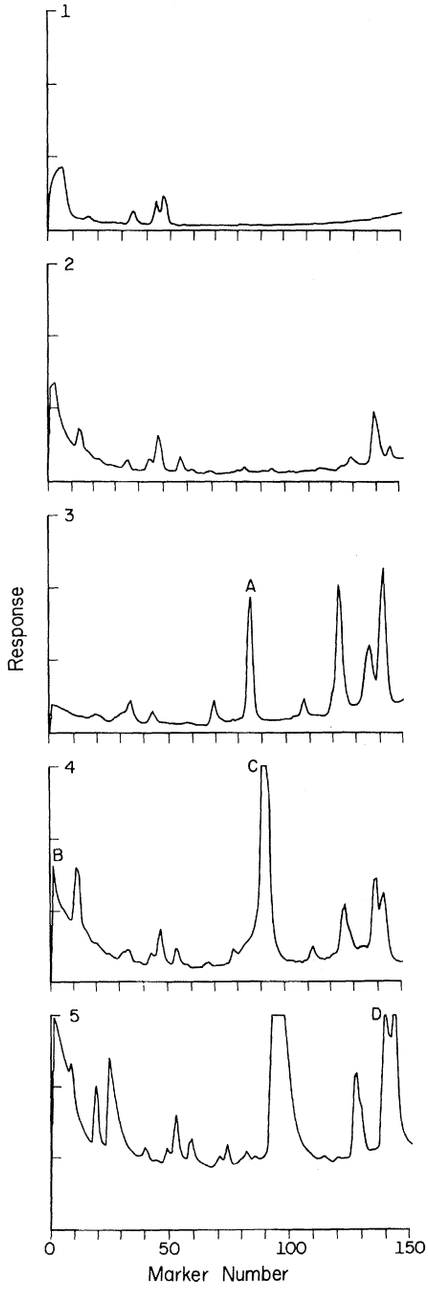
Halohydrocarbon Synthesis by Bromoperoxidase

Abstract. *An enzyme extracted from marine red algae,* Bonnemaisonia hamifera, *is capable of incorporating bromine into a number of organic substrates in the* pH *range 5 to 8. At* pH *7.3, incubation of partially purified preparations of bromoperoxidase with hydrogen peroxide, bromide ion, and 3-oxooctanoic acid leads to the formation of three volatile brominated hydrocarbons: dibromomethane, bromoform, and 1-pentyl bromide. The presence of significant quantities of halometabolites including volatile halohydrocarbons in marine organisms, ocean waters, and the upper atmosphere may result from peroxidase-catalyzed halogenation reactions.*

During an expedition of the research vessel *Alpha Helix* to Baja, California, in 1974, more than 1000 samples of marine organisms were collected and assayed for halometabolites (*1*). This work demonstrated the widespread occurrence of organic compounds containing bromine and chlorine and indicated that expanded studies on the chemistry of marine natural products would lead to the discovery and characterization of many new halometabolites. About 25 percent of the organisms collected on the Baja expedition yielded lipid extracts that contained more than 10 μg of organic halogen per gram (wet weight) of tissue; red algae (Rhodophyta) were found to be a particularly rich source of halogen-containing organic compounds. These compounds include a diverse array of halogenated terpenes, hydrocarbons, and phenols (*2, 3*). Recently, members of the family Bonnemaisoniaceae (Nemaliales) have been shown to produce a variety of halogenated ketones (*4–6*). Dibromo-, tribromo-, and tetrabromo-2-heptanones and bromoiodo-2-heptanones have been isolated from the odorous alga *Bonnemaisonia hamifera*.



