Synthesis of Brominated Heptanones and Bromoform by a Bromoperoxidase of Marine Origin†

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ABSTRACT: The presence of naturally occurring volatile halohydrocarbons in marine organisms, seawater, and the upper atmosphere has prompted a search for their biosynthetic origin. An earlier report documented the preparation of an enzyme extract from a marine algae which catalyzed the formation of dibromomethane, tribromomethane, and 1-bromopentane from 3-oxooctanoic acid. This report did not establish a pathway nor did it examine potential intermediates involved in the synthesis of the halometabilities (Theiler, R., Cook, J., Hager, L., & Siuda, J. (1978) *Science (Washington, D.C.) 202*, 1094–1096). This paper shows that an extract of the green marine algae, *Penicillus capitatus*, which contains a potent bromoperoxidase activity, is capable of catalyzing the incorporation of bromide ion into organic combination in the presence of 3-oxooctanoic acid. By use of gas chromatography and mass spectroscopy, it has been possible to identify tribromomethane, 1-bromo-2-heptanone, 1,1-dibromo-2-heptanone, and 1,1,1-tribromo-2-heptanone as products of this reaction. The properties of the enzymatically synthesized products have been compared to authentic compounds and found to be identical. The mono- and dibromoheptanones can be utilized as precursors for the enzymatic formation of tribromoheptanone, but the final hydrolysis of the tribromoheptanone to bromoform appears to be a nonenzymatic reaction with the *P. capitatus* extracts.

A wide variety of halometabolites have been isolated from marine organisms (Lunde, 1973; Siuda & DeBernardis, 1973; White, 1974; Hager et al., 1976; Hewson & Hager, 1979). Those compounds of particular interest in this study are the halogenated methyl ketones having the general structure CX_3COCX_2R where X may be either a halogen or a hydrogen atom and R may be either an alkyl residue or a hydrogen atom.

Halogenated ketones of this structure have been isolated as natural products from *Bonnemaisonia hamifera* (Siuda et al., 1975; Rinehart et al., 1975), *Asparagopsis taxiformis* (Fenical, 1975; Burreson et al., 1975; McConnell & Fenical, 1977a), *Asparagopsis armata* (Fenical, 1974; McConnell & Fenical, 1977a), *Bonnemaisonia nootkana* (McConnell & Fenical, 1977b), and *Bonnemaisonia asparagoides* (McConnell & Fenical, 1977c). A recent review article describes the natural products isolated from the *Bonnemaisonia* (McConnel & Fenical, 1980).

These naturally occurring halo ketones are of importance in terms of both the biology and the chemistry of marine algae. First, the antimicrobial activity of extracts obtained from *B. hamifera* (Siuda et al., 1975) and *A. taxiformis* and *A. armata* (McConnell & Fenical, 1977a) has been traced to these halo ketones. This activity is presumably due to the ability of these ketones to act as alkylating agents similar to bromo- and iodoacetic acid. Specific halogenated ketones isolated from *B. hamifera* have been shown to have activity against the growth of bacteria and fungi at concentrations as low as 100 μg/mL (Siuda et al., 1975). Second, the environmental impact resulting from the pollution of the seas and the upper atmosphere with halomethanes and other volatile halometabolites (Lovelock et al., 1973; Lovelock, 1975) must be due in part to biological halogenation. The formation of halomethanes and haloforms has been speculated to occur in conjunction with the biosynthesis of these halogenated ketones (Burreson et al., 1975; McConnell & Fenical, 1977a). On a dry weight basis, it has been found that *A. taxiformis* contains 1% bromoform (Burreson et al., 1975) in addition to the high concentrations of the halo ketones.

The first proof of the biosynthetic capability of an enzyme system of marine origin for synthesizing volatile halohydrocarbons was provided by Theiler et al. (1978a). These investigators incubated an enzyme extract from *B. hamifera* with bromide ion, hydrogen peroxide, and 3-oxooctanoic acid and identified dibromomethane, 1-bromopentane, and bromoform as products of the reaction. These halogenated hydrocarbons were postulated to have been formed via the intermediates shown in Figure 1.

In this paper we show that halo ketones are produced as intermediates in a peroxidative bromination reaction. Furthermore, we show that the production of bromoform can result from the spontaneous hydrolysis of the trihalo ketone formed in the enzyme reaction. In contrast to the *B. hamifera* system, no significant amounts of bromomethane, dibromomethane, or 1-bromopentane were formed when the bromoperoxidase preparation from *Penicillus capitatus* was used as a catalyst for the synthesis of the halo ketones.

Experimental Procedures

Materials. Dibromomethane and 2-heptanone were purchased from Eastman Chemical Co. Bromoform was obtained from Aldrich Chemical Co. The organic solvents used in the various extraction procedures (pentane, petroleum ether, and dichloromethane) were all glass distilled and were obtained from Burdick & Jackson Labs. The dichloromethane used for the extraction of the enzymatically formed brominated ketones and bromoform did not contain these compounds as contaminants. This fact was established by control experiments conducted under normal enzyme assay conditions. Methyl undecanoate was purchased from Sigma Chemical Co. Monochlorodimedone was synthesized according to the procedure of Hager et al. (1966). 3-Oxooctanoic acid was a gift from J. F. Siuda and was synthesized according to a modified procedure of Stallberg-Stenhagen (1945). The β-keto acid was purified by crystallization from petroleum ether and

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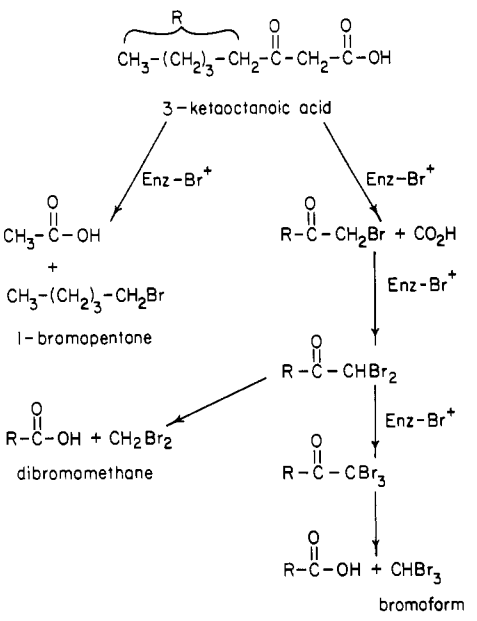

FIGURE 1: Postulated scheme for bromoform formation.

distilled water. All inorganic reagents were of analytical reagent grade. Gas chromatography (GC) packings were purchased from Supelco.

