Tans, G., van Zutphen, H., Comfurius, P., Hemker, H. C., & Zwaal, R. F. A. (1979) *Eur. J. Biochem. 95*, 449–457.
van de Waart, P., Bruls, H., Hemker, H. C., & Lindhout, T. (1983) *Biochemistry 22*, 2427–2432.
van de Waart, P., Hemker, H. C., & Lindhout, T. (1984) *Biochemistry 23*, 2838–2842.
van Rijn, J. L. M. L., Govers-Riemslag, J. W. P., Zwaal, R. F. A., & Rosing, J. (1984) *Biochemistry 23*, 4557–4564.

Characterization of Vanadium Bromoperoxidase from *Macrocystis* and *Fucus*: Reactivity of Vanadium Bromoperoxidase toward Acyl and Alkyl Peroxides and Bromination of Amines†

Helena S. Soedjak and Alison Butler*
Department of Chemistry, University of California, Santa Barbara, California 93106
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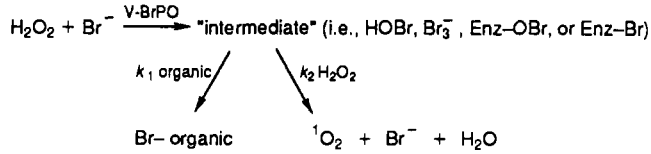
ABSTRACT: Vanadium bromoperoxidase (V-BrPO) has been isolated and purified from the marine brown algae *Fucus distichus* and *Macrocystis pyrifera*. V-BrPO catalyzes the oxidation of bromide by hydrogen peroxide, resulting in the bromination of certain organic acceptors or the formation of dioxygen. V-BrPO from *F. distichus* and *M. pyrifera* have subunit molecular weights of 65 000 and 74 000, respectively, and specific activities of 1580 units/mg (pH 6.5) and 1730 units/mg (pH 6) for the bromination of monochlorodimedone, respectively. As isolated, the enzymes contain a substoichiometric vanadium/subunit ratio; the vanadium content and specific activity are increased by addition of vanadate. V-BrPO (*F. distichus*, *M. pyrifera*, and *Ascophyllum nodosum*) also catalyzes the oxidation of bromide using peracetic acid. In the absence of an organic acceptor, a mixture of oxidized bromine species (e.g., hypobromous acid, bromine, and tribromide) is formed. Bromamine derivatives are formed from the corresponding amines, while 5-bromocytosine is formed from cytosine. In all cases, the rate of the V-BrPO-catalyzed reaction is much faster than that of the uncatalyzed oxidation of bromide by peracetic acid, at pH 8.5, 1 mM bromide, and 2 mM peracetic acid. In contrast to hydrogen peroxide, V-BrPO does not catalyze formation of dioxygen from peracetic acid in either the presence or absence of bromide. V-BrPO also uses phenylperacetic acid, *m*-chloroperoxybenzoic acid, and *p*-nitroperoxybenzoic acid to catalyze the oxidation of bromide; dioxygen is not formed with these peracids. V-BrPO does not catalyze bromide oxidation or dioxygen formation with the alkyl peroxides ethyl hydroperoxide, *tert*-butyl hydroperoxide, and cuminyl hydroperoxide.

Vanadium bromoperoxidase (V-BrPO)[1] was first discovered in the marine brown alga *Ascophyllum nodosum* (Vilter, 1984). This enzyme has been shown to catalyze the bromination of monochlorodimedone (2-chloro-5,5-dimethyl-1,3-dimedone, MCD) using hydrogen peroxide as an oxidant of bromide (Wever et al., 1985). In the absence of an organic substrate, V-BrPO catalyzes the formation of dioxygen (Everett & Butler, 1989). Bromide or iodide is required for dioxygen formation, which is a reaction best described as the halide-assisted disproportionation of hydrogen peroxide. V-BrPO does not catalyze the direct disproportionation of hydrogen peroxide (Everett & Butler, 1989), contrary to the iron heme haloperoxidases which have catalatic activity (Manthey & Hager, 1981; Thomas et al., 1970). Kinetic investigations of the rate of dioxygen formation and MCD bromination catalyzed by V-BrPO indicate that both reactions proceed via the formation of a common intermediate (Everett & Butler, 1989; Everett et al., 1990a,b), although the identity of the exact intermediate has not been identified with certainty. The production of the intermediate is rate limiting (Scheme I). The dioxygen formation pathway has been shown to be competitive with MCD bromination under certain conditions (Everett et al., 1990a). Moreover, the dioxygen produced is in the singlet excited state (Everett et al., 1990a). The striking feature of singlet oxygen production by V-BrPO is its exceptional stability, which is not inactivated by singlet oxygen or oxidized bromine derivatives (Everett et al., 1990a). By contrast, iron heme haloperoxidases (e.g., lactoperoxidase and chloroperoxidase) are strongly inactivated by turnover of hy-

Scheme I



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* To whom correspondence should be addressed.

