catalyzed reaction is the conversion of **4** to **5** (or a tautomer of **5**) with the concomitant reduction of O_2 to H_2O_2. One indication of this is that the initial rate of disappearance of O_2, as measured by an O_2 electrode, is the same within experimental error as the initial rate of disappearance of the diaminouracil, as measured by its UV absorption, when the reactions are carried out under identical conditions. For example, with **4a** (0.25 mM) as the reactant in 40 mM borate buffer, pH 8.6, 25 °C, 0.25 mM O_2, 20 μM EDTA, and 23 μM Cu(II), the initial rate is 3.7 μM/s when measured by O_2 uptake and 3.5 μM/s when measured by the decrease in absorption at 281 nm (for **4a**, ϵ = 10900 M^{-1} cm^{-1} at pH 8.6). Under the same conditions with 37 μM Cu(II), the initial rates measured by the two methods were 7.6 and 7.8 μM/s, respectively.

That H_2O_2 is a product is evident from the effects of catalase, some of which are illustrated in Figure 1. Not only is the initial rate of O_2 uptake decreased by a factor of 2 when catalase is present but more O_2 is observed if catalase is added to a reaction that has proceeded for some time without catalase. As shown in Figure 1, when 0.25 mM **4d** is autoxidized to completion in the presence of catalase (curve A), 0.125 mM O_2 reacts as expected. However, as also illustrated, in the absence of catalase the reaction stops after the O_2 uptake is 0.210 mM rather than 0.250 mM as would be expected if H_2O_2 is the only reduction product of O_2. Furthermore, only 0.085 mM O_2 is released when catalase is added. These results indicate that H_2O_2 can also act as an oxidant for **4**, and in the example given in Figure 1, 0.040 mM H_2O_2 has oxidized 0.040 mM **4d**. Additional experiments showed that Cu(II) is a catalyst for the reaction of H_2O_2 with **4**. Those in Figure 1 were performed with a relatively low Cu(II) concentration. When a high concentration (270 μM) of Cu(II) was used for the reaction of the same concentration (0.25 mM) of **4d** as in Figure 1, the total O_2 uptake in the presence of catalase was found to be 0.125 mM as before. However, in the absence of catalase the reaction stops after 0.158 mM O_2 reacts, and only 0.034 mM O_2 is produced when catalase is subsequently added.

From a number of such experiments it was found that the relationship of eq 4 (the subscripts mean the following: i, initial; r, reacted; f, final) is always obeyed irrespective of the Cu(II)

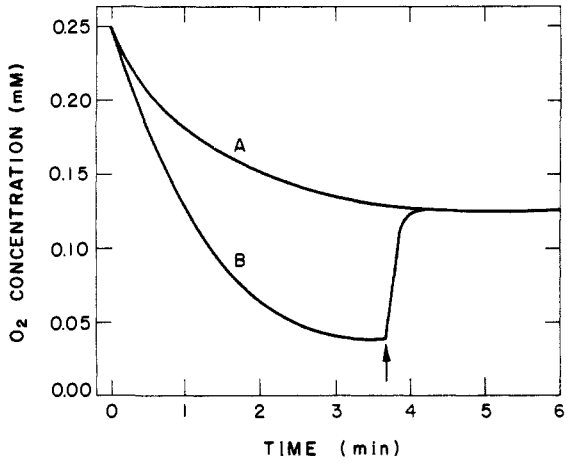


Figure 1. Oxygen uptake during the Cu(II)-catalyzed autoxidation of **4d**, in the presence and absence of catalase. Reaction conditions: volume, 3 mL; 25 °C; 40 mM CHES buffer, pH 8.6; 5 μM free Cu(II) [25 μM Cu(II) and 20 μM EDTA]. Initial reactant concentrations: 0.25 mM O_2 (air saturated) and 0.25 mM **4d**. In run A, 150 μg/mL catalase was present from the beginning of the reaction, while in B no catalase was present until the time indicated by the arrow when 150 μg/mL catalase was added.

concentration and whether catalase is present or not (of course, when catalase is present, $[H_2O_2]_f = 0$). These results indicate

$$[4]_i = 2[O_2]_r - [H_2O_2]_f \quad (4)$$

that **4** is being oxidized by only two electrons but it can be oxidized by either O_2 or H_2O_2, both of which are catalyzed by Cu(II). Since we were mainly interested in the oxidation of **4** by O_2 rather than by H_2O_2, the latter has not been studied further. In order to avoid any possible complications of the H_2O_2 reaction to the kinetics of the oxidation by O_2, all reported kinetic data (vide infra) were obtained from initial rates, under which conditions the H_2O_2 reaction is negligible.