1-Bromo-2-heptanone was prepared from hexanoyl chloride in a manner similar to a literature procedure for the preparation of 1-bromo-2-tridecanone (Lutz & Wilson, 1947). A solution of 4.6 g of hexanoyl chloride in 100 mL of ether was cooled to 0 °C, and excess ethereal diazomethane was added. The ice bath was removed, and the solution was allowed to stand at room temperature for 10 h. One-half of the resulting solution of 1-diazo-2-heptanone was stirred and cooled at 0 °C as 3 mL of 48% hydrobromic acid was added over 30 min. The mixture was stirred at 0 °C for 20 min and at room temperature for 9 h. The ether layer was washed with saturated sodium bicarbonate and water, dried (Na_2SO_4), and concentrated. Distillation of the remaining oil in a Kugelrohr apparatus (90 °C, 0.1 mm) gave 1.6 g (48%) of a colorless liquid. Since GC analysis indicated the presence of some impurities, the material was further purified by chromatography on a column of silica gel with 5% ether–hexane as eluant and by preparative high-performance LC on a 3.9 mm × 30 cm column of μ-Porasil with ~1% ether–isooctane as eluting solvent. The purified monobromo ketone, a known compound (Catch et al., 1948), exhibited the following spectral properties: IR (neat) 1717 cm^{-1}; ^1H NMR ($CDCl_3$) δ 0.90 (t, 3, CH_3), 1.33 (m, 4, H at C-5 and C-6), 1.62 (quintet, 2, $CH_2CH_2C{=}O$), 2.63 (t, 2, $CH_2C{=}O$), 3.85 (s, 2, CH_2Br); mass spectrum (EI, 70 eV), *m/e* (relative intensity) 99 (52), 71 (36), 43 (100) (CI, isobutane; see Figure 3).

1,1-Dibromo-2-heptanone was prepared in a manner analogous to a literature procedure (Roedig, 1960). The other half of the ethereal solution of 1-diazo-2-heptanone (see above) was stirred and cooled at 0 °C as 2 mL of bromine was added dropwise. The ice bath was removed, and the solution was allowed to stand at room temperature for 9 h. The product was isolated, distilled [80 °C (0.5 mm); yield 1.8 g (39%)], and purified by column chromatography and preparative HPLC as described above for the monobromo ketone. The purified dibromo ketone, a known compound (Villeras, 1967), was homogeneous according to GC analysis and exhibited the followed spectral properties: IR (neat) 1725 cm^{-1}; ^1H NMR ($CDCl_3$) δ 0.89 (t, 3, CH_3), 1.31 (m, 4, H at C-5 and C-6), 2.74 (quintet, 2, $CH_2CH_2C{=}O$), 2.88 (t, 2, $CH_2C{=}O$), 5.72 (s, 1, $CHBr_2$); mass spectrum (EI, 70 eV), *m/e* (relative intensity) 99 (58), 71 (43), 43 (100) (CI, isobutane; see Figure 3).

1,1,1-Tribromo-2-heptanone was prepared from 1-heptyne by a known procedure (Myddleton et al., 1930, 1927). A solution of mercuric acetate was prepared by stirring 5.8 g (27 mmol) of mercuric oxide in 30 mL of glacial acetic acid at 40 °C until the solid had dissolved. The solution was stirred and heated at 70 °C as a solution of 1 g (10.4 mmol) of 1-heptyne in 10 mL of acetic acid was added. After 15 min, the solution was cooled to room temperature, a small amount of precipitate was removed by filtration, and the filtrate was diluted with water. The resulting precipitate of the mercury derivative was collected, dried (yield 1.22 g), and suspended in 100 mL of chloroform. Bromine (0.33 mL) was added, the mixture was stirred at room temperature for 5 h, and the precipitate was removed by filtration. The filtrate was washed with dilute hydrochloric acid and water, dried (Na_2SO_4), and evaporated. Purification of the remaining oil by preparative HPLC as described above for the monobromo ketone gave 0.4 g of the tribromo ketone which appeared as a single peak on GC analysis. The spectral characteristics of 1,1,1-tribromo-2-heptanone are as follows: IR (neat) 1750 cm^{-1}; ^1H NMR ($CDCl_3$) δ 0.90 (t, 3, CH_3), 1.38 (m, 4, H at C-5 and C-6), 1.75 (quintet, 2, $CH_2CH_2C{=}O$), 3.14 (t, 2, $CH_2C{=}O$); mass spectrum (EI, 70 eV), *m/e* (relative intensity) 99 (38), 71 (37), 43 (100) (CI, isobutane; see Figure 3).

Quantitation of Bromoperoxidase Activity. Bromoperoxidase activity was assayed by the procedure described earlier (Theiler et al., 1978b) by using an extinction coefficient at 292 nm of 2.0×10^4 at pH 7.0 for monochlorodimedone. It was found that the order of addition of substrates to the enzyme was important in order to obtain a linear rate in this assay system. The enzyme had to be first incubated with hydrogen peroxide before the bromination reaction was initiated by the addition of potassium bromide. Final substrate concentrations in the assay reaction were 90 μM hydrogen peroxide, 50 mM potassium bromide, and 75 μM monochlorodimedone. Protein concentrations were determined by the Lowry method (Lowry et al., 1951) by using lysozyme as a standard. The hydrogen peroxide used in the assays was quantitated via the oxidation of iodide to triiodide (Cotton & Dunford, 1973; Ovenston & Rees, 1950).

