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The Chlorination of Barbituric Acid and Some of Its Derivatives by Chloroperoxidase

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Barbituric acid and some of its derivatives are presented as new substrates for the chloroperoxidase from *Caldariomyces fumago*. These compounds are rapidly converted to the 5-chloro or 5,5-dichloro derivatives, in very high yield. The reaction path is discussed and the kinetics of the reactions are investigated. It is shown that neither the concentration nor the structure of the organic substrate has any influence on the rate of halogenation. The enzymatic chlorination of 1-methyl-5-phenylbarbituric acid does not proceed in a stereoselective manner. The results are compared with the present theories concerning the enzymatic reaction mechanism, and the current research on this topic is evaluated. The available data do not as yet permit a definitive choice of reaction mechanism.

INTRODUCTION

Chloroperoxidase from *Caldariomyces fumago* (EC 1.11.1.10, chloride: hydrogen-peroxide oxidoreductase) is a widely studied enzyme capable of halogenating a variety of organic compounds by means of hydrogen peroxide and chloride, bromide, or iodide ions. Examples are β-dicarbonyl species such as monochlorodimedon (*1, 2*), alkenes (*3–5*), cyclopropanes (*6*) and the heterocycles thiazole (*7*), antipyrine (*8*), and NADH (*9*).

The reaction mechanism of the enzyme is still a matter of controversy. There is some evidence that a complex is formed involving an oxidized form of the enzyme, a halide ion, and the organic substrate (*10, 11*). Other results imply that the halogenation is performed by an enzyme-made hypohalous acid (*12, 13*). A third mechanism was published recently involving hypohalous acid or elemental halogens and radical intermediates (*14*).

Due to our continuing interest into the chemistry of heterocycles and the use of (immobilized) enzymes in organic syntheses (*15*) we investigated the potential application of chloroperoxidase (CPO) in heterocyclic chemistry. In this paper, we wish to present the results on the CPO-mediated halogenation of barbituric acid and some of its derivatives. Moreover, we want to report on our studies concerning the kinetics of these halogenation reactions and on their implications

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for the enzymatic reaction mechanism. Part of this work has already been published as a preliminary communication (*16*).

MATERIALS AND METHODS

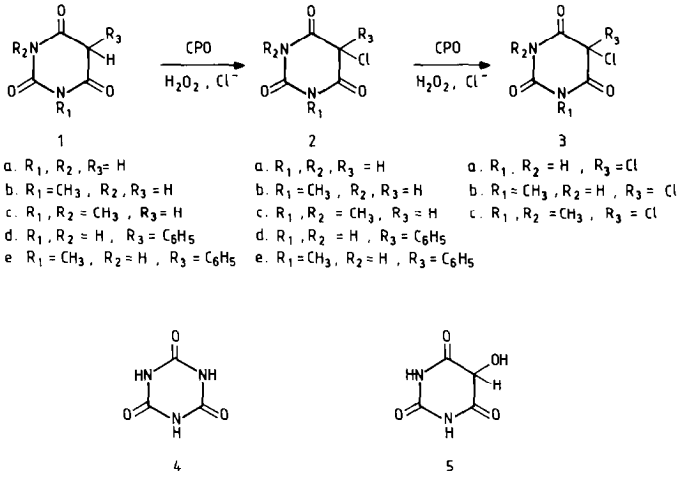
General. Chloroperoxidase (crude type) from *Caldariomyces fumago* was obtained from Sigma Chemical Company or was a gift from E. de Boer and Dr. R. Wever from the Laboratory of Biochemistry, University of Amsterdam, The Netherlands. The specific activity of the enzyme was 400–600 μmol monochlorodimedone · mg protein^{-1} · min^{-1}, depending on the batch used. Protein concentrations were determined by the Lowry method (*17*) using bovine serum albumin as standard. Monochlorodimedone (MCD) was also purchased from Sigma. 1,3-Dimethylbarbituric acid was obtained from Fluka and the other substituted barbituric acids were synthesized from the corresponding malonic ester and urea derivatives according to the procedure of Dickey and Gray (*18*). 5-Chlorobarbituric acid and 5,5-dichlorobarbituric acid were obtained by means of Bock's method (*19*). Sodium hypochlorite solution was purchased from Janssen Chimica and was assayed by injecting an appropriate amount in 0.1 mM MCD, pH 2.7. The difference in A_{278} is proportional to the hypochlorite concentration, using $\Delta\varepsilon$ = 12200 M^{-1} · cm^{-1}. All chemicals were of the highest commercial grade.