Alloxan monohydrate (5,5-dihydroxybarbituric acid) is observed as a product when any one of **4a–d** is autoxidized and the reaction mixture allowed to sit for a few minutes. Both authentic alloxan monohydrate and the reaction product have identical R_f values on TLC (R_f 0.74 with polyamide-6 plates developed with methanol/acetic acid, 95:5) and paper chromatography (R_f 0.55 when developed with isopropyl ether/90% formic acid, 3:2, and visualized by dipping in an acetone solution of $AgNO_3$ followed by spraying with concentrated NH_4OH and exposing to UV). Furthermore, both the ultimate reaction product and authentic alloxan lack any distinguishing UV spectrum. When **4c** and **4d** are reacted, other products that are present after several minutes are *N*-methylaniline and *N*-ethylaniline, respectively, as determined by TLC of the product mixtures on silica gel-G (visualized by spraying with a 1% ethanolic solution of 2,6-dichloroquinone-4-chlorimide). Both authentic *N*-methylaniline and the product from **4c** have an R_f of 0.24 when developed with benzene and an R_f of 0.62 when developed with benzene/methanol, 19:1. Similarly, *N*-ethylaniline and the product from **4d** have an R_f of 0.65 with benzene/methanol, 19:1, and 0.73 with chloroform/methanol, 16:1.

It is clear, however, that the foregoing compounds are not the initial products of oxidation of **4**. Shown in Figure 2 are the time courses of some UV changes observed when **4a** is autoxidized. Prior to initiation of the oxidation **4a** has an absorption maximum at 281 nm that disappears within 1 min of initiation under the reaction conditions of Figure 2. As shown in the figure, immediately following initiation a new broad absorption at 330 nm appears, but this soon decays to give rise to a second absorption centered at 240 nm that in turn ultimately decays. After 20 min, the spectrum of the solution shows no well-defined absorptions and alloxan can be detected on workup (see above). The results in Figure 2 indicate that there are at least two successive intermediates formed following the autoxidation of **4a** prior to the formation of alloxan. Virtually identical results are obtained with **4b**. The absorptions due to the phenyl groups of **4c** and **4d**

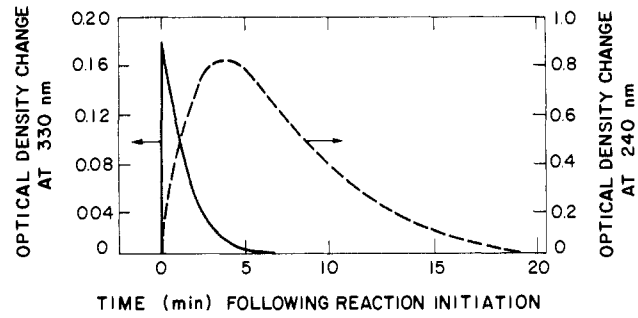


Figure 2. Some spectral changes at 330 (—) and 240 (---) nm following the Cu(II)-catalyzed autoxidation of **4a**. Reaction conditions: volume, 3 mL; 25 °C; 40 mM CHES buffer, pH 8.6; 25 μM free Cu(II) [45 μM Cu(II) and 20 μM EDTA]. Initial reactant concentrations: 0.25 mM O_2 (air saturated) and 0.25 mM **4a**. The spectral changes were recorded by using as a blank a solution containing all of the foregoing except Cu(II).

Table I. Effects of EDTA, Total Cu(II), and Free Cu(II) Concentrations on the Rate of Autoxidation of **4d**[a]

[EDTA], μM	total [Cu(II)], μM	free [Cu(II)], μM	init rate, μM/s
20	25	5	2.10
40	45	5	2.00
80	85	5	1.93
150	155	5	1.95

[a] Reaction conditions: 25 °C, 40 mM CHES buffer, pH 8.6, air saturated; 0.25 mM **4d**; 150 μg/mL catalase.

preclude a similar study of their reactions, but all other evidence suggests that they react in the same way that **4a** and **4b** do. Dryhurst and co-workers[5] have observed results very similar to those of Figure 2 upon the electrochemical oxidation of **4a** and have proposed that the intermediates have structures **5** and **1** or tautomers of these compounds. Furthermore, Wessiak and Bruice[2b] have shown that another **1** derivative hydrolyzes readily in aqueous solution. Therefore, the spectral results, taken in conjunction with the finding of alloxan and the amines as the ultimate products, strongly indicate that the Cu(II)-catalyzed autoxidation is in fact the **4** to **5** conversion with alloxan and the amines being formed in subsequent slower hydrolytic steps.

Kinetic Studies—General Observations. As indicated earlier, trace metal ions catalyze the autoxidation of **4**, but this can be eliminated by the presence of small amounts of EDTA. Consequently, for all kinetic runs, solutions of **4** were prepared in buffers containing EDTA (usually 20 μM), and the reactions were initiated by adding sufficient Cu(II) to have some free Cu(II) available as a catalyst. In preliminary experiments it was shown that only free Cu(II) catalyzes the reaction. No autoxidation is observed if the total Cu(II) concentration is less than that of EDTA, and, with differing amounts of total Cu(II) and EDTA present (Table I), the rate is constant if the amount of uncomplexed or free Cu(II) remains constant. In all subsequent treatments of the kinetic data the amount of Cu(II) available for catalysis was considered to be the total copper concentration minus the EDTA concentration.