Source of Bromoperoxidase Activity. Bromoperoxidase was isolated from *P. capitatus* collected on shoals off Molasses Key in the Florida Keys in early October 1979. This green algae had earlier been determined to be a rich source of bromoperoxidase (Hewson & Hager, 1980). The algae was stored frozen and returned to the lab where 2-kg aliquots were thawed, suspended in 10 L of 10 mM phosphate–citrate buffer at pH 7.0, and then homogenized in a Brinkman homogenizer with probe generator PT25. The homogenate was centrifuged at 10000*g* for 30 min, and the supernatant fraction was concentrated 20-fold on an Amicon hollow fiber cartridge. A 25–60% ammonium sulfate fraction was prepared by the addition of solid ammonium sulfate to the crude algae extract. The 25–60% ammonium sulfate precipitate was dissolved in and dialyzed against 100 mM potassium–citrate buffer, pH 7.0, prior to use.

Enzymatic Production of Halogenated Heptanones. The enzymatic reaction mixtures were incubated in sealed 100-mL serum bottles which contained 100 mL of 100 mM potassium phosphate–citrate buffer, at various pH values, 50 mM potassium bromide, 1 mM 3-oxooctanoic acid, and 0.7 unit/mL bromoperoxidase. Aliquots of hydrogen peroxide were supplied

by the mechanical injection of 1 mL of 5 M hydrogen peroxide over a 90-min period at a rate of approximately 90 nmol every 90 s. The final protein concentration in the reaction mixture was approximately 1.3 mg/mL. During incubation, the temperature was maintained at 25 °C, and the reaction was vigorously stirred with a magnetic stirring bar. During incubation, the serum bottle was sealed with a silicone rubber serum cap backed with Teflon sheet. One-milliliter aliquots of the reaction mixture were withdrawn at 15-min intervals through an intramedic tube attached to a hypodermic needle which penetrated through the serum cap. These 1-mL aliquots were transferred to 1-mL Pierce reacti-vials which were also sealed with Teflon-backed rubber septums with the Teflon facing inward. After transfer, these aliquots were incubated for an additional 10-min period in order to ensure complete utilization of the hydrogen peroxide. Following the 10-min incubation period, 100 μL of dichloromethane was added to each vial, and the reaction mixture was vigorously extracted by agitation on a vortex mixer for two 30-s periods. The resulting emulsion was broken by centrifugation. A 50-μL aliquot of the dichlormethane layer was removed and diluted with an equal volume of dichloromethane which contained an internal standard of 140 μg/mL methyl undecanoate. A control reaction mixture which contained no hydrogen peroxide was supplemented with known concentrations of brominated hydrocarbon standards and was extracted in a similar fashion in order to check on the accuracy of the quantitation procedure. All samples were stored in a freezer at −20 °C in 0.3-mL Pierce reacti-vials with Teflon-backed septums prior to analysis by gas chromatography.

Brominated Heptanones as Substrates. Reaction mixtures similar to those described above were incubated in sealed Kimax 8-mL sample vials. Starting with a sample volume of 7 mL, 1 mL of 0.35 M hydrogen peroxide was added over a 90-min period at a rate of approximately 90 nmol every 90 s. The final concentrations of the reaction components were 100 mM potassium phosphate–citrate buffer, pH 7.6, 1 mM brominated heptanone, 50 mM potassium bromide, and 0.89 unit/mL bromoperoxidase activity. The protein concentration in these reaction mixtures was approximately 1.6 mg/mL. As before, these reaction mixtures were sealed with Teflon-backed rubber septums, and product analysis was carried out according to the procedure described in the previous section.

Quantitation by Gas Chromatography. 2-Heptanone, bromoform, 1-bromopentane, and the brominated heptanones were quantitated on 3% OV-1 (Supelco) coated on 80/100 and packed in a 3.7-m glass column. A Varian 3700 gas chromatograph equipped with FID was programmed for a temperature arise from 100 to 200 °C at 10 °C/min for these analyses.

Bromoform, 1-bromopentane, and dibromomethane were chromatographed on Supelco 0.2% Carbowax 1500 coated on 60/80 Carbopack C and packed in a 3.7-m column. For these analyses, the gas chromatograph was programmed for a temperature rise from 100 to 170 °C at 20 °C/min. After 170 °C was reached, this temperature was maintained for 8 min. Quantitation of the area under the various elution peaks was performed on a Spectra-Physics 4100 computing integrator.

Identification of Products. The products of the incubations were identified by a comparison of their gas chromatographic retention times and mass spectra with those of authentic samples.

For mass spectral analyses, samples of the reaction mixtures obtained from incubations with 3-oxooctanoic acid and 1-bromo-2-heptanone were extracted with 3 volumes of dichloromethane, dried over anhydrous magnesium sulfate, and concentrated by rotary evaporation at 0 °C to a volume of approximately 100 μL. It was inadvertently discovered that dichloromethane solutions of 1-bromo-2-heptanone and 1,1-dibromo-2-heptanone kept in overnight contact with molecular sieves were not stable. The monobromo ketone undergoes rearrangement to a mixture of 1-bromo- and 3-bromo-2-heptanone while the dibromo ketone is partially isomerized to its 1,3-dibromo isomer. Gas chromatograms of the isomeric mixtures of the bromo ketones are shown in Figure 2.

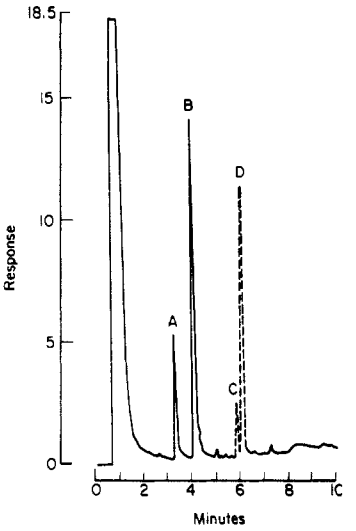

FIGURE 2: Gas chromatorgraphic separation of the rearrangement products resulting from the incubation of 1-bromo-2-heptanone and 1,1-dibromo-2-heptanone with molecular sieves. Chromatography was carried out on a 3% OV-1 column. The solid curve traces the gas chromatographic separation of 3-bromo-2-heptanone (peak A) and 1-bromo-2-heptanone (peak B) after incubation of 1-bromo-2-heptanone with molecular sieves. The dashed curved traces the separation of 1,1-dibromo-2-heptanone (peak C) from 1,3-dibromo-2-heptanone (peak D) after incubation of the 1,1-dibromo-2-heptanone with molecular sieves.