[1] Abbreviations: BrPO, bromoperoxidase; Capso, 3-(4-aminocyclohexyl)-2-hydroxy-1-propanesulfonic acid; ClPO, chloroperoxidase; MCD, monochlorodimedone; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Mops, 3-(*N*-morpholino)-2-hydroxypropanesulfonic acid; taurine, 2-aminoethanesulfonic acid; Tes, 2-[[tris(hydroxymethyl)methyl]amino]ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane.

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drogen peroxide, producing singlet oxygen in the presence of bromide (Everett et al., 1990a).

Since the discovery of vanadium bromoperoxidase in the brown alga *A. nodosum*, vanadium bromoperoxidase has been isolated from other Phaeophyta [e.g., *Laminaria saccharina* (de Boer et al., 1986)] and Rhodophyta [*Ceramium rubrum* (Krenn et al., 1987); *Corallina pilulifera* (Krenn et al., 1989b)] as well as a terrestrial lichen (Plat et al., 1987). An iron heme bromoperoxidase has also been isolated from *Penicillus capitatus* (Chlorophyta) (Manthey & Hager, 1981). Brominated compounds are well-known constituents of many marine algae. Rhodophyta contain the most diverse array of brominated compounds, while only a few halogenated compounds have been isolated from Phaeophyta. *A. nodosum* and *Fucus vesiculosis* are two species of brown algae that produce large quantities (i.e., ca. 10^{10} g/year) of volatile, halogenated organic compounds such as $CHBr_3$, $CHBr_2Cl$, $CHBrCl_2$, and CH_2Br_2 (Gschwend et al., 1985). The biological function of halogenated hydrocarbons is not known, although their antibiotic properties have been noted (Gschwend et al., 1985). Oxidation of amines could also produce antimicrobial products, via the formation of the bromamine derivative, as has been proposed (Nieder & Hager, 1985; Kanofsky, 1989c).

To investigate the nature of bromoperoxidases present in other marine brown algae and to compare the reaction chemistry of these bromoperoxidases with that of V-BrPO from *A. nodosum*, we have purified and characterized the bromoperoxidase from the Phaeophyta *Fucus distichus* and *Macrocystis pyrifera*. We have compared the nature of the peroxide that is required for bromination and dioxygen formation catalyzed by V-BrPO and iron heme BrPO. We have established that bromamine species are formed by a V-BrPO-catalyzed reaction using peracetic acid as the oxidant of bromide but not hydrogen peroxide. Detection of brominated amines suggests that enzymatic bromination could occur through the formation of an enzyme-bound bromonium moiety.

Materials and Methods

Isolation and Purification of Bromoperoxidase from Fucus and Macrocystis. *F. distichus* was collected in Monterey, CA, in July 1988. *M. pyrifera* was collected off the coast of Goleta, CA, in November 1989. *A. nodosum* was collected at Kornwerderzand, Holland, in April 1989. Bromoperoxidases from *Fucus* and *Macrocystis* were purified as previously described (Wever et al., 1985; Everett et al., 1990a) with the incorporation of the following modifications. Solid $BaCl_2$ (to a final concentration of 50 mM) was used in place of $CaCl_2$ to effect a more efficient precipitation of alginic acids, other polysaccharides, and polyphenols. After centrifugation at 12600*g* the clear supernatant was concentrated in an Amicon hollow-fiber cartridge (H5P10-43). The concentrate was batch loaded onto DE-52 (Whatman), packed in a column and eluted by gradient with 0.1 M Tris–sulfate, pH 8.3, containing 0.1–0.6 M potassium chloride for *Fucus* or with 0.1 M Tris–sulfate, pH 8.3, containing 0.2–0.8 M potassium chloride for *Macrocystis*. The fractions containing bromoperoxidase activity were pooled, washed with 0.1 M Tris–sulfate, pH 8.3, to remove excess potassium chloride, and concentrated by ultrafiltration. The bromoperoxidase was further purified by separation on a Sephacryl S-200 (Pharmacia) column by elution with 0.1 M Tris–sulfate, pH 8.3. The fractions containing the highest specific bromoperoxidase activity were pooled. In some experiments, it was necessary to remove Tris buffer from the stock enzyme solutions. This was accomplished by ultrafiltration with doubly distilled water on a Centricon (30000 MW cutoff; Amicon). V-BrPO from *A. nodosum* was purified as previously described (Everett et al., 1990).

Gel Electrophoresis. SDS–polyacrylamide gel electrophoresis was carried out with 12% polyacrylamide slab gels (Laemli, 1979). Protein staining was performed with Coomassie brilliant blue R-250 (Laemli, 1979) or with silver stain (Bio-Rad). Low molecular weight (i.e., 14400–94000) standard proteins from Bio-Rad were used for molecular weight determinations. The protein samples for molecular weight determination were denatured by boiling; the gels were run under reduced, denaturing conditions. Haloperoxidase activity was detected on gels run under nondenaturing conditions with *o*-dianisidine staining in the presence of iodide and hydrogen peroxide (Vilter & Glombitza, 1983). Staining for carbohydrate was performed under denaturing conditions with periodic acid silver (Dubray & Bezard, 1982), alcian blue (Wardi & Michos, 1972), or thymol–H_2SO_4 (Gander, 1984) reagents.