As might be expected for a metal ion catalyzed reaction, the rate varies considerably, depending on what buffer is used and what other additives are present. Some representative data are summarized in Table II. Those buffers, such as the glycine derivatives, that can effectively chelate the metal ion inhibit the reaction markedly. On the other hand, halide ions, especially chloride, increase the rate to some extent. The halide ion effect, however, depends on the concentration; at higher concentrations (greater than 0.2 M) inhibition is seen.[1b] Simple amines or ammonium ions (including NH_3/NH_4^+) also have an activating

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Table II. Effects of Buffers and Added Salts on the Cu(II)-Catalyzed Autoxidation of **4** at pH 8.6[a]

buffer[b]	added salt	init rate, μM/s 4a	4b	4c	4d
glycine/NaOH		0	0	0	0
BICINE/NaOH		0.16	0.58	0.08	0.10
TRICINE/NaOH		0.17	0.60	0.09	0.10
Tris-HCl		0.15	0.74	0.13	0.14
HEPES/NaOH		7.4	14.1	3.9	6.4
CHES/NaOH		11.3	20	6.8	7.4
NH_3/HCl		18	21	9.1	9.9
H_3BO_3/NaOH		4.8	9.7	3.0	3.3
H_3BO_3/NaOH	NaF	4.0	12.3	3.8	4.7
H_3BO_3/NaOH	NaCl	15.1	19	6.8	9.6
H_3BO_3/NaOH	NaBr	13.7	19	6.7	7.7
H_3BO_3/NaOH	NaI	9.9	13.8	6.3	7.2
H_3BO_3/NaOH	NH_4Cl	14.3	19	6.8	9.3
H_3BO_3/NaOH	LiCl	15.6	19	7.5	8.5
H_3BO_3/NaOH	KCl	14.1	19	7.3	9.5

[a] Reaction conditions: 25 °C, 40 mM air-saturated buffers, pH 8.6; 0.25 mM **4**; 200 mM added salt when present; 5 μM free Cu(II) [25 μM total Cu(II) and 20 μM EDTA]. [b] Abbreviations used: BICINE, *N*,*N*-bis(2-hydroxyethyl)glycine; TRICINE, *N*-[tris(hydroxymethyl)-methyl]glycine; Tris, tris(hydroxymethyl)aminomethane; HEPES, *N*-2-(hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; CHES, 2-(*N*-cyclohexylamino)ethanesulfonic acid.

effect, but there is no change in rate when Na^+ ions are replaced by Li^+ or K^+. Since H_2O_2 at concentrations of 0.1 mM or greater stimulates the reaction as well,[1b] most of the subsequent data were obtained with catalase present.

In addition to buffer and salt effects, the reaction is also pH dependent, the magnitude and direction of which depends on what buffer is used.[1b] Nevertheless, Cu(II)-catalyzed autoxidation can be observed from pH 6 to 11. Because of the complex pH, buffer, and salt effects, no attempt was made to collect sufficient data to be able to analyze them quantitatively. Rather, all subsequent data were obtained at one pH (8.6) by using either 40 mM borate or CHES buffer with no added salt. These were chosen because they seemed to cause few complications and they allowed a relatively rapid Cu(II)-catalyzed reaction. Although the actual rates in borate buffer are approximately a factor of 2 slower than in CHES, the general results observed with one buffer were found to be the same as those obtained which the other under otherwise identical conditions. When CHES buffer was used at pH 8.6, it was found that varying the concentration of CHES from 5 to 200 mM has no detectable effect on the initial rate when all other conditions are the same.

Kinetic Dependence on the Concentrations of O_2, Cu(II), and 4. As illustrated by the results shown in Figure 3, the reaction is first order in the O_2 concentration under all conditions investigated. These include experiments in borate and CHES buffers, with or without catalase, and at high or low concentrations of free Cu(II). The lines in the figure were calculated from least-square analyses of the data; their slopes range from 0.92 to 1.09 (average 1.01) with correlation coefficients of 0.993 or better.