The dichloromethane solutions of the enzymatic products and authentic brominated ketones were analyzed by tandem gas chromatography and mass spectrometry (GC/MS) on a Varian MAT311A mass spectrometer interfaced with a Varian Model 2700 gas chromatograph equipped with one or the other of the two columns described above. Although bromoform was readily identified by its characteristic electron impact (EI) mass spectrum, the EI mass spectra of the bromo ketones unfortunately exhibited no peaks for either molecular ions or bromine-containing fragments. Therefore, most of the GC/MS analyses were conducted by chemical ionization (CI) with isobutane. This method yielded characteristic quasi-molecular ions (M + 1) for the mono-, di-, and tribromo ketones (see Figure 3). The multiplicity of the quasi-molecular ion in the CI/MS immediately reveals the number of bromine atoms present. Thus, the quasi-molecular ions of the monobromo, and tribromo ketones appear as a 1:1 doublet (*m/e* 193 and 195), a 1:2:1 triplet (*m/e* 271, 273, and 275), and a 1:3:3:1 quartet (*m/e* 349, 351, 353, and 355), respectively, owing to the statistical distribution of the two bromine isotopes. The CI mass spectra of the three bromo ketones obtained by synthesis and the enymatically produced bromo ketones were identical.

The EI mass spectrum of 1,3-dibromo-2-heptanone was useful in the identification of this rearranged dibromo ketone. The fragmentation pattern corresponded to that reported by Siuda et al. (1975), exhibiting peaks for the expected acylonium and alkyl fragments arising by α cleavage. In contrast, the EI mass spectra of 1-bromo-, 1,1-dibromo-, and 1,1,1-

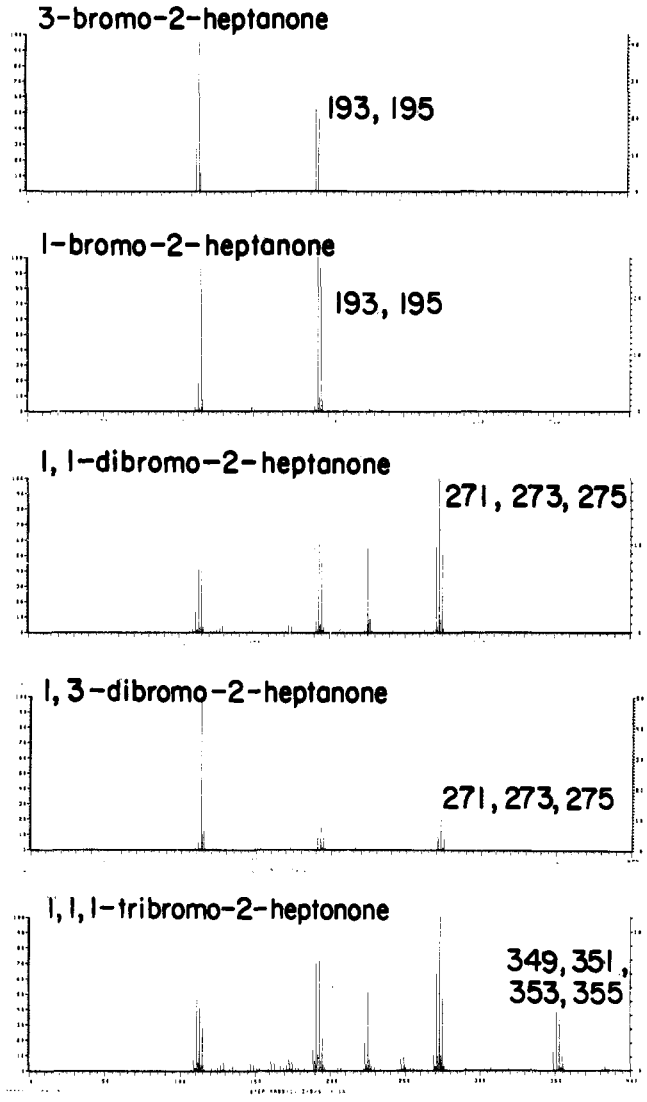

FIGURE 3: Chemical ionization mass spectra of brominated heptanones.

tribromo-2-heptanone showed a peak at *m/e* 99 for the non-brominated fragment, $C_5H_{11}CO^+$. The absence of a peak at *m/e* 99 in the EI spectrum of the isomeric monobromo ketone produced upon contact with molecular sieves is consistent with the structure 3-bromo-2-heptanone.

Results

Enzymatic Bromination Activity. A bromoperoxidase preparation obtained from *P. capitatus* was used as the enzyme source for catalyzing the bromination of 3-oxooctanoic acid. The production of brominated heptanones in the enzymatic reaction was monitored by gas chromatography as seen in Figure 4. The possible production of other brominated hydrocarbons was monitored as shown in Figure 5. Bromoform, 1-bromo-2-heptanone, 1,1-dibromo-2-heptanone, and 1,1,1-tribromo-2-heptanone were all formed from 3-oxooctanoic acid. The formation of the brominated heptanones as a function of time and pH is shown in Figure 6. It should be noted that at pH 7 and 7.6, steady-state levels of the mono-, di-, and trihalo ketones are established during the first 15 min of the reaction, and approximately 66% of the total amount of bromination also occurs during this initial time period. In contrast, bromoform synthesis, at both pH 7 and 7.6, is essentially linear for at least the first 75 min of the reaction. Enzymatic bromination at pH 8 follows a quite different pattern. Bromoform synthesis is negligible at pH 8, and almost all of the