UV spectra and kinetic measurements were performed on an Aminco–Chance DW-2 split-beam spectrophotometer. Mass spectra were recorded on an AEI MS 902 instrument or a VG Micromass 7070 F apparatus (direct probe mode). Circular dichroism spectra were run on a Jobin–Yvon Auto-Dichrograph Mark V. HPLC analysis was carried out on a Varian 5000 instrument (see below for experimental details).

HPLC measurements. The reaction of CPO with barbituric acid and its derivatives could be monitored by HPLC using reversed-phase, ion-pair chromatography. The stationary phase was a Spherisorb-S 10 ODS column. The mobile phase was a mixture of 20% methanol and 80% water containing 10 mM potassium phosphate and 5 mM nonyltrimethylammonium bromide, pH 7.3 (28% methanol for **1e**). The eluent flow was 1.0 ml/min. An aliquot of the enzymatic reaction was taken and directly injected into the chromatograph. The barbituric acids were detected by means of their UV absorption at 265 nm (245 nm for **1c**).

Kinetic measurements. Specific activities of the enzyme were determined under the standard assay conditions (*2*): 100 mM H_3PO_4/KOH, pH 2.7, 20 mM KCl, 0.24 mM H_2O_2, 0.1 mM MCD, 25°C. To 2.5 ml of this solution was added 100–150 ng CPO and the absorption at 278 nm was followed, using $\Delta\varepsilon$ = 12,200 M^{-1} · cm^{-1}. These conditions were also applied in all other kinetic measurements and in the experiments where there was no need for isolation of the product; for the compounds **1a–c** 0.48 mM H_2O_2 was used. All determinations were carried out in triplicate.

The following molar absorption coefficients ($\Delta\varepsilon$, M^{-1} · cm^{-1}) were used at pH



SCHEME 1. Reaction pattern of the halogenation of barbituric acid (**1a**) and some of its derivatives by CPO/H_2O_2/Cl^-. Cyanuric acid (**4**) and 5-hydroxybarbituric acid (**5**) were not chlorinated by the enzyme.

2.7: 5-chlorobarbituric acid, 13,800 at 268 nm; 5-phenylbarbituric acid, 15,800 at 267 nm.

Circular dichroism. (*CD*). The following mixture was made: 2.44 ml of a solution of 50 μM 1-methyl-5-phenylbarbituric acid in 0.1 M H_3PO_4/KOH, pH 2.7, containing 20 mM KCl and 60 μl freshly prepared 10 mM H_2O_2 in doubly-distilled water. To this was added 440 ng CPO, and the reaction was monitored with a UV spectrophotometer at 268 nm. When the reaction was complete, a CD spectrum was recorded between 210 and 330 nm. A reference spectrum was made of the same solution by replacing the hydrogen peroxide solution with 60 μl 0.1 M H_3PO_4/KOH, pH 2.7. This spectrum was substrated from the first one, yielding the final CD spectrum of the enzymatic conversion.

Isolation of enzymatic products. Since the barbituric acids are much too soluble in water, isolation of the products by direct extraction with organic solvents is not very effective; therefore the halogenation reaction was performed in the following way. The standard reaction medium was replaced by dilute HCl, pH 2.7, containing 5 mM KCl and 0.24 mM H_2O_2. When needed, some extra H_2O_2 was added. A suitable amount of CPO was injected into the solution, typically 0.5 μg per milliliter of reaction medium containing 1.0 mM barbituric acid derivative. When UV spectroscopy indicated that the reaction was complete the reaction mixture was lyophilized and the product was extracted from the solid material with distilled methanol. In this way, a more or less salt-free product was obtained, which was analyzed by mass spectroscopy. The mass spectra of all compounds were identical to those of authentic specimen.