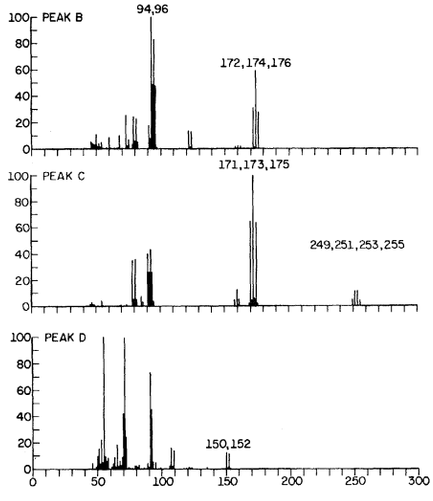
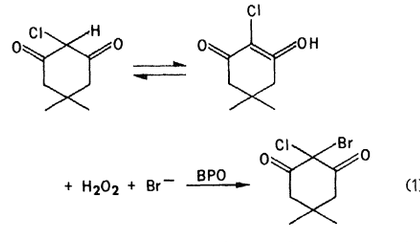


Fig. 1 (left). Gas chromatographic traces of the ether extracts of the bromoperoxidase reaction incubated at *p*H 5.6 (trace 2), *p*H 6.6 (trace 3), *p*H 7.3 (trace 4), and *p*H 7.3 with a twofold increase in the amount of hydrogen peroxide added to the reaction mixture (trace 5). Trace 1 represents a blank control that was incubated for 60 minutes in the presence of all reagents except bromoperoxidase. The reaction conditions are described in the text. Gas chromatography was carried out on columns of 0.2 percent Carbowax 1500 on Carbopak C at temperatures between 100° and 220°C, programmed to rise at 10°C per minute. This is a total ion current summation plot of amplitude versus spectral scan number. In these experiments, bromoform retention was approximately 9 minutes; thus marker 100 (spectral scan number) corresponds to a retention time of 9 to 10 minutes. The various runs were made on different days at slightly different rates of gas flow; thus identical peaks are not perfectly aligned in the five traces. However, all peaks were examined by mass spectrometry at a scan speed of 2.2 seconds per decade (0.5-second return time) so that correct assignments could be made in all cases. The computer scans were not started until the solvent peak had largely subsided. Fig. 2 (right). Mass spectra of peaks B, C, and D in Fig. 1. All mass spectra are corrected for background. Data were collected only for ions above *m/e* 45.

One objective of our study of marine halometabolites has been to examine the mechanisms responsible for the biosynthesis and metabolism of these compounds. We have assumed that the biogenesis of marine halometabolites must encompass halogenation reactions similar to those of chloroperoxidase (*7*). Chloroperoxidase, which has been purified from the fungus *Caldariomyces fumago*, is capable of introducing iodine, bromine, and chlorine atoms into organic molecules through an electrophilic substitution mechanism (*8*). In this study we examined biological halogenation reactions in *B. hamifera*. Crude extracts of this organism exhibit a powerful enzymatic brominating activity (*9*).

Samples of *B. hamifera* were collected in the vicinity of Punta Mejia off the northernmost point of Isla Angel de la Guarda in the Gulf of California in March 1974 and were stored at −20°C. The extraction and partial purification of a halogenating enzyme from *B. hamifera* has been reported (*9*). As shown in reaction 1



(1)

the rate of formation of 2-bromo-2-chloro-5, 5-dimethyl-1, 3-cyclohexanedione (bromochlorodimedon) from 2-chloro-5, 5-dimethyl-1, 3-cyclohexanedione (monochlorodimedon) formed the basis of the standard assay for bromoperoxidase (*BPO*) activity (*10*). Bromoperoxidase is unable to oxidize either Cl^- or F^- but can effectively oxidize I^- and Br^-. The ability of the enzyme to catalyze the oxidation of halogen anions was measured spectrophotometrically by monitoring both the formation of I_3^- from I^-

0036-8075/78/1208-1094$00.50/0

at 350 nm and the direct halogenation of monochlorodimedon by Br^- at 292 nm. With monochlorodimedon as a substrate, the enzyme was found to be active over the *p*H range 5 to 8. The *p*H optimum for bromination of monochlorodimedon was 5.4; however, the optimum may vary depending on the nature of the acceptor molecule.