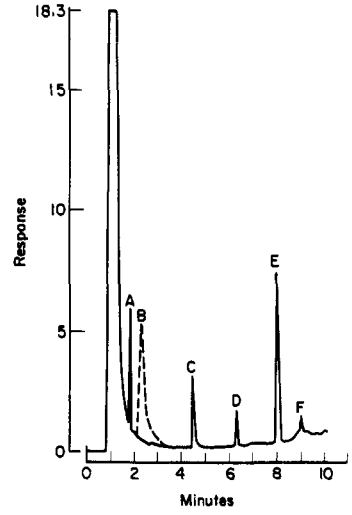

FIGURE 4: Gas chromatographic separation of biosynthetic bromoform and brominated heptanones. The enzymatic reactions were carried out at pH 7.6 with 3-oxooctanoic acid as the halogen acceptor according to the conditions described under Experimental Procedures. Chromatography was carried out on a 3% OV-1 column. Peaks A, C, D, and F are respectively bromoform, 1-bromo-2-heptanone, 1,1-dibromo-2-heptanone, and 1,1,1-tribromo-2-heptanone formed in the enzymatic reaction. Peak E represents the methyl undecanoate internal standard. Peak B is a 2-heptanone standard chromatographed in a separate experiment. The GC detector sensitivity was set 1 × 10^{-11} A/mV at an attenuation setting of 16.

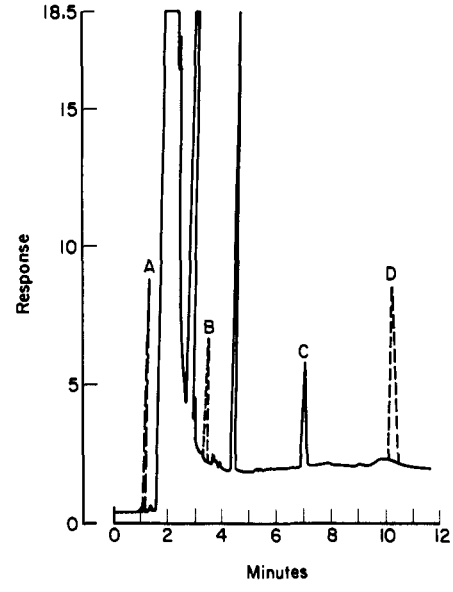

FIGURE 5: Gas chromatographic separation of bromoform from bromomethane, dibromomethane, and 1-bromopentane. The enzymatic reaction to produce bromoform was carried out at pH 7.6 with 3-oxooctanoic acid as the halogen acceptor according to the conditions described under Experimental Procedures. Chromatography was carried out on a 0.3% Carbowax 1500 Carbopack C column. Peak C represents the enzymatically synthesized bromoform. Peaks A, B, and D are respectively bromomethane, dibromomethane, and 1-bromopentane standards which were chromatographed in a separate experiment. The GC detection sensitivity was set 1 × 10^{-11} A/mV at an attenuation setting of 4.

enzymatic bromination activity can be accounted for in terms of the formation of 1-bromo-2-heptanone.

The results indicate that the pH optimum for both total bromine incorporation and bromoform production occurs at a pH value of 7.6 as shown in Figure 7. Since bromoperoxidase is known to catalyze the denaturation of enzymes via the incorporation of bromine into tyrosine residues of proteins (McElvany, 1980), aliquots of the various reaction mixtures were removed and assayed for enzyme activity after the 90-min

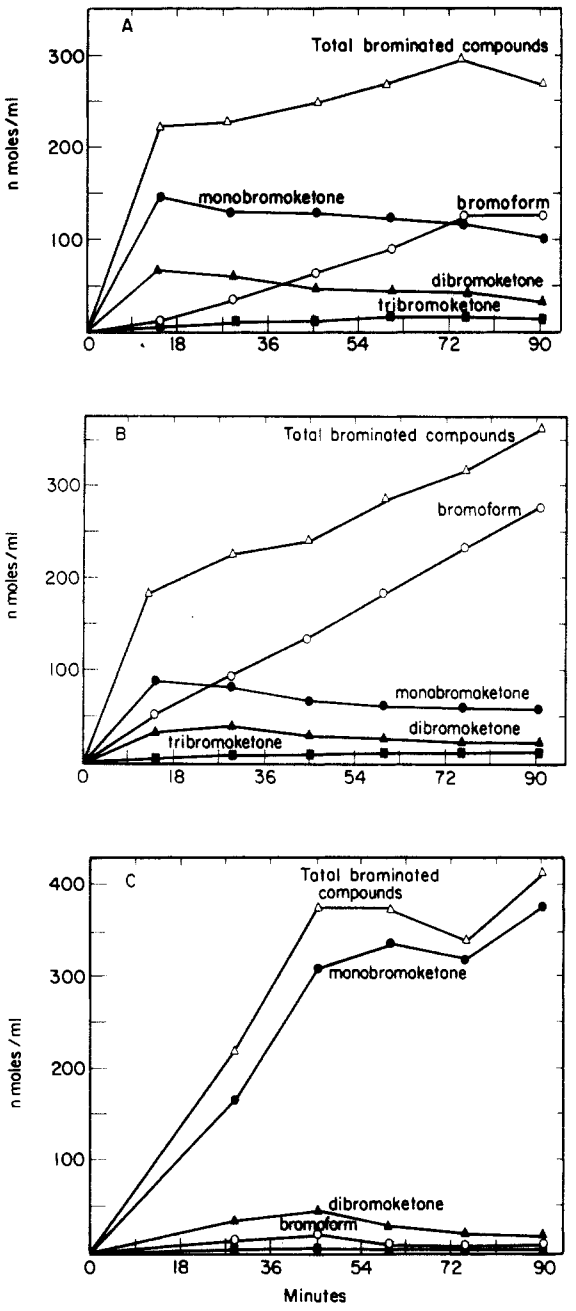

FIGURE 6: Enzymatic bromination and product accumulation as a function of time and pH. The enzymatic reactions were carried out at pH 7 (panel A), pH 7.6 (panel B), and pH 8 (panel C) with 3-oxooctanoic acid as the substrate. The reactions were stopped at the indicated time intervals, extracted, and quantitated according to the conditions described under Experimental Procedures. The curves trace the formation of 1,1,1-tribromo-2-heptanone (■), 1,1-dibromo-2-heptanone (▲), 1-bromo-2-heptanone (●), bromoform (○), and the sum of all brominated derivatives (△) as a function of time.