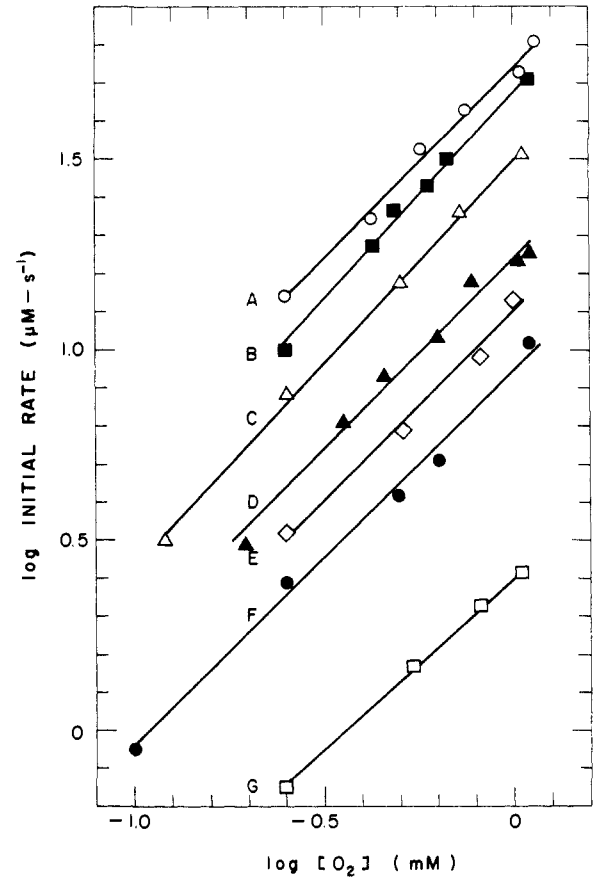


Figure 3. Kinetic dependence of the rate of the Cu(II)-catalyzed autoxidation of **4** on the O_2 concentration. General reaction conditions: 25 °c, 40 mM borate or CHES buffer, pH 8.6, 0.25 mM **4**, 20 μM EDTA, 5 μM free Cu(II) unless indicated otherwise, 150 μg/mL catalase when present. Key: A, CHES buffer, **4d** reactant, catalase present and 45 μM free Cu(II); B, borate buffer, **4b** reactant, catalase absent; C, CHES buffer, **4d** reactant, catalase absent; D, borate buffer, **4a** reactant, catalase absent; E, borate buffer, **4d** reactant, catalase absent; F, CHES buffer, **4d** reactant, catalase present; G, borate buffer, **4a** reactant, catalase present.

Summarized in Table III are some results indicating the effects that the concentrations of Cu(II) and **4** have on the rate of autoxidation at low Cu(II) concentrations with catalase present. The reasonable constancy of the k_3 values (defined as in eq 5)

$$\text{init rate} = k_3[O_2][\text{Cu(II)}][\mathbf{4}] \quad (5)$$

for each of the three different derivatives indicates that, under such conditions, the reaction is third order overall, i.e., first order in each of Cu(II) and **4** as well as in O_2. At higher reactant concentrations, however, the reaction ultimately becomes zero order in both Cu(II) (Figure 4a) and **4d** (Figure 4b) either with

Table III. Effects of the Concentrations of Free Cu(II) and **4** on the Rate of Autoxidation of **4**[a]

free[b] [Cu(II)], μM	[4], mM	init rate (V, μM s^{-1}) and third-order rate const (k_3[c], mM^{-2} s^{-1}) for the reaction of 4a V	4a k_3	4c V	4c k_3	4d V	4d k_3
5	0.05	0.17	2.7	0.095	1.5	0.11	1.8
5	0.10	0.29	2.3	0.21	1.7	0.23	1.8
5	0.15	0.49	2.6	0.26	1.4	0.32	1.7
5	0.20			0.38	1.5	0.44	1.8
5	0.25	0.75	2.4	0.53	1.7	0.55	1.8
2	0.25	0.33	2.6			0.31	2.5
9	0.25	1.8	3.2	0.79	1.4	1.26	2.2
13	0.25	2.8	3.5	1.30	1.6	1.9	2.4
17	0.25	3.9	3.7	1.7	1.6	2.9	2.7

[a] Reaction conditions: 25 °C, 40 mM borate buffer, pH 8.6, air saturated, 150 μg/mL catalase, 20 μM EDTA. [b] The total [Cu(II)] is that given plus 20 μM (this latter amount is complexed to the EDTA and is catalytically inactive). [c] Defined in eq 5.

Table IV. Effect of D_2O on the Rate of Autoxidation of **4d**[a]

[Cu(II)], μM	init rate μM/s, in H_2O	in D_2O	k_{H_2O}/k_{D_2O}
5	7.7	3.5	2.2
17	12.0	4.2	2.9
25	14.0	4.7	3.0
250	27	5.7	4.7

[a] Reaction conditions: 25 °C, 40 mM CHES buffer, pH or pD 8.6, air saturated, 250 μM **4d**.

or without catalase being present. Such results imply that some complex of Cu(II) with **4** is the species that is autoxidized (see Discussion).

Effect of Superoxide Dismutase. Since superoxide seemed like a possible intermediate in the autoxidation, the effects of superoxide dismutase on the rate were investigated. However, even at relatively high concentrations (200 μg/mL), superoxide dismutase has no effect on the rates of autoxidation of any of the derivatives, in either the presence or absence of catalase. These experiments were carried out under conditions similar to those given in the footnote of Table II (borate buffer).

Effect of D_2O on the Rate of Autoxidation of 4d. As illustrated by the results shown in Table IV, the reaction proceeds considerably more slowly in D_2O than in H_2O, at both high and low concentrations of Cu(II).

Reaction of Cu(II) with 4a under Anaerobic Conditions. Since one mechanism for the Cu(II)-catalyzed autoxidation would involve reduction of Cu(II) to Cu(I) by **4** followed by reoxidation of Cu(I) by O_2, some experiments were performed in the absence of O_2 to determine whether Cu(II) alone could oxidize **4**. However, even with a large excess of Cu(II) over **4** no evidence for **4** oxidation under such conditions was obtained. For example, in some experiments monitored by UV, the 281-nm absorption shown by **4a** (70 μM in 40 mM CHES buffer, pH 8.6) disappears within a few minutes when 250 μM Cu(II) is added in the presence of O_2. However, in the absence of O_2, the absorption at 281 nm increases in a few seconds and remains constant for more than 1 h when the same amount of Cu(II) is added. The lack of a decrease in absorption at 281 nm indicates that **4a** is not being oxidized; presumably, the increase in absorption is due to the formation of some complex involving Cu(II) and **4a**.