Reactions with HOCl. To 1 mmol substrate in water/HCl, pH 2.7, or buffer were added 2 mmol (**1a–c**) or 1 mmol (**2d,e**) of HOCl in four portions with stirring.

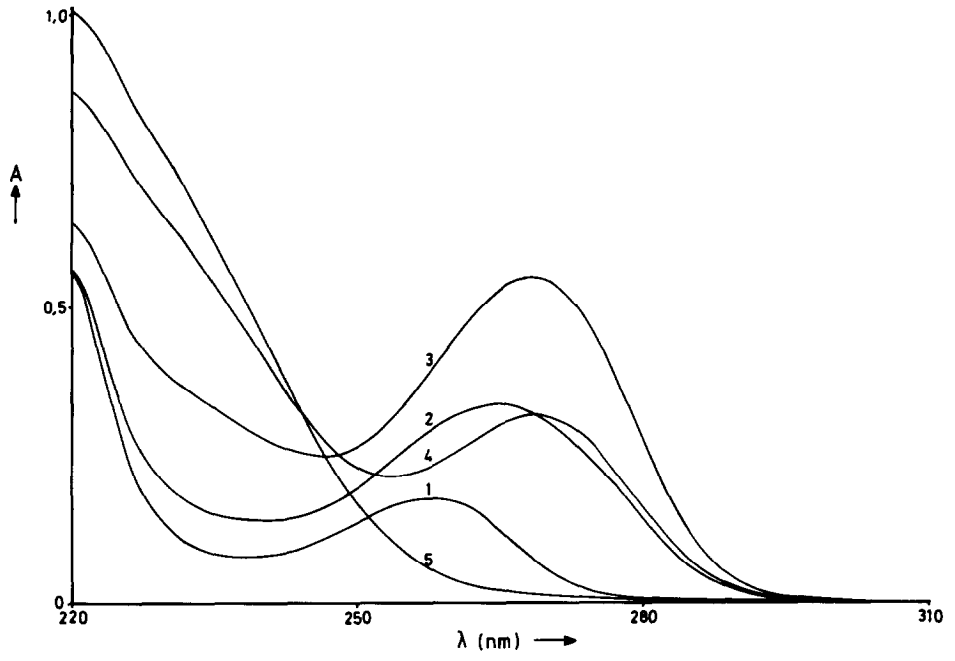


FIG. 1. UV absorption spectra as recorded during the reaction of barbituric acid (**1a**) with chloroperoxidase. The quartz cuvette was filled with 2.5 ml of a solution containing 0.1 mM **1a**, 0.48 mM H_2O_2, 20 mM KCl, and 0.1 M H_3PO_4/KOH, pH 2.7. To this 114 ng of CPO was added, and UV spectra were recorded with appropriate time intervals. 1, t = 0 min (pure **1a**); 2, t = 0.5 min; 3, t = 2.0 min; 4, t = 5.0 min; 5, t = 8.0 min (reaction is complete).

The pH of the solution was maintained at 2.7 by adding dilute HCl when needed. After 15 min incubation the reaction mixture was either lyophilized and extracted with distilled methanol or analyzed by HPLC (see above).

RESULTS

Reaction of CPO with Barbituric Acid and Its Derivatives

When barbituric acid (**1a**) is incubated with CPO under standard conditions, a rapid change in the UV spectrum is visible (see Fig. 1). During the first 2 min of the reaction the original absorption peak of **1a** at 258 nm shifts to a higher wavelength (268 nm) with a concomitant increase of the absorption intensity. After this period the peak at 268 nm slowly disappears, indicating the consecutive formation of a second product (peak at 210 nm, not shown in the figure). The same observation is made when monitoring the reaction by reversed-phase, ion-pair HPLC (see Fig. 2).