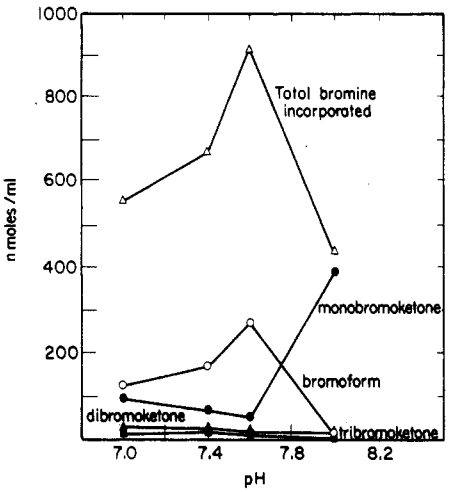

FIGURE 7: Effect of pH on product accumulation. The enzymatic reactions were carried out for 90 min at the pH values indicated with 3-oxooctanoic acid as the substrate according to the conditions described in Figure 6. The curves trace the amount of 1,1,1-tribromo-2-heptanone (■), 1,1-dibromo-2-heptanone (▲), 1-bromo-2-heptanone (●), bromoform (○), and total brominated products (△) synthesized as a function of the pH of the incubation medium.

incubation period. No enzymatic bromination activity was lost at pH 7.0 during the incubation period and at least 50% of the original activity was retained at pH 8.0. The loss of activity at pH 8.0 could partially explain the inability of the enzyme to catalyze the further bromination of 1-bromo-2-heptanone as seen in Figure 6 (panel C), but it is not likely to be the total explanation.

Brominated Heptanones as Substrates for Bromoform Synthesis. For determination of whether mono-, di-, and tribromoheptanones were intermediates in the formation of bromoform, these compounds were individually tested as substrates. The results which are listed in Table I show that 3-oxooctanoic acid and all three brominated heptanones are substrates for the production of bromoform as previously predicted (Theiler et al., 1978a). However, the results also show that bromoform production from 3-oxooctanoic acid is much greater than bromoform production from an equivalent amount of 1-bromo-2-heptanone. Other facets of the reaction are also revealed in Table I. The control reaction mixtures show that 1-bromo-2-heptanone is the only brominated heptanone which can be totally recovered from a 90-min incubation reaction mixture. 1,1-Dibromo-2-heptanone and 1,1,1-tribromo-2-heptanone appear to be quite unstable at pH 7.6 and disappear from their respective reaction mixtures. 1,1-Dibromo-2-heptanone in a potassium phosphate–citrate buffer at pH 7.6 disappears completely in 26 h in the absence of enzyme and without any detectable dibromomethane formation. This observation rules out a modified haloform-type reaction to account for the loss of the dibromo ketone. In an analogous fashion, in the absence of enzyme, 1-bromo-2-heptanone completely disappears from reaction mixtures in 97 h, and no bromomethane is found. This again rules out a haloform-type hydrolytic reaction to account for the disappearance of the monobromo ketone. There also appears to be some interaction of the brominated heptanones with the proteins present in the reaction mixture. This is clearly established by the disappearance of 63% of the 1-bromo-2-heptanone when it was incubated for 90 min in the presence of the crude enzyme preparation. A third point shown in Table I is that bromoform can be formed nonenzymatically from the tribromo ketone. 1,1,1-Tribromo-2-heptanone clearly hydroylzes to bromoform at pH 7.6 in the absence of enzyme. Some reductive debromination of the tribromo ketone to 1,1-dibromo-2-heptanone is also evident from the data.

Heptanone as a Substrate for Bromoform Synthesis. The possible decarboxylation of 3-oxooctanoic acid to heptanone and the subsequent bromination of heptanone to form the mono-, di-, and tribromo ketones were examined by testing heptanone as a substrate under the assay conditions used for bromoform synthesis. The results of these experiments are given in Table II.

The incubation of heptanone with the crude *P. capitatus* bromoperoxidase preparation, hydrogen peroxide, and bromide did not lead to the formation of detectable levels of 1-bromo-2-heptanone or 1,1-dibromo-2-heptanone. Traces of

Table I: Bromoform and Bromoheptanone Synthesis from Various Substrates

substrate added	condition[a]	products formed or substrate recovered (nmol) monobromoheptanone	dibromoheptanone	tribromoheptanone	$CHBr_3$	total
monobromoheptanone (7000 nmol)	a	1960	168	112	479	2719
	b	8456	0	0	0	8456
	c	3108	0	0	0	3108
	d	7658	0	0	0	7658
dibromoheptanone (7000 nmol)	a	0	406	63	1113	1582
	b	0	1400	0	0	1400
	c	0	1666	0	0	1666
	d	0	2310	0	0	2310
tribromoheptanone (7000 nmol)	a	0	266	1673	910	2549
	b	0	126	420	882	1428
	c	0	189	861	1274	2324
	d	0	70	70	623	763
β-oxooctanoic acid (7000 nmol)	a	357	133	77	1260	1827
	b	0	0	0	0	0
	c	0	0	0	0	0
	d	0	0	0	0	0

[a] The experimental conditions are those given under Experimental Procedures: (a) complete, (b) complete minus enzyme, (c) complete minus H_2O_2, and (d) complete minus enzyme and H_2O_2.