Autoxidation of 3-Me-4c. In order to obtain information concerning what groups on **4** the Cu(II) might be binding, 6-(*N*-methylanilino)-3-methyl-5-aminouracil (3-Me-**4c**) was prepared, and its reactivity characteristics in the autoxidation system were compared to those of **4c**. It was found[1b] that under a number of different conditions (various O_2, Cu(II), and 3-Me-**4c** concentrations) the rates of autoxidation of 3-Me-**4c** were identical within experimental error with the rates of reaction of **4c** itself under the same conditions.

Discussion

The finding that Cu(II) is such an effective catalyst for the autoxidation of **4** is, in and of itself, a significant finding, because Cu(II) is the metal ion involved in the amine oxidase reaction. Such a result is considered reasonable evidence for a specific role for enzyme-bound copper in the reoxidation of the reduced enzyme as originally hypothesized.[2a] The fact that H_2O_2 is the product of O_2 reduction in both the model and enzymic reactions adds further credence to this suggestion. Furthermore, the evidence that **5** (or its tautomer) is the initial product formed from the oxidation of **4** lends support the possibility that eq 2 or 3 may represent the overall mechanism of the enzymic reaction.

Of several conceivable mechanisms one could envisage for the reaction, many can be eliminated by the current results. As indicated in the previous section, any mechanism involving the oxidaton of **4** by Cu(II) followed by reoxidation of the Cu(I) by O_2 is eliminated as a possibility by the finding that Cu(II) does not oxidize **4** in the absence of O_2. In addition this result indicates that a mechanism for the reaction similar to that believed to hold for the copper ion catalyzed autoxidation of ascorbic acid[6] cannot

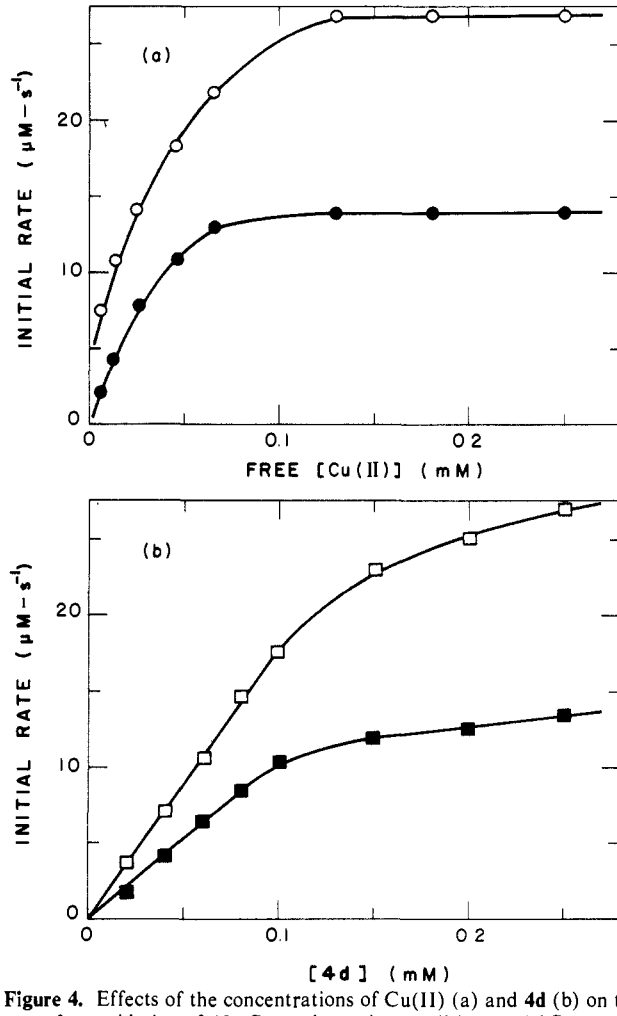


Figure 4. Effects of the concentrations of Cu(II) (a) and **4d** (b) on the rate of autoxidation of **4d**. General reaction conditions: 25 °C, 40 mM CHES buffer, pH 8.6, air saturated, 20 μM EDTA and 150 μg/mL catalase when present (filled symbols). Specific conditions: (○) 0.25 mM **4d**, no catalase; (●) 0.25 mM **4d** with catalase; (□) 0.25 mM free Cu(II), no catalase; (■) 0.25 mM free Cu(II) with catalase.

be the correct one either. In that case, Cu(I) is believed to be the actual catalyst, formed in a preliminary step by reduction of Cu(II) by ascorbic acid. Both of the above types of mechanism are also eliminated by the detailed kinetic results, because such mechanisms require half-order dependencies on the concentrations of one or more of the reactants and that is not observed. A final piece of evidence against any mechanism involving reoxidation of a Cu(I) species by O_2 is the observation that superoxide dismutase has no effect on the rate; superoxide is the expected product of O_2 reduction by Cu(I).