Various nucleophilic substrates have been examined as potential halogen acceptors in the synthesis of halometabolites by bromoperoxidase. Since brominated heptanones are the major natural halogen-containing compounds produced by *B. hamifera* (*4*), the β-ketoacids, 3-oxooctanoic acid, and 3-oxohexanoic acid were examined for acceptor activity.

By analogy to chloroperoxidase reactions, the following reaction sequence could be visualized for the formation of brominated heptanones from 3-oxooctanoic acid,

$$\text{Enz-Br}^+ + \text{HO}_2\text{C-CH}_2\text{-}\overset{\overset{\text{O}}{\|}}{\text{C}}\text{-(CH}_2)_4\text{-CH}_3 \rightarrow$$

$$\text{CO}_2 + \text{Br-CH}_2\text{-}\overset{\overset{\text{O}}{\|}}{\text{C}}\text{-(CH}_2)_4\text{-CH}_3 + \text{H}^+ \quad (2)$$

$$\text{Enz-Br}^+ + \text{Br-CH}_2\text{-}\overset{\overset{\text{O}}{\|}}{\text{C}}\text{-(CH}_2)_4\text{-CH}_3 \rightarrow$$

$$\text{Br}_2\text{-CH-}\overset{\overset{\text{O}}{\|}}{\text{C}}\text{-(CH}_2)_4\text{-CH}_3 + \text{H}^+ \quad (3)$$

$$\text{Enz-Br}^+ + \text{Br}_2\text{-CH-}\overset{\overset{\text{O}}{\|}}{\text{C}}\text{-(CH}_2)_4\text{-CH}_3 \rightarrow$$

$$\text{Br}_3\text{-C-}\overset{\overset{\text{O}}{\|}}{\text{C}}\text{-(CH}_2)_4\text{-CH}_3 + \text{H}^+ \quad (4)$$

where Enz stands for enzyme. Various nucleophiles were examined as halogen acceptors by incubation with bromoperoxidase, hydrogen peroxide, and radioactive $^{77}Br^-$ (*9*). It was found that β-ketoacids and cyclic β-diketones were particularly good acceptors, whereas substituted phenols were significantly less effective (*9*).

To analyze the exact chemical structure of the enzymatically synthesized halometabolites, reactions were carried out in vitro with 3-oxooctanoic acid as a substrate. Reaction mixtures contained 0.8 mmole of potassium phosphate–citrate buffer, 1 mmole of Br^-, 50 μmole of 3-oxooctanoic acid, and 750 units (*11*) of bromoperoxidase in a total volume of 20 ml. The reaction was sustained by stepwise addition of 15 μmole of H_2O_2 at 2-minute intervals for 60 minutes. The reaction mixtures were then extracted with three volumes of diethyl ether and the organic phase was dried over $MgSO_4$. Ether solutions were concentrated at 5° to 10°C to a small volume (0.1 to 0.3 ml). Reaction products were analyzed and identified with the aid of a Varian MAT 311A mass spectrometer interfaced with a Varian model 2700 gas chromatograph. When the bromoperoxidase reaction was carried out at *p*H 5.6 and the products were chromatographed on glass columns (6 or 12 feet long; inner diameter, 2 mm) containing 3 percent OV-1 on Supelcoport 80–100, both 1-bromo-2-heptanone and 1,1-dibromo-2-heptanone were confirmed as major reaction products (*12*). 2-Heptanone and trace amounts of 1,1,1-tribromoheptanone were also present in the reaction mixture. The formation of tribromoheptanone as a product of the bromoperoxidase reaction encouraged us to search for conditions that would support the enzymatic synthesis of bromoform since the cleavage of trihaloketones at a carbon-carbon bond is known to occur under basic conditions. In the bromoperoxidase reaction the tribromoheptanone could yield bromoform and caproic acid according to

$$\text{Br}_3\text{C-}\overset{\overset{\text{O}}{\|}}{\text{C}}\text{-(CH}_2)_4\text{-CH}_3 + \text{OH}^- \rightarrow$$

$$\text{HCBr}_3 + \text{CH}_3\text{-(CH}_2)_4\text{-}\overset{\overset{\text{O}}{\|}}{\text{C}}\text{-O}^- \quad (5)$$