At t = 0 min, only a peak of **1a** appears (t_r = 4.3 min). After 0.5 min reaction

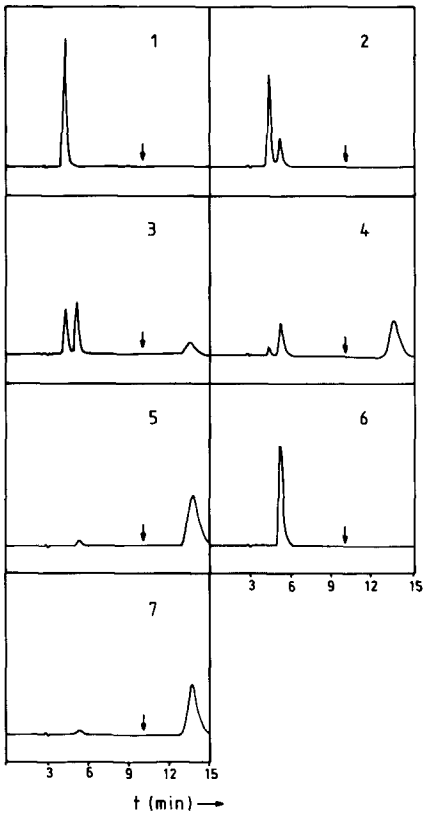


FIG. 2. HPLC chromatograms as recorded during the reaction of **1a** with CPO. See Fig. 1 for experimental details. Ten microliters of the enzymatic reaction medium was injected directly into the chromatograph (see Materials and Methods). Eight minutes after each injection, the absorption scale was changed from 0.055 to 0.005 AUFS (indicated by arrow). 1, t = 0 min (pure **1a**); 2, t = 0.5 min; 3, t = 2.0 min; 4, t = 5.0 min; 5, t = 8.0 min (reaction is complete); 6, authentic **2a**; 7, authentic **3a**.

time, the peak of the first product becomes visible (t_r = 5.2 min) and at t = 2 min, the second product starts to form (t_r = 13.6 min). After 8 min reaction time no further changes in the chromatogram are observed, and only a large peak of the second product and a small one of the primary compound are visible.[2]

The UV spectra and the retention times of the products coincide perfectly with authentic 5-chlorobarbituric acid (**2a**) and 5,5-dichlorobarbituric acid (**3a**), indicating that **1a** undergoes chlorination at C-5. That these compounds are indeed formed is confirmed by the mass spectra of **2a** and **3a**, isolated from the enzyme-mediated chlorination of **1a**, which were fully identical to those obtained from

[2] It should be noted that the described chromatographic system separates various barbituric acid derivatives excellently, whereas other stationary phases (silica, silica modified with propionitril or octadecyl groups, or ion-exchange columns) either give no separation or unacceptably long retention times.

TABLE 1

YIELDS OF THE ENZYMATIC AND CHEMICAL CHLORINATION OF **1a** AND SOME OF ITS DERIVATIVES[a]

		Yield (%)	
Starting material	Product	$CPO/H_2O_2/Cl^-$	HOCl
1a	**3a**	99	90
1b	**3b**	98	85
1c	**3c**	94	67
1d	**2d**	98	50[b]
1e	**2e**	98	94

[a] The yields are determined by HPLC.

[b] Could be increased to 72% by adding extra 0.4 equivalents HOCl.

authentic samples. The reaction sequence is depicted in Scheme 1. Additional evidence for the suggested reaction path is given by the fact that authentic **2a** is converted by the enzyme into **3a** at a high rate, showing that the first chlorination takes place at C-5 and a subsequent chlorination at the same position. The yield of **3a** is 99%, based on the HPLC measurements. No N-halogenated product is detectable; this is supported by our observation that cyanuric acid (**4**) shows no reactivity toward the enzyme.

Comparison of the enzyme-mediated chlorination with the standard chemical procedures for chlorination of **1a** (*19–21*) show that the yield is higher in the enzymatic reaction.[3] However, pure 5-monochlorobarbituric acids like **2a–c** cannot be prepared directly by the enzymatic procedure: if the reaction is stopped halfway or if only one equivalent of H_2O_2 is used a mixture of starting material and monochloro and dichloro product is obtained. It has been reported that by combining the enzymatic conversion with an electrochemical reduction, the initially produced **3a** can be reduced quantitatively into **2a** in one step, allowing the isolation of pure **2a** (*22, 23*).