Table II: Bromoform and Bromoheptanone Synthesis from 2-Heptanone[a]

substrate added	products formed or substrate recovered (nmol) monobromoheptanone	dibromoheptanone	tribromoheptanone	2-heptanone	$CHBr_3$	total
2-heptanone (7000 nmol)	0	0	35	7630	112	7777
3-oxooctanoic acid (7000 nmol)	1267	490	94	0	602	2453

[a] The experimental conditions are those given under Experimental Procedures. The reaction mixture was incubated at pH 7.0 for 75 min with 1.02 units of bromoperoxidase/mL.

bromoform and 1,1,1-tribromo-2-heptanone were formed in the reaction mixture. No brominated hydrocarbons were formed when the enzyme or hydrogen peroxide was omitted from the reaction mixture. It should also be noted that 2-heptanone, the unbrominated decarboxylation product of 3-oxooctanoic acid, was not detected in the controls when 3-oxooctanoic acid was used as a substrate for bromoform synthesis (see Table I).

Comparison of Crude and Purified Bromoperoxidase Preparations. All of the experimental results consistently showed that no brominated products were formed unless all three substrates (bromide ion, hydrogen peroxide, and an organic halogen acceptor) and the crude *P. capitatus* bromoperoxidase preparation were present in the reaction mixture. In addition, control experiments showed that denatured enzyme preparations were not effective as catalysts for the bromination reaction. When highly purified preparations of bromoperoxidase[1] were used as a replacement for the crude *P. capitatus* extracts, the same bromination patterns and products were found. This finding suggests that a single brominating enzyme catalyzes the entire sequence of reactions which leads to the accumulation of the various products.

Nonenzymatic Rearrangements of 1-Bromo- and 1,1-Dibromo-2-heptanone. As mentioned previously under Experimental Procedures, 1-bromo- and 1,1-dibromo-2-heptanone rearrange to mixtures containing the isomeric 3-bromo- and 1,3-dibromo-2-heptanones when stored overnight in contact with molecular sieves. There is considerable precedent for the $\alpha \rightarrow \alpha'$ rearrangement of bromo ketones (Warnoff et al., 1978), and the mechanism in this case probably involves enolization, allylic rearrangement, and ketonization. The same rearrangement was also observed to be catalyzed by the silicic acid which was used to purify these compounds in an earlier investigation (Siuda et al., 1975). The facility of the rearrangement of these bromo ketones suggests the possibility that some of the putative naturally occurring ketones bearing bromine at C-3 could in fact arise by isomerization of 1-bromo ketones during the purification and/or analysis of the extracts. However, the isolation of 1,1,3,3-tetrabromo-2-heptanone provides evidence that bromination at C-3 must occur to some extent in the red algae, *B. hamifera* (Siuda et al., 1975).

Discussion

The incubation of 3-oxooctanoic acid with bromoperoxidase preparations from *P. capitatus* gives rise to many products. The interpretation of the data is unfortunately complicated by several possible chemical reactions known to occur with halogenated ketones. The observed instability of 1,1-dibromo-2-heptanone and 1,1,1-tribromo-2-heptanone in aqueous buffer solution at neutral or slightly alkaline pH may be in part the result of Favorski rearrangements of these bromo ketones to bromo acids. The resulting bromo acids would not have been detected by the extraction procedures and purification methods used in these experiments. Favorski arrangements are known to occur with halogenated ketones in weakly alkaline solutions (Rappe & Andersson, 1965), and bromo acids of this type have been isolated from *Bonnemaisonia motkana* (McConnell & Fenical, 1977b). 1,1,1-Tribromo-2-heptanone is also unstable under neutral and alkaline incubation conditions and undergoes spontaneous hydrolysis to yield bromoform. Another likely nonenzymatic reaction for the bromo ketones would be the alkylation of sulfhydryl groups, or possibly other nucleophilic sites, on amino acid residues in the enzymes and proteins present in the reaction mixtures. These reactions would proceed in a manner analogous to the reaction of iodoacetic acid with proteins. The reduction of the tribromo ketone to 1,1-dibromo-2-heptanone might occur by a nonenzymatic transfer of bromine to a

[1] Provided by John Manthey. The purified bromoperoxiase preparation electrophoresed as a single band in polyacrylamide gels.

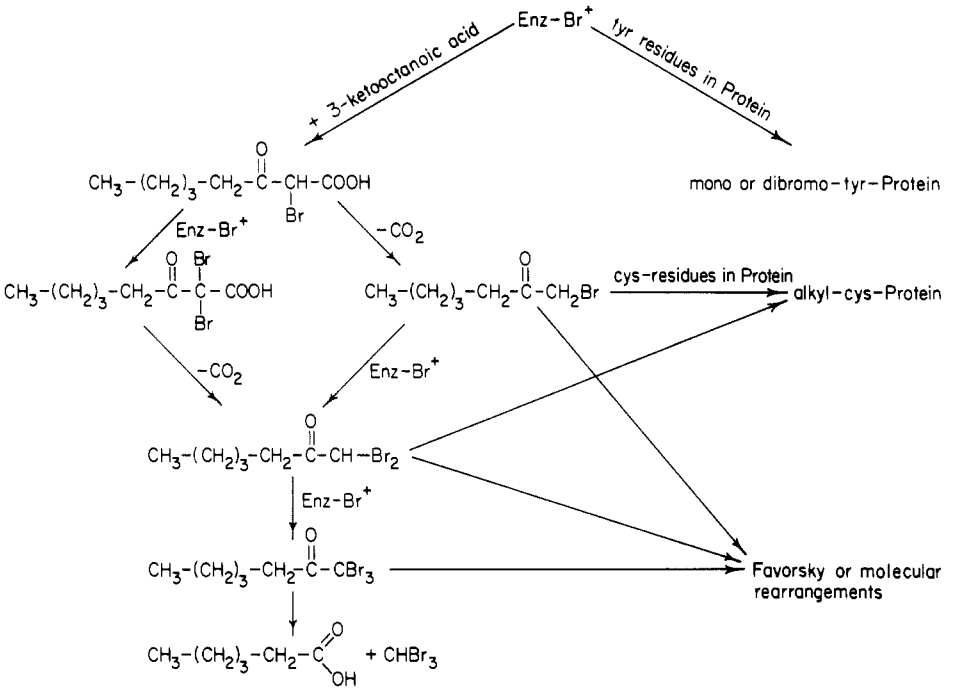

FIGURE 8: Summary of enzymatic and nonenzymatic reactions.

sulfhydryl nucleophile. The various combinations of enzymatic and nonenzymatic reaction pathways are summarized in Figure 8.