The kinetic results suggest that the reaction proceeds by a mechanism of the type shown in eq 6. In order to derive a rate expression for this mechanism, one needs to define the terms in eq 7–9. Under conditions where the concentrations of Cu(II),

$$\text{Cu(II)} + \mathbf{4} \overset{K}{\rightleftharpoons} \text{Cu(II)}\text{-}\mathbf{4} \xrightarrow[k]{O_2} \text{products} \qquad (6)$$

$$K = \frac{[\mathbf{4}][\text{Cu(II)}]}{[\text{Cu(II)}\text{-}\mathbf{4}]} \qquad (7)$$

$$[\text{Cu(II)}]_T = [\text{Cu(II)}] + [\text{Cu(II)}\text{-}\mathbf{4}] \qquad (8)$$

$$[\mathbf{4}]_T = [\mathbf{4}] + [\text{Cu(II)}\text{-}\mathbf{4}] \qquad (9)$$

4 and, Cu(II)–**4** are comparable, it is difficult to derive an exact expression relating the rate to $[Cu(II)]_T$, $[\mathbf{4}]_T$, and $[O_2]$. However, when either $[Cu(II)]_T$ or $[\mathbf{4}]_T$ is in large excess over the other,

(6) Shtamm, E. V.; Purmal, A. P.; Skurlatov, Y. I. *Int. J. Chem. Kinet.* **1979**, *11*, 461–494.

then a simple relation results. Thus, if $[\mathbf{4}]_T \gg [Cu(II)]_T$, then $[\mathbf{4}] \simeq [\mathbf{4}]_T$ and eq 10 results, whereas if $[Cu(II)]_T \gg [\mathbf{4}]_T$, then $[Cu(II)] \simeq [Cu(II)]_T$ and eq 11 ensues. Qualitatively, the results

$$\text{rate} = \frac{k[O_2][Cu(II)]_T[\mathbf{4}]_T}{K + [\mathbf{4}]_T} \quad (10)$$

$$\text{rate} = \frac{k[O_2][Cu(II)]_T[\mathbf{4}]_T}{K + [Cu(II)]_T} \quad (11)$$

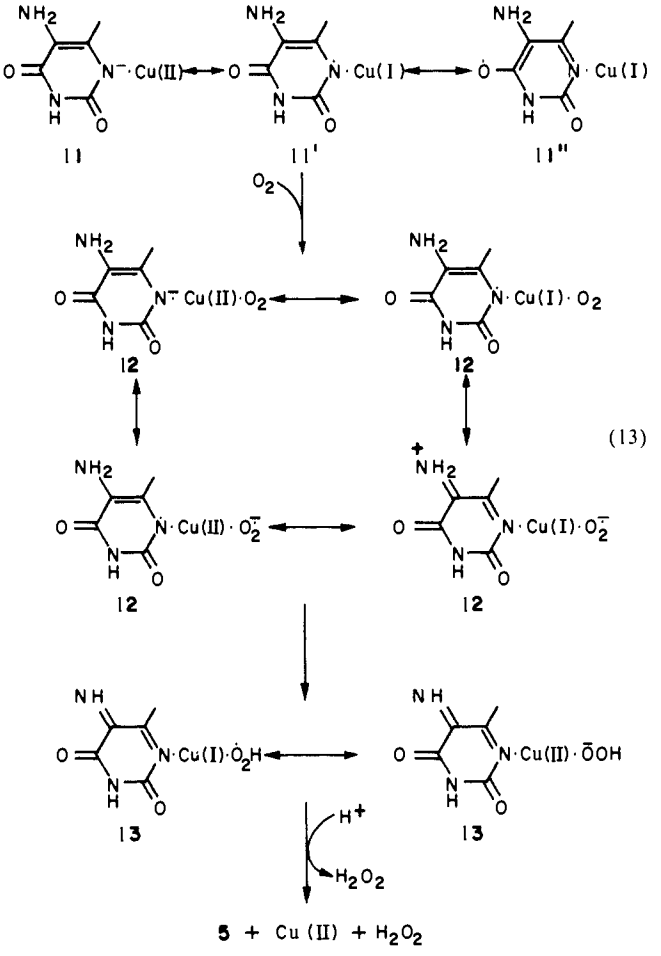
summarized in Table III and Figures 3 and 4 are in agreement with these expressions. Thus, under all conditions, the reaction is first order in the O_2 concentration (Figure 3), at low $[Cu(II)]_T$ the reaction is third order overall (Table III), and at high concentrations of either Cu(II) or **4** the rate becomes independent of the concentration of that reactant (Figure 4). The reason that the reaction is first order in $[\mathbf{4}]_T$ even when it is in excess (Table III) is probably because those reactions were performed in borate buffer. It can be shown that if borate complexes to the Cu(II) as expected, the rate expression obtained when $[\mathbf{4}]_T \gg [Cu(II)]_T$ is that given in eq 12 where K' is a dissociation constant of a Cu(II)–borate complex. This predicts that the reaction would remain first order in $[\mathbf{4}]_T$ to a higher concentration of **4** than would otherwise be expected.

$$\text{rate} = \frac{kK'[O_2][Cu(II)]_T[\mathbf{4}]_T}{KK' + K'[\mathbf{4}]_T + K[\text{borate}]} \quad (12)$$