The enzymatic chlorination reaction was also investigated with substituted barbituric acids as substrates. 1-Methylbarbituric acid (**1b**) and 1,3-dimethylbarbituric acid (**1c**) are converted via their 5-chloro derivatives **2b** and **2c** into the 5,5-dichloro compounds **3b** and **3c** (see Scheme 1) in yields comparable to those obtained with **1a** (see Table 1). The 5-phenylbarbituric acids **1d** and **1e** are also found to be very good substrates for the enzyme, giving the corresponding 5-chloro derivatives **2d** and **2e**, respectively, in very good yields. Remarkably, 5-hydroxybarbituric acid (**5**) seems to resist the enzymatic chlorination reaction since the UV spectrum of **5** did not show any change.

[3] For example, **3a** was synthesized by treating **1a** with chlorine in water (*19, 20*). No yield was given, but in our hands it was 56%.

TABLE 2

RELATIVE REACTION RATES OF THE ENZYMATIC CHLORINATION OF VARIOUS SUBSTRATES[a]

Compound	Relative reaction rate[b]
MCD	100 ± 3[c]
1d	98 ± 6
2a	110 ± 5

[a] Determined under standard assay conditions (see Materials and Methods).

[b] Arbitrary units; the value for MCD was set at 100; $n = 2$; each value is the mean of three determinations.

[c] Corresponds to 400 μmol MCD · mg protein^{-1} · min^{-1}.

Kinetics of the CPO-Mediated Chlorination of Barbituric Acids

To gain further insight into the mechanism of the enzyme-mediated reaction, we studied the kinetics of the chlorination of barbituric acid (**1a**) and some of its derivatives (**1b–e**).

Comparison of the enzymatic chlorination rate of MCD with those determined with **1d** and **2a** shows that the velocities of the three reactions differ only to a very small extent (see Table 2). The presence of methyl groups on the barbituric acid nitrogen atoms also has no effect on the rate of chlorination (Table 3). Since we deal with two competing reactions (**1** → **2** and **2** → **3**) we determined the total time

TABLE 3

TIME NEEDED FOR COMPLETE CONVERSION (**1** → **3**) OF SOME BARBITURIC ACIDS[a]

Compound	Conversion time[b]
1a	100 ± 3[c]
1b	101 ± 4
1c	111 ± 4

[a] Determined under standard assay conditions by monitoring the UV absorption at 268 nm, except that $[H_2O_2] = 0.48$ mM.

[b] Arbitrary units; the value for **1a** was set at 100; $n = 2$; each value is the mean of three determinations.

[c] Corresponds to 150 s.

in which the reaction **1** → **3** is completed. The results thus obtained indicate that the barbituric acids show considerable flexibility in their structure regarding their reactivity toward CPO. It appears that they do not influence the reaction rate, suggesting that the active site of the enzyme can accommodate compounds with rather different electronic and steric influences. This result is further supported by the fact that the concentration of neither MCD, nor of **1d** and **2a** in the range of 2 to 100 μM, has any effect on the enzymatic reaction rate, so no K_m value could be obtained for these substrates.

Additional information is obtained from inhibition experiments. As mentioned previously, cyanuric acid (**4**) and 5-hydroxybarbituric acid (**5**) are not reactive toward the enzyme. Since both compounds contain the β-dicarbonyl function common to all good substrates for CPO, there is the possibility of a nonproductive binding. However, we find that **4** and **5** in concentrations up to 200 μM are not able to inhibit the enzymatic chlorination of MCD.