Bromoperoxidase Activity Assay. Bromoperoxidase activity was measured spectrophotometrically by the bromination of monochlorodimedone: $\Delta\epsilon = 19900\ M^{-1}\ cm^{-1}$ at 290 nm (Hewson & Hager, 1980). The standard assay conditions consisted of 0.1 M phosphate buffer, pH 6.5 (for *Fucus*) or pH 6.0 (for *Macrocystis*), containing 0.2 M Na_2SO_4, 0.1 M KBr, and 50 μM MCD. The reaction was initiated by the addition of H_2O_2 to a final concentration of 2 mM. The specific activity is expressed in units per milligram, which is defined as the micromoles of MCD brominated per minute per milligram of bromoperoxidase.

Preparation and Quantitation of Peracetic Acid. Peracetic acid was prepared by reaction of acetic anhydride with 30% aqueous hydrogen peroxide. In a typical reaction 4.87 g of acetic anhydride (i.e., 47.71 mol) was added dropwise to a stirred solution of 1 g of 30% aqueous H_2O_2 (i.e., 8.82 mol of H_2O_2 and 38.89 mol of H_2O) containing concentrated H_2SO_4 (~0.1 g) over a period of about 40 min, while the temperature was maintained below 30 °C:

$$(CH_3CO)_2O + H_2O_2 \rightarrow CH_3COOOH + CH_3COOH$$

$$(CH_3CO)_2O + H_2O \rightarrow 2CH_3COOH$$

$$CH_3COOOH + CH_3COOH \rightarrow (CH_3CO)OO(COCH_3) + H_2O \text{ (slow side reaction)}$$

The peracetic acid prepared this way (~3 M) was free of hydrogen peroxide and contained only a small amount (i.e., <2%) of diacyl peroxide, as determined by the iodide titration methods described by Swern (1970). After storage for 1 day at 4 °C, hydrogen peroxide was not formed, although the content of diacyl peroxide increased to ca. 5%.

Dioxygen Measurements. Rates of dioxygen formation were measured with a Yellow Springs Instrument (YSI) oxygen probe (YSI 5331) and monitor (YSI 5300). The reaction medium was sparged with nitrogen gas to reduce the dioxygen concentration so that the oxygen probe would not become saturated during the course of the reaction. The reaction was initiated by the addition of V-BrPO. The rate of dioxygen formation was calculated directly from a plot of the percent dioxygen in solution versus time with an oxygen concentration of 0.247 mM in air-saturated water as the standard (Thomas et al., 1970). The reported rates have also been corrected for an apparent background rate of dioxygen formation or drift in the electrode of 0.2–0.4% min. All glassware was soaked with NoChromix, and the buffers were passed through a Bio-Rex Chelex ion exchange membrane before use. If these precautions were not taken, high rates of oxygen formation occurred sometimes in the absence of enzyme.

Preparation of Bromamine Derivatives of Taurine, Tris, and Capso and Determination of Bromamine Extinction Coefficients. The brominated derivatives of taurine, Tris, or Capso were prepared by incubating 3 mM of the amine for 1 h in 0.1 M phosphate buffer, pH 8.5, containing 1 mM Br^-, 30 nM V-BrPO, 0.2 M Na_2SO_4, and 1 mM peracetic acid. The reaction was followed spectrophotometrically by monitoring the absorption maximum of the bromamines. After 1 h the absorbance was constant, indicating the reactions were complete. The concentration of each bromamine was determined by titration against the sulfhydryl compound 5-thio-2-nitrobenzoic acid (TNB; Thomas, 1979) or iodide (Stelmaszynska & Zgliczynski, 1978). One mole of bromamine oxidizes 2 equiv of TNB, forming 1 mol of the disulfide compound 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB). TNB was prepared by reducing DTNB with a 2-fold excess of sodium borohydride (Thomas, 1979) in 50 mM phosphate, pH 6.0. The concentration of TNB was determined by the absorbance at 412 nm ($\epsilon = 1.36 \times 10^4$ M^{-1} cm^{-1}; Ellman, 1959). The concentration of the brominated amine derivatives was calculated from half the difference between the concentrations of TNB added and that remaining. Alternatively, the bromamine was standardized against oxidation of iodide to triiodide, which was determined spectrophotometrically ($\epsilon = 2.64 \times 10^4$ M^{-1} cm^{-1} at 353 nm; Thompson, 1986).

General Procedures and Reagents. The hydrogen peroxide (30% aqueous solution; Fisher Scientific) concentration was determined spectrophotometrically following the formation of triiodide as described by Cotton and Dunford (1973). Protein concentrations were determined by the Bio-Rad method using bovine serum albumin (Bio-Rad) as a standard. Spectrophotometric measurements were performed on a Kontron Uvikon 860 spectrophotometer. MCD was purchased from Sigma. All other chemicals were reagent grade. The concentration of vanadium was determined by graphite furnace atomic absorption spectroscopy on a Varian AA5 spectrometer.