To detect the formation of volatile haloforms, bromoperoxidase was incubated in vitro with 3-oxooctanoic acid, hydrogen peroxide, and bromide ion at several *p*H values. Volatile halocarbons were resolved and detected by gas chromatography of ether extracts of the reaction mixture followed by mass spectral analysis. These experiments showed the absence of detectable quantities of bromoform in the reactions at *p*H 5.6. However, gas chromatography of ether extracts of the reaction mixture after incubation of bromoperoxidase at *p*H 6.6 showed tribromomethane (Fig. 1, peak A). At *p*H 7.3 both dibromomethane (Fig. 1, peak B) and tribromomethane (peak C) were produced in significant quantities. Analysis of peak heights on the gas chromatography trace indicated that the ratio of tribromo- to dibromomethane was about 10:1 at *p*H 7.3.

The mass spectral analysis of peak *B* (Fig. 2) shows the molecular ions of dibromomethane [mass-to-charge ratio (*m/e*) 172, 174, and 176] split into the typical 1:2:1 distribution by the bromine isotopes of *m/e* 79 and 81. Strong ion intensities at *m/e* 94 and 96 show the presence of ion fragments $CH_2{}^{79}Br^+$ and $CH_2{}^{81}Br^+$. The mass spectral analysis of peak *C* shows the molecular ions of bromoform (*m/e* 249, 251, 253, and 255) in a ratio of 1:3:3:1. Strong ion intensities at *m/e* 171, 173, and 175 show the presence of ion fragments $^{79}BrCH^{79}Br^+$, $^{79}BrCH^{81}Br^+$, and $^{81}BrCH^{81}Br^+$, respectively.

In addition to the brominated methanes, the analysis revealed the presence of a third volatile brominated derivative (Fig. 1, peak D). This unknown compound has been identified as pentyl bromide on the basis of a comparison of the mass spectral properties of the unknown peak with those of authentic pentyl bromide. The mass spectral analysis of peak D (Fig. 2) shows the molecular ions of pentyl bromide at *m/e* 150 and 152.

The enzymatic formation of dibromomethane, bromoform, and pentyl bromide from 3-oxooctanoic acid can be rationalized on the basis of multiple halogenations on both of the carbon atoms alpha to the ketone. Multiple bromination followed by hydrolysis could then give rise to the observed mixture of brominated derivatives. Dibromomethane and bromoform could arise from the hydrolysis of the brominated heptanones outlined in reactions 3 and 4. The pentyl bromide could arise from the hydrolysis of an oxooctanoic acid derivative that has been brominated in position 4. This reaction is

$$\text{CH}_3\text{-(CH}_2)_3\text{-CHBr-}\overset{\overset{\text{O}}{\|}}{\text{C}}\text{-CH}_2\text{-COOH} +$$

$$\text{H}_2\text{O} \rightarrow \text{CH}_3\text{-(CH}_2)_3\text{-CH}_2\text{Br} + \text{HOOC-CH}_2\text{-COOH} \quad (6)$$

Further experimentation will be required to determine whether the hydrolytic reaction is enzyme-catalyzed or spontaneous under the reaction conditions employed.