Stereochemistry of the Chlorination Reaction

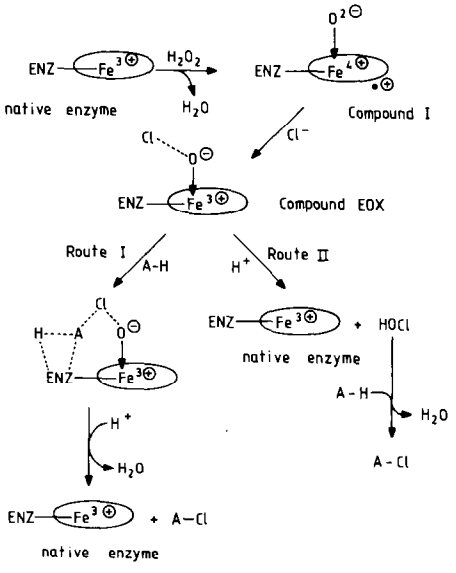
To determine whether the enzyme-mediated halogenation shows some stereoselectivity, we investigated the halogenation of 1-methyl-5-phenylbarbituric acid (**1e**). Compound **1e** has a chiral C-5 atom. Resolution of the two enantiomers in **1e** is not possible, however, because of the rapid enolization at C-5 in protic solvents. When the hydrogen at C-5 in **1e** is replaced by a halogen atom, the configuration is fixed and possibly one isomer is formed preferentially in a stereoselective reaction. To examine whether CPO is capable of stereoselective halogenation, the enzymatic chlorination of **1e** is monitored by means of circular dichroism. However, the CD spectrum shows no absorption, clearly indicating that a racemic mixture is obtained and thus no stereoselective reaction has occurred. This result is in agreement with investigations performed by other authors (*3, 4, 24*).

Reactions with HOCl

When the barbituric acids are incubated with HOCl, we find that the products are the same as in the enzymatical reactions, but yield and purity are generally much lower: **1a–c** give mixtures of **2a–c** and **3a–c**. The results are summarized in Table 1. The yields of the 5,5-dichlorobarbituric acids are not improved by adding more than 2 equivalents HOCl, because this results in partial degradation of the heterocyclic compounds.

DISCUSSION

The chloroperoxidase from *C. fumago* is, as shown in these studies, capable of smoothly converting barbituric acid and some of its derivatives into the corresponding 5-chloro or 5,5-dichloro compounds. The very high yields in which the products are obtained make the enzymatic reaction competitive with chemical syntheses.



SCHEME 2. The reaction mechanism of *Caldariomyces fumago* chloroperoxidase. The protein part of the enzyme is represented by ENZ, the heme group is depicted as Fe^{+x} (x = 3 or 4) in the center of an ellipse, for reasons of clarity. In Compound I, the iron atom has a 4+ oxidation state and the porphyrin ring is oxidized to a radical cationic species. The covalent bonds which are generally accepted in literature are indicated by solid lines between the atoms; if there is any doubt dashed lines are used. A–H = organic substrate, A–Cl = chlorinated product.

The reaction mechanism of the enzyme is depicted in Scheme 2. Native CPO is transformed by H_2O_2 to an enzyme form named Compound I. This highly oxidized species contains an iron(IV) ion and a radical cation located in the porphyrin ring of the heme group (*25*). Compound I is reduced by Cl^- to a species called Compound EOX, containing an oxygen atom which is attached to the iron and a chlorine atom which is located in the proximity of the heme, but is not an iron ligand (*26*). Two alternative pathways are advanced concerning the following steps: route I, suggesting that the organic substrate (A–H) binds to the enzyme to form a complex which splits into the native enzyme, the organic product, and a molecule of water, and/or route II, in which Compound I decomposes to native CPO and a molecule of hypochlorous acid, being the active halogenating species in this reaction.

Evidence for the occurrence of route I was based on kinetic measurements: (i) the ratio of the reaction velocities of thiourea and MCD was different for CPO/H_2O_2/Cl^- and HOCl (*11*); (ii) the reaction constant for the enzymatic conversion of MCD was 200 times higher than the reaction constant for MCD and HOCl (*10*); (iii) a K_m value was found for MCD and 2-methyl-4-propylcyclopentanedione (*4*), indicating some kind of interaction between the organic substrate and the enzyme active site.[4]

[4] These data are most probably incorrect, *vide infra*.