Results and Interpretation

Characterization of Bromoperoxidases from Fucus and Macrocystis. Under nondenaturing conditions the purified bromoperoxidases from *Fucus* and *Macrocystis* gave rise to one major band after polyacrylamide gel electrophoresis. This band stained both for protein and for peroxidase activity. The peroxidase activity, which was detected by staining with *o*-dianisidine and H_2O_2, was not observed in the absence of iodide, indicating that the enzyme lacks true peroxidase activity. The requirement of bromide or iodide to detect peroxidase activity staining for other bromoperoxidases has also been reported (Vilter & Glombitza, 1983; Yamada et al., 1985). SDS-denatured polyacrylamide gels of V-BrPO from *Fucus* and *Macrocystis* were stained for carbohydrate with periodic acid silver, alcian, blue, or thymol–H_2SO_4 reagents. V-BrPO from *A. nodosum* is also a glycoprotein (Krenn et al., 1989a).

The subunit molecular mass of the bromoperoxidase from *Fucus* and *Macrocystis* was estimated from the reduced SDS–polyacrylamide gel electrophoresis. The molecular weights for bromoperoxidases from *Fucus* and *Macrocystis* were found to be 65 000 and 74 000, respectively, which agree with the subunit molecular weight from *A. nodosum*, 65 000 (Everett & Butler, 1989), *L. saccharina*, 67 000 (de Boer et al., 1986), *C. pilulifera*, 64 000 (Itoh et al., 1986), etc.

Metal Content of Bromoperoxidase. The presence of vanadium in bromoperoxidases purified from *Fucus* and *Macrocystis* was established by atomic absorption spectroscopy. The *Fucus* enzyme contained 0.30 vanadium ions per enzyme subunit as isolated, while the *Macrocystis* enzyme contained 0.38 vanadium ion per enzyme subunit as isolated. Substoichiometric ratios of V/subunit as isolated were also observed for V-BrPO isolated from other algae [e.g., *A. nodosum* (de Boer et al., 1986a), *L. saccharina* (de Boer et al., 1986b), and *C. pilulifera* (Krenn et al., 1989b)]. Incubation of the enzymes with vanadate increased the specific activity of both enzymes. In samples in which excess vanadate was removed by treatment with Chelex, atomic absorption data showed 1 equiv of vanadium per enzyme subunit for the *Fucus* enzyme, which is also consistent with the behavior of V-BrPO from *A. nodosum*. The *Macrocystis* bromoperoxidase had 1.5 equiv of vanadium per subunit after addition of excess vanadate, followed by Chelex treatment. Vanadium can be removed from both enzymes (as confirmed by atomic absorption) by dialyzing the enzymes against citrate–phosphate buffer, pH 4.0, containing 1 mM EDTA. The presence of phosphate was required for the complete removal of vanadium. The apoenzyme was inactive; however, the bromoperoxidase activity could be fully restored by incubating the apoenzyme with vanadate. Vanadate-reconstituted BrPO was used for all reactivity studies. Aqueous vanadate and nonspecifically bound bromoperoxidase do not have bromoperoxidase activity. The UV-vis spectra clearly indicate the absence of a Soret absorption due to Fe(III) heme, consistent with V-BrPO from *A. nodosum*.

Specific Activities of V-BrPO from Fucus and Macrocystis. The specific activity of MCD bromination for V-BrPO from *F. distichus* and *M. pyrifera* is 1580 and 1730 units/mg, respectively, at pH 6.5 and pH 6.0, respectively, under the standard assay conditions (see Materials and Methods). The specific activity of V-BrPO from *F. distichus* and *M. pyrifera* is much higher than that for V-BrPO from *A. nodosum* determined under the same conditions (i.e., 170 units/mg at pH 6.5).[2] The origin of the 10-fold difference in specific activity between the V-BrPO from *Fucus* and *Macrocystis* and that from *Ascophyllum* is under investigation.

Dioxygen Formation and pH Dependence of V-BrPO from Fucus and Macrocystis. In the absence of MCD or other substrate for bromination, V-BrPO from *F. distichus* and *M. pyrifera* catalyze the formation of dioxygen. Bromide or iodide is required for dioxygen formation. The rate of the bromide-assisted disproportionation of hydrogen peroxide is markedly pH dependent as shown in Figure 1 for V-BrPO from *F. distichus* and Figure 2 for V-BrPO from *M. pyrifera*. Figures 1A and 2A show the pH dependence as a function of bromide at a constant hydrogen peroxide concentration (i.e., 5 mM). Bromide is both a substrate and an inhibitor. Bromide inhibition is most pronounced at lower pH, although, for V-BrPO from *F. distichus*, pH 8 is the only pH that bromide inhibition is not observed. The *Ascophyllum* (Wever et al., 1985) and *Macrocystis* enzymes are not as sensitive to bromide inhibition as the *Fucus* enzyme. Figures 1B and 2B show the pH dependence as a function of hydrogen peroxide at a constant bromide concentration (i.e., 50 mM). Hydrogen peroxide is also both a substrate and an inhibitor, although the effect is most pronounced at higher pH. The pH profiles of MCD bromination catalyzed by V-BrPO from *A. nodosum* (Wever et al., 1985; Soedjak and Butler, unpublished observations) and *L. saccharina* (de Boer et al., 1986) show that bromide and hydrogen peroxide are both substrates and inhibitors, consistent with the behavior of dioxygen formation

[2] The specific activity of V-BrPO from *A. nodosum* is 120 units/mg with the bicinchoninic acid assay to determine the protein concentration, whereas the specific activity with the Bio-Rad protein assay is 170 units/mg.