Despite the fact that the results are qualitatively in agreement with the mechanism of eq 6, there is a pecularity that indicates that the reaction cannot be as simple as that. The peculiarity is that the rate of the reaction becomes independent of one of the reactant concentrations (Figure 4) at less than a 1:1 molar stoichiometry of the reactants; the simple mechanism of eq 6 predicts that such should not occur until the concentration of the reactant being varied is either equal to or greater than the concentration of the other reactant. One possible rationale for this effect is that Cu(II) and **4** may form not just one complex but many, including polymeric complexes. If only one or a small number of such complexes react with O_2 then one can understand, at least in principle, the observed results. As the concentration of Cu(II) or **4** increases beyond a certain point, large amounts of unreactive complexes may be formed, and this would make the reaction go more slowly then predicted if only reactive complexes were formed. Because of the complexity of these possibilities and because of our lack of knowledge concerning what specific complexes may be formed, no attempt was made to derive an exact expression for such a mechanism.

If, as the data suggest, the reaction proceeds by a mechanism of the type shown in eq 6, then the actual oxidation step could be either a bimolecular reaction of O_2 with a Cu(II)–**4** complex or, more likely, a transformation involving a weak ternary complex of O_2 and the reactive Cu(II)–**4** species. A possible mechanism by which this step may occur is outlined in eq 13. In this mechanism the copper is illustrated as binding to the N-1 site of **4** because this seems like the most probable binding site. However, similar mechanisms could be written if it were bound at other sites on **4**. The observation that 3-Me-**4c** reacts at essentially the same rate as **4c** indicates that the copper does not bind to N-3.

Since the hydrogen on N-1 of **4** is fairly acidic, it is expected that when bound to copper the proton would be lost as illustrated in structure **11** of eq 13 (in the illustrations of eq 13, a dot between the Cu and a ligand merely represents a complex between the two, whereas a superscript dot on one of the ligands represents a structure having a radical on that ligand). The substituted pyrimidine ring should be strongly electron donating so that other resonance contributors (as examples, **11′** and **11″**) involving Cu(I) forms will contribute to the overall structure. If they contribute sufficiently, it seems possible that the copper would bind O_2 in its inner coordination sphere to give **12**; a few structures that would contribute to the stabilization of such a complex are illustrated in eq 13. The actual oxidation of **4** and reduction of O_2 would be consummated by proton migration from the NH_2 group of **12**



(13)

to O_2 to give **13**. The observation of a D_2O solvent isotope effect (Table IV) suggests that this is the slow step in the reaction. Dissociation of **13** to **5**, Cu(II), and H_2O_2 would complete the overall autoxidation reaction.

The role of the copper ion in such a mechanism has been previously discussed in detail.[2a,7] In one of its important functions, it binds to both **4** and O_2, thereby providing a mechanism for transmitting electrons from one reactant to the other in the actual oxidation step (**12** to **13** conversion). Equally importantly, the spin inversion problem usually associated with O_2 to H_2O_2 conversions[7] is completely avoided by having Cu(II) as a catalyst. When Cu(II) (which has one unpaired electron) reacts with triplet O_2, it is spin allowed to obtain an adduct such as **12** with one unpaired electron. Furthermore, the product **13** has the copper in essentially the Cu(II) state and thus will also have an unpaired electron. Consequently, the **12** to **13** conversion involves no change in spin and would be expected to occur rapidly as it apparently does.

There is considerable evidence that a mechanism similar to that in eq 13 probably holds for the step involving O_2 in the amine oxidase catalyzed reaction as well. During the enzymic reaction, O_2 apparently binds directly to the copper,[8] and no evidence has ever been obtained for the copper changing valence during the reaction.[2a] Therefore, regardless of whether the overall mechanism is that shown in eq 2 or 3, the copper probably functions as illustrated in eq 13.

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Conclusion

Cu(II) was found to be an effective catalyst for the autoxidation of several aminouracils. The most likely mechanism for the reaction is one in which a ternary complex of the aminouracil, Cu(II), and O_2 is formed and transformed in a two-electron mechanism into a complex of oxidized aminouracil, Cu(II), and peroxide. In this mechanism the Cu(II) functions to transfer electrons from one ligand to another, and also it allows a two-electron reduction of O_2 to occur in one step. This model reaction bears many striking similarities to reactions catalyzed by the copper-containing amine oxidases, and thus the step involving O_2 in the enzymic reactions is thought to proceed by a similar mechanism.

Experimental Section

General Procedures. Melting points were determined on a Thomas-Hoover melting point apparatus and are uncorrected. Elemental analyses were performed by M-H-W Laboratories, Phoenix, AZ. Routine UV and visible spectra were obtained on a Gilford spectrophotometer, Model 240, or a Hitachi Model 100-80. All pHs of solutions were measured (using a Fisher pH meter, Model 144) at the temperature they were to be used.