Support for route II comes from the fact that the products obtained from both enzymatic and HOCl reactions (*2, 5, 6*) as well as their ratios in case of mixture (*13*), are identical. No stereoselectivity was found with the enzymatic reactions (*3, 4, 24*). Without addition of an organic substrate, the enzyme produces HOCl (*11*), and studies on the catalase reaction of the enzyme also pointed to HOCl involvement (*12*). Recently, a combination of free radicals and molecular halogens was suggested to play a role in the halogenation reaction (*14*).

The results presented in this publication show that CPO is an enzyme with a broad substrate specifity. Barbituric acid (**1a**) and a number of its derivatives are chlorinated by the enzyme in very good yields. HOCl has the same regioselectivity as CPO, since with HOCl the same chloro compounds are obtained, although both yield and purity of the products are usually far lower than in the enzymatic reaction. In contrast to what is reported in the literature (*4*), the concentration of MCD (or any barbituric acid derivative, not shown) has no influence on the enzymatic reaction rate in the range 2–100 μM. Moreover, even the nature of the organic substrate is not important to the enzyme, as can be concluded from the results mentioned in Tables 2 and 3.[5] Potential inhibitors such as **4** and **5** have no effect, and the enzyme-mediated chlorination of **1e** is not stereoselective.

Although it is tempting to conclude from our work that CPO from *C. fumago* reacts via route II, generating free HOCl, caution should be exercised. The fact that both CPO and HOCl show the same regioselectivity, and complete lack of stereoselectivity, cannot be considered as a definite proof for enzymatically generated free HOCl since it remains possible that the enzyme has a HOCl-like reactivity, for instance via heme-bound hypochlorite. In addition, most studies mentioned in the literature deal with substrates which are far less reactive than MCD and which use much more enzyme and longer reaction times than is necessary for standard assay conditions. It is possible that under reaction conditions in which the enzyme cannot find a suitable substrate HOCl is produced as if there were no substrate at all.

The fact that **1e** is not chlorinated stereoselectively cannot be considered as evidence for route I because the difference between the enantiotopic faces of the substrate is possibly too small to exert influence. Moreover, it is questionable whether stereoselectivity is an intrinsic property of the enzyme, as shown in the conversion of cyclopentanedione (its natural substrate, *27*) to caldariomycin, in which there is no need for stereoselective control.

The kinetic measurements presented in this study do not allow a decision whether route I or II is most favored either. The fact that the concentration of the organic substrate has no influence on the rate-determining step does not exclude route II. The reaction between MCD or barbiturates and HOCl is possibly so fast that the first two steps in the enzymatic reaction sequence, which are independent of the organic substrate concentration, determine the overall reaction velocity. The same reasoning is valid for route I: if step 1 or 2 is much slower than the

[5] The fact that **4** and **5** are not converted by the enzyme seems contradictory to this conclusion; however, neither of these compounds are halogenated by HOCl, so these results do not support any mechanism.

subsequent steps, the concentration of the organic substrate will not affect the overall reaction velocity. The fact that the rate constant for the enzymatic chlorination step could only be determined under nonstandard assay conditions with stopped-flow techniques is evidence for this view (*10*). A K_m value for MCD is reported (*4*), but our results indicate that it cannot have been determined at pH 2.7 using 20 mM KCl and 0.24 mM H_2O_2.

We conclude that most of the literature data concerning the reaction mechanism of CPO, including our own, do not permit a choice between route I or II. However, even if route II is proven to be correct, the steady-state concentration of HOCl in the reaction medium is so low that CPO is still a smooth halogenating agent allowing the synthesis of various chlorinated barbituric acids in very high yields. Therefore the use of chloroperoxidase as a synthetic tool in organic chemistry will remain a subject of current investigation.