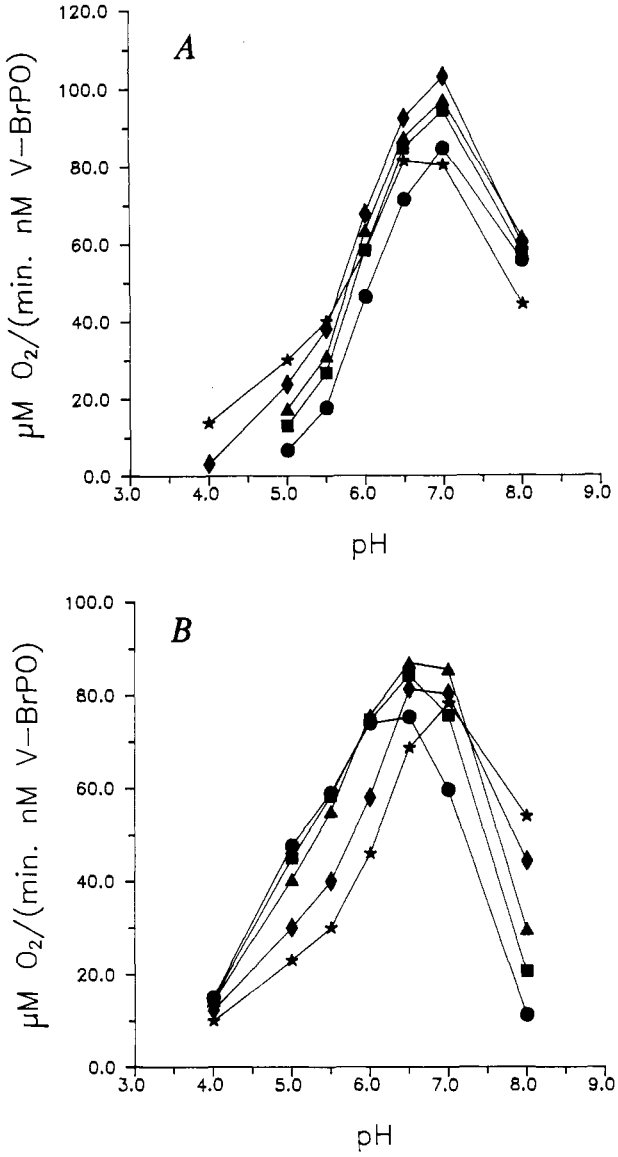

FIGURE 1: pH dependence of dioxygen formation for V-BrPO from *Fucus*. (A) pH dependence at 50 mM bromide as a function of hydrogen peroxide concentration: (●) 0.1 mM H_2O_2; (■) 0.25 mM H_2O_2; (▲) 0.50 mM H_2O_2; (♦) 1.00 mM H_2O_2; (★) 5.00 mM H_2O_2. (B) pH dependence at 5 mM hydrogen peroxide as a function of bromide concentration: (●) 5.0 mM Br^-; (■) 10.0 mM Br^-; (▲) 16.7 mM Br^-; (♦) 50.0 mM Br^-; (★) 100.0 mM Br^-. The assay solutions were buffered in 0.1 M sodium citrate for pH 4.0 and 5.5 and in 0.1 M phosphate for pH 6.0 and above. Sodium sulfate was used to adjust the ionic strength to 0.97 M. The concentration of V-BrPO was 0.3 nM.