Kinetic Experiments. Except for a few runs followed by standard UV techniques, most of the data reported here obtained by following O_2 uptake on a Yellow Springs Instrument Co. biological oxygen monitor (Model 53) containing a YSI 5331 oxygen probe. All reactions were run at 25 °C. Solutions (3-mL total volume) of the buffer, **4**, and EDTA in the reaction vial were temperature equilibrated with the probe in place, and the autoxidation was initiated by injection of a 5-μL volume of the metal ion solution. Each initial rate of O_2 uptake listed here is the average of at least three runs with not more than 5 or 10% variation.

Materials. All of the chemicals used were reagent-grade quality or better; most of the inorganic salts were Gold Label ultrapure (99.99%), from Aldrich Chemical Co. 2-Thiobarbituric acid, D_2O, DCl, NaOD, 2,4,6-trichloropyrimidine, bovine liver catalase (2X crystallized), and bovine blood superoxide dismutase were purchased from Sigma Chemical Co. 5,6-Diaminouracil, purchased from U.S. Biochemical Co., was recrystallized from 1 M H_2SO_4. The distilled water used in this work was doubly distilled and deionized. Most buffers were prepared by mixing appropriate ratios of the acid or base and its salt, but with HEPES, CHES, BICINE, and TRICINE the pH was adjusted to 8.6 with 1 M NaOH. CHES buffer in D_2O (pD 8.6) was prepared by dissolving the appropriate amount of CHES in D_2O and adjusting the meter reading to 8.2 with an NaOD solution. Since the pH meter was initially standardized against aqueous buffers, the pD of such a solution is thus 8.6.[9]

6-(*N*-Methylamino)-5-aminouracil (4b). To a boiling solution of 8.5 g (0.05 mol) of 6-(*N*-methylamino)-5-nitrosouracil[10] in 150 mL of water was added gradually 17.0 g (0.10 mol) of sodium hydrosulfite, and the boiling was continued for 15 min. After filtration while hot, the solution was cooled to room temperature whereupon crystals of the aminouracil separated. The product was collected by filtration, washed with ice water, and dried. Data: yield, 6.0 g (77%); mp, 252 °C; λ_{max} (in 0.1 N NaOH) 275 nm (ϵ 4200 M^{-1} cm^{-1}). Anal. Calcd for $C_5H_8N_4O_2$: C, 38.46; H, 5.13; N, 35.90. Found: C, 38.13; H, 5.01; N, 35.68.

6-(*N*-Alkylanilino)-5-aminouracil (4c, 4d). To a filtered solution of 3.83 g (0.02 mol) of 6-chloro-5-nitrouracil[11] in 150 mL of absolute ethanol was added a solution of 0.02 mol of *N*-alkylaniline (alkyl = methyl, ethyl) and 2.0 g (0.02 mol) of triethylamine in 50 mL of ethanol. After the mixture was refluxed for 10 min, 100 mL of ethanol was removed by distillation and the mixture cooled in an ice bath. The crystals that formed were separated by filtration, washed successively with cold ethanol and ether, and dried. To a boiling suspension of 0.01 mol of this 6-(*N*-alkylanilino)-5-nitrouracil in 40 mL of water was added gradually 3.4 g (0.02 mol) of sodium hydrosulfite and the boiling continued until the yellow color disappeared. After filtration while hot, the solution was cooled to room temperature whereupon the product (**4c**, **4d**) crystallized. In the case of **4c** the product was recrystallized from ethanol. Characteristics of **4c**: mp 256 °C; λ_{max} (in 0.1 N NaOH) 322 nm (ϵ 12 700 M^{-1} cm^{-1}), 238 (ϵ 15 600). Anal. Calcd for $C_{11}H_{12}N_4O_2$: C, 56.89; H, 5.17; N, 24.09. Found: C, 56.62; H, 5.45; N, 23.63. Characteristics of **4d**: mp 194–196 °C; λ_{max} (in 0.1 N NaOH) 324 nm (ϵ 11 500 M^{-1} cm^{-1}), 239 (ϵ 13 900). Anal. Calcd $C_{12}H_{14}N_4O_2$: C, 58.54; H, 5.69; N, 22.76. Found: C, 58.78; H, 5.78; N, 22.27.

6-(*N*-Methylanilino)-3-methyl-5-aminouracil (3-Me-4c). This was prepared by the same procedure as used for **4c** except that the starting material was 6-chloro-3-methyl-5-nitrouracil.[12] Characteristics: mp 163–164 °C; λ_{max} (in 0.1 N NaOH) 321 nm (ϵ 13 900 M^{-1} cm^{-1}), 238 (ϵ 16 200). Anal. Calcd for $C_{12}H_{14}N_4O_2$: C, 58.54; H, 5.69; N, 22.76. Found: C, 58.77, H, 5.66; N, 22.90.

Registry No. 4a, 3240-72-0; **4b**, 80277-74-3; **4c**, 62348-44-1; 3me-**4c**, 62348-50-9; **4d**, 62348-45-2; Cu, 7440-50-8; PhNHMe, 100-61-8; PhNHEt, 103-69-5; D_2, 7782-39-0; 6-(*N*-methylamino)-5-nitrosouracil, 56128-57-5; 6-chloro-5-nitrouracil, 6630-30-4; 6-chloro-3-methyl-5-nitrouracil, 878-86-4; amine oxide, 9059-11-4.

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