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REFERENCES

1. Hager, L. P., Morris, D. R., Brown, F. S. and Eberwein H. (1966) *J. Biol. Chem.* **241,** 1769.
2. Hallenberg, P. F. and Hager, L. P. (1978) *in* Methods in Enzymology, Vol. 52, p. 521, Academic Press, Orlando, FL.
3. Geigert, J., Neidleman, S. L., Dalietos, D. J., and DeWitt, S. K. (1983) *Appl. Environ. Microbiol.* **45,** 366.
4. Ramakrishnan, K., Oppenhuizen, M. E., Saunders, S., and Fisher, J. (1983) *Biochemistry* **22,** 3271.
5. Yamada, H., Itoh, N., and Izumi, Y. (1985) *J. Biol. Chem.* **260,** 11962.
6. Geigert, J., Neidleman, S. L., and Dalietos, D. J. (1983) *J. Biol. Chem.* **258,** 2273.
7. Neidleman, S. L., Cohen, A. I., and Dean, L. (1969) *Biotechnol. Bioeng.* **11,** 1227.
8. Ashley, P. L., and Griffin, B. W. (1981) *Arch. Biochem. Biophys.* **210,** 167.
9. Griffin, B. W., and Haddox, R. (1985) *Arch. Biochem. Biophys.* **239,** 305.
10. Lambeir, A. M., and Dunford, H. B. (1983) *J. Biol. Chem.* **258,** 13558.
11. Libby, R. D., Thomas, J. A., Kaiser, L. W., and Hager, L. P. (1982) *J. Biol. Chem.* **257,** 5030.
12. Griffin, B. W. (1983) *Biochem. Biophys. Res. Commun.* **116,** 873.
13. Lee, T. D., Geigert, J., Dalietos, D. J., and Hirano, D. S. (1983) *Biochem. Biophys. Res. Commun.* **110,** 880.
14. Griffin, B. W., and Ashley, P. L. (1984) *Arch. Biochem. Biophys.* **233,** 188.
15. Namely, our work on aldehyde oxidase, Angelino, S. A. G. F., and van der Plas, H. C. (1984) *in* Bio-Organic Heterocycles (van der Plas, H. C., Ötvös, L., and Simonyi, M., Eds.), pp. 285–317. Akadémiai Kiadó, Budapest, and our work on xanthine oxidase, Naeff, H. S. D. van der Plas, H. C., Tramper, J., and Müller, F. (1985) *Quant. Struct. Act. Relat.* **4,** 161, and references cited therein.
16. Franssen, M. C. R., and van der Plas, H. C. (1984) *Recl. Trav. Chim. Pays-Bas* **103,** 99.

17. LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L., AND RANDALL, R. J. (1951) *J. Biol. Chem.* **193,** 265.
18. DICKEY, J. B., AND GRAY, A. R. (1938) *Org. Syn.* **18,** 8.
19. BOCK, W. (1923) *Ber. Dtsch. Chem. Ges.* **56,** 1222.
20. MACBETH, A. K., NUMAN, T. H., AND TRAILL, D. (1926) *J. Chem. Soc.,* p. 1248.
21. ZIEGLER, E., SALVADOR, R., AND KAPPE, TH. (1962) *Monatsh. Chem.* **93,** 1376.
22. LAANE, C., PRONK, W., FRANSSEN, M. C. R., AND VEEGER, C. (1984) *Enzyme Microb. Technol.* **6,** 165.
23. LAANE, C., WEYLAND, A., AND FRANSSEN, M. C. R. (1986) *Enzyme Microb. Technol.* **8,** 345.
24. KOLLONITSCH, J., MARBURG, S., AND PERKINS, L. M. (1970) *J. Amer. Chem. Soc.* **92,** 4489.
25. RUTTER, R., HAGER, L. P., DHONAN, H., HENDRICH, M., VALENTINE, M., AND DEBRUNNER, P., (1984) *Biochemistry* **23,** 6809.
26. KREJCAREK, G. E., BRYANT, R. G., SMITH, R. J., AND HAGER, L. P. (1976) *Biochemistry* **15,** 2508.
27. BECKWITH, J. R., AND HAGER, L. P. (1963) *J. Biol. Chem.* **238,** 3091.