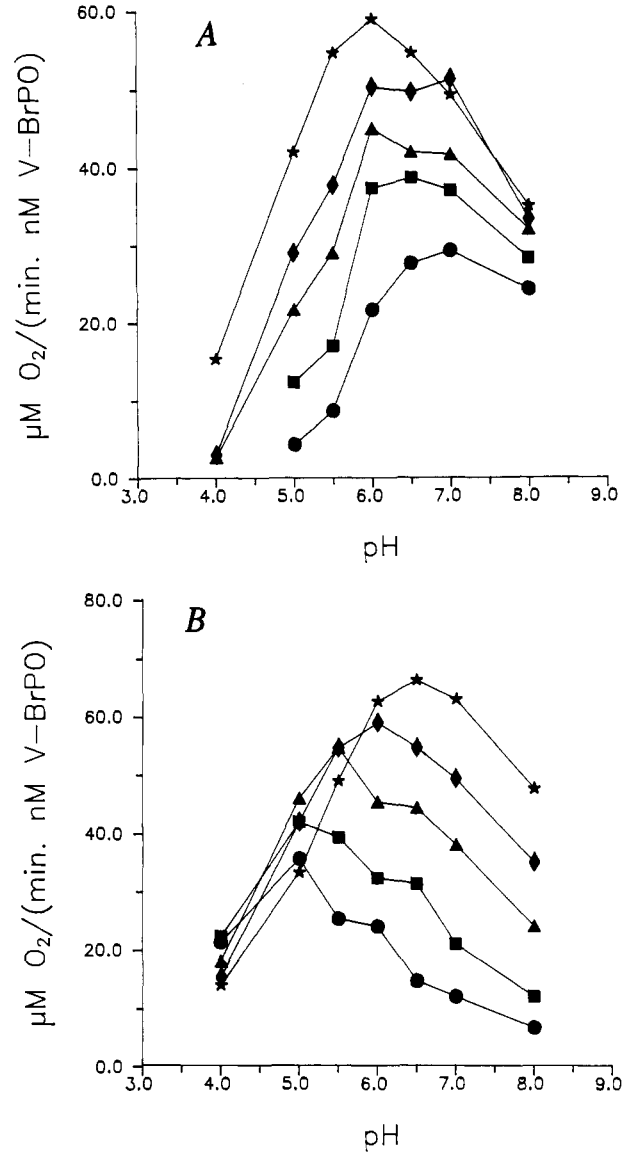

FIGURE 2: pH dependence of dioxygen formation for V-BrPO from *Macrocystis*. (A) pH dependence at 50 mM bromide as a function of hydrogen peroxide concentrations: (●) 0.1 mM H_2O_2; (■) 0.25 mM H_2O_2; (▲) 0.50 mM H_2O_2; (♦) 1.00 mM H_2O_2; (★) 5.00 mM H_2O_2. (B) pH dependence at 5 mM hydrogen peroxide as a function of bromide concentration: (●) 5.0 mM Br^-; (■) 10.0 mM Br^-; (▲) 16.7 mM Br^-; (♦) 50.0 mM br^-; (★) 100.0 mM Br^-. The same buffer solutions and reactions conditions were employed as described in the caption for Figure 1.

shown in Figures 1 and 2. We have shown that dioxygen formation (i.e., the bromide-assisted disproportionation of hydrogen peroxide) competes effectively with MCD bromination and the effect is most significant at high pH (Everett et al., 1990b). Thus the pH profile of dioxygen formation is a better measure of the pH dependence of V-BrPO since the rate of MCD bromination is anomalously low at higher pH due to the competing dioxygen formation reaction.

Investigation of the Nature of the Peroxide Used by V-BrPO (A. nodosum, F. distichus, and M. pyrifera), Peracetic Acid. We have investigated the reactivity of V-BrPO isolated from *A. nodosum*, *F. distichus*, and *M. pyrifera* toward peracetic acid in the presence of bromide and in the presence or absence of an organic halogen acceptor. Unlike hydrogen peroxide, peracetic acid oxidizes bromide, producing a mixture of hypobromous acid, bromine, and tribromide ion (identified by λ_{max} at 267 nm) of which the rate and equilibrium position of these oxidized bromine species depend on pH and bromide concentration. V-BrPO (from a stock solution stored in water) catalyzes the rate of formation of the 267-nm absorption maximum at pH 8.5, indicating that V-BrPO can use peracetic acid as a source of peroxide. It is important to note that the enzymatic formation of $HOBr/B_2/Br_3^-$ absorbing at 267 nm is only observed with enzyme stored in water or non-amine-containing buffers. At lower pH, the difference between the rates of the uncatalyzed and V-BrPO-catalyzed reactions becomes very small because the rate of the uncatalyzed oxidation increases. Addition of excess bromide at the end of the V-BrPO-catalyzed and uncatalyzed reactions increased the absorbance at 267 nm, substantially, which is consistent with a shift in the equilibrium distribution of $HOBr/Br_2/Br_3^-$ toward formation of Br_3^-. Thus, we conclude that in the absence of a halogen acceptor HOBr or a mixture of HOBr, Br_2, or Br_3^- is produced in the chemical and the V-BrPO-catalyzed

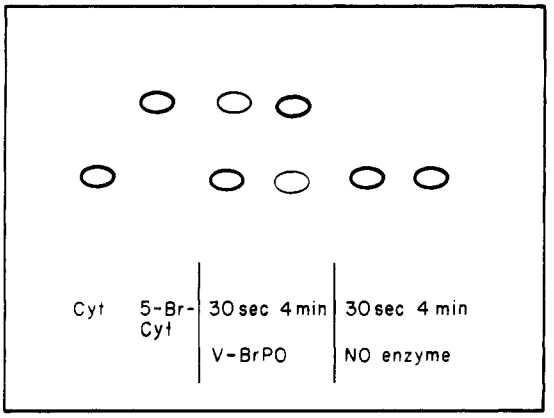

FIGURE 3: TLC analysis of V-BrPO (*F. distichus*) catalyzed cytosine bromination with peracetic acid as the oxidant of bromide oxidation. Lanes 1 and 2 (from the left) are standards of cytosine and 5-bromocytosine, respectively. Lanes 3 and 4 are the V-BrPO-catalyzed reaction at 30 s and 4 min, respectively, under conditions of 0.1 M phosphate buffer, pH 8.5, 5 mM cytosine, 5 mM bromide, 5 mM peracetic acid, and 500 nM V-BrPO (*Fucus*). Lanes 5 and 6 are the uncatalyzed reaction under the same conditions as in lanes 3 and 4 (i.e., no V-BrPO). Samples were applied on a TLC silica coated plate (Kodak) 30 s or 4 min after the reaction was initiated. The TLC plate was developed in 1-butanol–ethanol–water at a ratio of 2:1:1.

oxidations of bromide by peracetic acid. Both hypobromous acid and tribromide have absorption maxima at 267 nm with extinction coefficients of 87 M^{-1} cm^{-1} and 36 100 M^{-1} cm^{-1}, respectively; the absorption maximum of bromine is at 395 nm (ϵ = 177 M^{-1} cm^{-1}) (Thompson, 1986).