We propose that bromomethanes found in natural waters (*13*) and as constituents of at least one species of edible red algae (*14, 15*) are the products of enzyme-catalyzed bromination of ketones. Since bromoperoxidase is also capable of oxidizing iodide, it is reasonable to assume that the enzyme can induce the synthesis of a number of iodomethanes that are known to occur in significant concentrations in ocean waters (*16*). The reaction of iodo- or bromomethanes with chloride in seawater could also give rise to chloromethanes (*17*). In addition, Pedersen (*18*) reported the isolation from

the red algae *Cystoclonium purpurem* of a peroxidase that can use both bromide and chloride as halogenation substrates. Peroxidases capable of oxidizing chloride could give rise to chloromethanes by a mechanism similar to the bromoperoxidase reaction. Peroxidative halogenation with either I^-, Br^-, or Cl^- is therefore responsible not only for the synthesis of a wide variety of marine halometabolites but possibly also for contributing large quantities (*16*) of the more volatile halogenated hydrocarbons to ocean waters and the environment. In light of recent environmental concern over halohydrocarbon-catalyzed destruction of ozone (*19*), it would be important to measure the production of halocarbons by marine organisms and compare it with industrial halocarbon production.

RICHARD THEILER
J. CARTER COOK
LOWELL P. HAGER
Department of Biochemistry, University of Illinois at Urbana-Champaign, Urbana 61802
JEROME F. SIUDA
Department of Medicinal Chemistry, School of Pharmacy, University of Pittsburgh, Pittsburgh, Pennsylvania 15261

References and Notes

1. L. P. Hager, R. H. White, P. F. Hollenberg, D. L. Doubek, R. C. Brusca, R. Guerrero, in *Food-Drugs from the Sea*, H. H. Webber and G. D. Ruggieri, Eds. (Marine Technology Society, Washington, D.C., 1976), pp. 421–428.
2. J. F. Siuda and J. F. DeBernardis, *Lloydia* **36**, 107 (1973).
3. W. J. Fenical, *J. Phycol.* **11**, 245 (1975).
4. J. F. Suida, G. R. VanBlaricom, P. D. Shaw, R. D. Johnson, R. H. White, L. P. Hager, K. L. Rinehart, Jr., *J. Am. Chem. Soc.* **97**, 937 (1975).
5. A. F. Rose, J. A. Pettus, Jr., J. J. Sims, *Tetrahedron Lett.* **1977**, 1847 (1977).
6. O. J. McConnell and W. Fenical, *ibid.*, p. 1851; *ibid.*, p. 4159.
7. D. R. Morris and L. P. Hager, *J. Biol. Chem.* **241**, 1763 (1966).
8. L. P. Hager, P. F. Hollenberg, T. Rand-Meir, R. Chiang, D. L. Doubek, *Ann. N.Y. Acad. Sci.* **244**, 80 (1975).
9. R. F. Theiler, J. F. Siuda, L. P. Hager, in *Drugs and Food from the Sea*, P. N. Kaul, Ed. (Univ. of Oklahoma Press, Norman, in press).
10. L. P. Hager, D. R. Morris, F. S. Brown, H. Eberwein, *J. Biol. Chem.* **241**, 1769 (1966).
11. One unit of bromoperoxidase activity is defined as the amount of enzyme that catalyzes the formation of 1 μmole of bromochlorodimedon per minute under standard assay conditions.
12. J. F. Siuda and L. P. Hager, in preparation.
13. A. A. Stevens and J. M. Symons, *Proc. Am. Water Works Assoc. Water Qual. Technol. Conf.* **26**, 1 (1975).
14. B. J. Burreson and R. E. Moore, *Tetrahedron Lett.* **1975**, 473 (1975).
15. O. McConnell and W. Fenical, *Phytochemistry* **16**, 367 (1977).
16. J. E. Lovelock and R. J. Maggs, *Nature (London)* **241**, 194 (1973).
17. O. C. Zafiriou, *J. Mar. Res.* **33**, 75 (1975).
18. M. Pedersen, *Physiol. Plant.* **37**, 6 (1976).
19. M. J. Molina and F. S. Rowland, *Nature (London)* **249**, 810 (1974); R. J. Cicerone, R. S. Stolaski, S. Walters, *Science* **185**, 1166 (1974).
20. We thank the staff of the Mass Spectrometer Laboratory for valuable assistance. Supported by NSF grant PCM 76-12547 and by NIH grant HEW PHS CA 11388 for the University of Illinois Mass Spectrometer Laboratory.