It was proposed in an earlier publication (Theiler et al., 1978a) that the production of bromoform from 3-oxooctanoic acid by the action of bromoperoxidase occurs by successive bromination of a series of bromo ketones as shown in Figure 1. If this pathway were the main route to the tribromo ketone and bromoform, one would expect that the production of bromoform from 2-heptanone and the three bromo ketones would be greater than from 3-oxooctanoic acid. In fact, the opposite result was observed. Larger amounts of bromoform were formed from 3-oxooctanoic acid than from either 2-heptanone (Table II) or the bromo ketones (Table I). This is particularly evident in the case of the monobromo ketone. When equivalent amounts of 3-oxooctanoic acid and 1-bromo-2-heptanone were incubated with the bromoperoxidase preparation under identical conditions, 2.5 times more bromoform was produced from 3-oxooctanoic acid. It is evident from these results that 1-bromo-2-heptanone cannot be a major intermediate in the conversion of 3-oxooctanoic acid to bromoform. This apparent inconsistency is readily explained by assuming that 3-oxooctanoic acid undergoes mono- and dibromination prior to decarboxylation (see Figure 8). Although according to this concept the monobromo and dibromo β-keto acids would be the primary intermediates in the reaction sequence, these unstable compounds would decarboxylate readily to yield their respective bromo ketones. Thus although we do not present direct evidence for the intermediacy of the bromo β-keto acids, the results suggest the simultaneous operation of two paths. The major route would proceed via the bromo β-keto acids while the minor route would involve decarboxylation and subsequent enzymatic bromination of the corresponding ketones.

Another slightly different mechanism for bromination should also be mentioned. It is known that the reaction of nonenolizable β-keto acids with bromine is a useful synthetic method for the regiospecific preparation of unsymmetrical bromo ketones (Corey & Burke, 1955; Corey et al., 1955). These reactions presumably take place by rapid bromination of a short-lived enol intermediate formed by decarboxylation of the β-keto acid (Pederson, 1936). Whether the enol intermediates which arise by decarboxylation of the various β-keto acids would have a sufficient lifetime to be substrates for bromination under the rather dilute conditions of these incubations is uncertain.

In contrast to the bromoperoxidase preparation isolated from *B. hamifera* (Theiler et al., 1978a), no 1-bromopentane or dibromomethane was detected in the products obtained from incubations with the enzyme extract from *P. capitatus*. However, in the present work, these brominated hydrocarbons could have been present in concentrations below the detection limit of the assay (0.4 nmol/mL for dibromomethane and 2 nmol/mL for 1-bromopentane). Alternatively it is quite possible that the bromoperoxidase preparation from *B. hamifera* has different specificities and gives products which differ from those formed in the *P. capitatus* reaction. One such difference is the greater amount of dibromotyrosine produced in the bromination of proteins by *B. hamifera* extract compared to bromotyrosine formation by the bromoperoxidase preparation form *P. capitatus*. On the other hand, the production of mono-, di-, and tribromoheptanones is quite similar with the enzyme extracts from the two different sources.

In summary, this research has shown that a bromoperoxidase preparation from *P. capitatus* is capable of brominating 3-oxooctanoic acid to 1-bromo-2-heptanone, 1,1-dibromo-2-heptanone, and 1,1,1-tribromo-2-heptanone. The tribromo ketone undergoes nonenzymatic hydrolysis to liberate bromoform. Although the mono- and dibromo ketones are substrates for bromination, the principal route to the bromo ketones and bromoform appears to involve bromination of 3-oxooctanoic acid and 2-bromo-3-oxooctanoic acid followed by decarboxylation. 2-Heptanone is a poor substrate compared to 3-oxooctanoic acid. Neither 1-bromopentane nor dibromomethane was detected in the incubation mixtures produced by the bromoperoxidase preparation from *P. capitatus*.

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Photoaffinity Inhibition of Rat Liver NAD(P)H Dehydrogenase by 3-(α-Acetonyl-*p*-azidobenzyl)-4-hydroxycoumarin†

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ABSTRACT: NAD(P)H dehydrogenase was purified in four steps from a homogenate of rat liver. The final step was affinity chromatography on Sepharose coupled to 3,3′-(*m*-hydroxybenzylidene)bis(4-hydroxycoumarin). The purified enzyme was inhibited competitively with respect to NADH by 3-(α-acetonyl-*p*-nitrobenzyl)-4-hydroxycoumarin (acenocoumarin) (K_i = 1.7 μM). The acenocoumarin was converted into an azide which was used to photoaffinity inhibit the enzyme. Following photolysis in the presence of the azide, the enzyme was inactivated in proportion to the concentration of azide present during irradiation. A maximum of 35–40% inhibition could be achieved by a single irradiation at 254 nm for 1.5 min. This inhibition was noncompetitive with respect to NADH. The inactivation was shown to be specific as acenocoumarin afforded complete protection against inactivation, irradiation was required to achieve inactivation, and the enzyme was unaffected by irradiation alone.

Affinity labeling is a technique which has the potential to identify and distinguish specific binding or catalytic sites on proteins (Singer, 1967). The photoaffinity labeling reagents represent a unique class of affinity labeling reagents (Knowles, 1972; Creed, 1974) since the reactive group is created in situ after the protein has been reacted with a ligand. Thus, in partially characterized multienzyme systems such as that involved in the vitamin K dependent γ-glutamyl carboxylation of the coagulation zymogens (Suttie, 1978), photoaffinity labeling reagents potentially could be used to identify and dissociate enzyme activities associated with the various steps in the process. There is mounting evidence that the epoxide reductase is the enzyme in this multienzyme carboxylation

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