The build up of $HOBr/Br_2/Br_3^-$ is observed when peracetic acid is used as the oxidant of bromide but not when hydrogen peroxide is the source of the peroxide, except under certain low-pH conditions. Under most conditions, hydrogen peroxide rapidly reduces oxidized bromine species, producing singlet oxygen (Kanofsky, 1989a–c). Peracetic acid, on the other hand, is not an efficient two-electron reductant; thus, the concentration of $HOBr/Br_2/Br_3^-$ can build up in solution. de Boer and Wever (1988) report observation of the 267-nm absorption produced by V-BrPO and hydrogen peroxide, but only under conditions of low pH (i.e., pH 5), high bromide concentration (i.e., 0.1 M), and very high enzyme concentration (i.e., 130 nM) and in the first second of the reaction. At low pH the rate of $HOBr/Br_2$ by hydrogen peroxide is reduced (Kanofsky, 1984).

Bromination of Cytosine. To determine whether V-BrPO uses peracetic acid to catalyze bromination of organic substrates, the enzymatic versus chemical bromination of cytosine was investigated. The brominated product, 5-bromocytosine, is stable and can be separated from cytosine by thin-layer chromatography (Itoh, 1987). Comparison of the rates of the V-BrPO-catalyzed and chemical bromination reactions of cytosine using peracetic acid was carried out under conditions of 5 mM cytosine, 5 mM bromide, and 5 mM peracetic acid, with or without 500 nM V-BrPO (*Fucus*) in 0.1 M phosphate buffer at pH 8.5, containing 0.2 M sulfate. 5-Bromocytosine is produced in much higher yield in the V-BrPO-catalyzed reactions than in the reactions without V-BrPO (Figure 3), establishing clearly that V-BrPO catalyzes bromination reactions with peracetic acid as an oxidant of bromide. To enhance the difference between the rate of V-BrPO-catalyzed reaction and the rate of the uncatalyzed reaction, and thus the amount of cytosine brominated, the reactions were carried out at high pH, low bromide and peracetic acid concentrations, very high enzyme concentration, and short reaction time.

Bromination of Amines. We have also found that V-BrPO catalyzes the bromination of primary (i.e., taurine and Tris), secondary (i.e., Capso and Tes), and tertiary (i.e., Hepes and Mops) amines, with peracetic acid as the oxidant. The bromamines were detected spectrophotometrically, and their spectra were compared to the absorption spectra of the bromamines prepared chemically, by reaction of tribromide with each amine compound. The absorption maxima of monobrominated amine derivatives generally fall in the range of 278–315 nm with extinction coefficients of 390–500 M^{-1} cm^{-1} (Galal-Gorchev & Morris, 1965; Johannesson, 1986; Yazdanbakhsh et al., 1987). Dibromoamine and tribromoamines have absorption maxima at higher energy and with much higher extinction coefficients. Brominated taurine (λ_{max} 286 nm), brominated Tris (λ_{max} 290 nm), and brominated Capso (λ_{max} 313 nm) are stable and readily detected spectrophotometrically. Bromotaurine formation was confirmed by comparison of the absorption maximum and extinction coefficient (see Table I) with values reported in the literature (i.e., λ_{max} 286 nm and ϵ 436 M^{-1} cm^{-1}) (Yazdanbakhsh et al., 1987); the extinction coefficients of Br-Tris and Br-Capso are quite similar (Table I). In addition, the absorption maxima of the brominated amines prepared enzymatically and chemically are identical, as shown in Figure 4 for the bromination of Tris. The rates of the amine brominations catalyzed by V-BrPO were significantly faster than the uncatalyzed rates, providing additional evidence that V-BrPO uses peracetic acid as an oxidant to effect bromination.

V-BrPO-catalyzed buildup of these bromamine compounds occurs when peracetic acid, but not hydrogen peroxide, is used

Table I: Summary of Absorption Maxima and Extinction Coefficients of the Brominated Amines

compd[a]	λ_{max} (nm)	titration method[b]	ϵ (M^{-1} cm^{-1})[c]
bromotaurine	286	KI	428 ± 19
		TNB	436 ± 7
Br-Tris	290	KI	451 ± 28
		TNB	503 ± 8
Br-Capso	313	KI	374 ± 4
		TNB	398 ± 9

[a] The brominated amine derivatives were prepared as described in the text. [b] The concentration of bromamine was determined by titration with iodide or 5-thio-2-nitrobenzoic acid (TNB) as described under Materials and Methods. [c] The standard derviation of three experiments is indicated.