14 April 1978; revised 8 August 1978

Subsynaptic Plate Perforations: Changes with Age and Experience in the Rat

Abstract. *The relative frequency of appearance of discontinuities in the postsynaptic thickening, or perforations in the subsynaptic plate, increased with age and experience. Rats reared from weaning in complex or social environments had a significantly higher proportion of occipital cortical synapses with perforations than did rats reared in isolation. In addition, the relative frequency of these perforations more than tripled between 10 and 60 days of age. Shifts in the frequency of perforations can occur independently of changes in the size of synapses. This result suggests a new potential mechanism of synaptic plasticity.*

Synapses in certain layers of the occipital cortex have longer postsynaptic thickenings in rats reared under environmental complexity (EC) than those reared under impoverished conditions in normal laboratory cages (IC) (*1*, *2*). The thickenings were measured because they reflected the area of the synaptic contact and thus, possibly, synaptic efficacy. In these studies, we measured along the length of the thickenings, ignoring the gaps which sometimes appeared in them, as seen in Fig. 1.

Peters and Kaiserman-Abramof (*3*) used serial sections to demonstrate that, in the third dimension, these gaps in the postsynaptic thickening corresponded to irregularly shaped perforations in a subsynaptic plate; they also found that these subsynaptic plate perforations (SSPP's) were more frequent in larger plates. We were intrigued by their suggestion that the edge of the subsynaptic plate might be the active site of the synapse and, thus, that perforations in the plate might functionally strengthen the synapse. Given this different possible anatomical measure of synaptic efficacy, we decided to analyze the percentage of synapses in which these SSPP's appeared in our EC and IC rats (*1*). In reanalyzing data from these animals, in new data from socially housed (SC) animals, and in a new group of EC-SC-IC triplet sets, we have noted that the percentage of occipital cortex synapses in which SSPP's appear is affected by the postweaning environment of the animal and that the frequency of SSPP's may vary independently of the size of the synapse. In addition, the frequency of SSPP's increased with age in a third study. To our knowledge, the results reported here are the first experimental evidence that SSPP's might have a functional role.

The initial observation involved four littermate pairs of male Long Evans hooded rats in which postsynaptic thickening differences were previously described (*1*). One member of each pair had been reared under EC and the other under IC. We compared the rate of occurrence of SSPP's of the type shown in Fig. 1 in round vesicle, asymmetric synapses (*4*) of layer 4 of the occipital cortex, in which the greatest and most consistent difference in the length of the postsynaptic thickening occurs (*1*, *2*). In this layer, an unweighted mean of 18.8 percent [standard error of the mean (S.E.M.) = 1.4 percent] of these synapses in EC animals contained SSPP's visible in transverse section, whereas only 11.9 percent (S.E.M. = 2.4 percent) of these synapses in the IC animals contained SSPP's. Each of the four EC rats had a higher percentage than its IC littermate [F (1, 6) = 5.96; $P \leq .05$].

Table 1. Total number of synapses and percentages in which SSPP's appear in postsynaptic thickening. Also included are comparisons of pairs of rats, which were reared for 30 days under the treatment conditions.

Layer	Treatment group						Comparison	
	EC		SC		IC			
	Synapses (No.)	Percent	Synapses (No.)	Percent	Synapses (No.)	Percent	EC > IC	SC > IC
				All synapses				
1	1941	11.40	1790	10.25	1806	8.63	7/11	7/11
3		14.84		11.78		9.69	7/11	8/11
4		13.38		13.59		11.78	7/11	8/11
				Large synapses				
1	1007	22.27	913	21.78	941	14.80	8/11	8/11
3		24.34		22.97		19.62	8/11	7/11
4		22.50		18.16		16.08	7/11	8/11

0036-8075/78/1208-1096$00.50/0