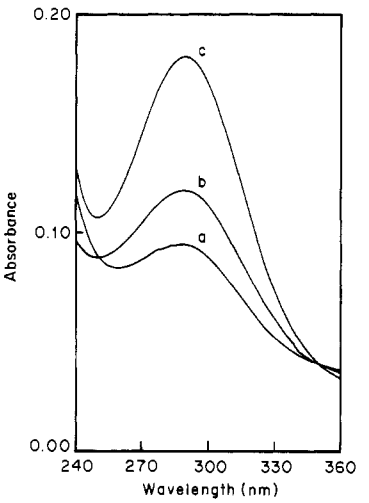

FIGURE 4: UV–vis spectrum of brominated Tris formed enzymatically and chemically: (a) 3 mM Tris, 2 mM bromide, and 1 mM peracetic acid; (b) 3 mM Tris and 0.13 mM tribromide; (c) 3 mM Tris, 2 mM bromide, 1 mM peracetic acid, and 30 nM V-brPO (*A. nodosum*). All reactions were carried out in 0.1 M phosphate buffer, pH 8.5. UV–vis spectra a and c were run after reaction for 30 min.

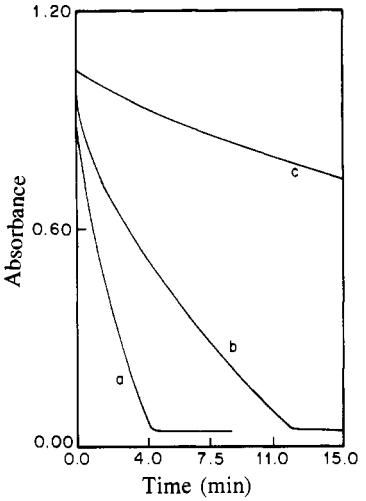

FIGURE 5: MCD bromination catalyzed by V-BrPO (*A. nodosum*) with peracetic acid: (a) reaction of MCD, peracetic acid, bromide, and V-BrPO; (b) reaction of MCD, peracetic acid, and bromide; (c) reaction of MCD, peracetic acid, and V-BrPO. The rate of MCD decomposition by peracetic acid was the same as in (c) in the absence of V-BrPO (data not shown). Reaction conditions were 1 mM peracetic acid and 50 μM MCD in 0.1 M phosphate buffer, pH 8.5, containing 0.2 M sulfate, ±2 mM bromide, and ±30 nM V-BrPO.

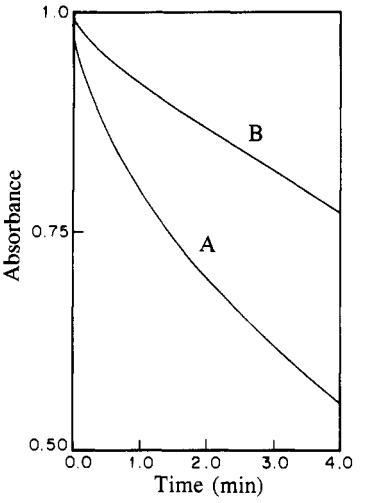

FIGURE 6: Comparison of the effect of hydrogen peroxide and peracetic acid on the rate of the V-BrPO-catalyzed bromination of MCD: (A) subtraction of spectrum 5c from spectrum 5a (50 μM MCD, 1 mM peracetic acid, ±2 mM bromide, and 30 nM V-BrPO (*A. nodosum*) in 0.1 M phosphate buffer, pH 8.5, containing 0.2 M sulfate); (B) reaction of 50 μM MCD, 1 mM hydrogen peroxide, 2 mM bromide, and 30 nM V-BrPO (*A. nodosum*) in 0.1 M phosphate buffer, pH 8.5, containing 0.2 M sulfate.

as the peroxide source. Hydrogen peroxide rapidly reduces the bromamines, producing dioxygen which is detected with an oxygen probe (data not shown). Dioxygen is not produced by reaction of bromamines with peracetic acid.

While taurine, Tris, and Capso produced stable brominated derivatives (Table I), bromination of Tes, Hepes, and Mops did not produce stable bromamine derivatives in the enzyme-catalyzed reaction with peracetic acid. Bromamine derivatives of Tes, Hepes, and Mops could be prepared by reaction with bromine as established spectrophotometrically (λ_{max} Br-Hepes, 313 nm; Br-Tes, 320 nm; Br-Mops, 296 nm), consistent with monobromamine complex formation, although they are not very stable. Nevertheless, by use of a diode array spectrophotometer, enzymatic bromination of Tes, Hepes, and Mops was observed with peracetic acid during the initial stage of the reaction. Formation of brominated Hepes and Mops is contrary to a report that tertiary amines do not form *N*-bromo derivatives in aqueous solution (Wajon & Morris, 1982).

Peracetic acid oxidizes the amines (Swern, 1949) in the absence of bromide, which is a potentially competing side reaction. Peracetic acid reacts completely within seconds with Hepes and completely within minutes with Mops and Tes. Peracetic acid reacts slowly with Tris, and no reaction was observed with Capso in 30 min. The qualitative reaction rate was determined by monitoring the concentration of unreacted peracetic acid.

Reactions with MCD. V-BrPO also catalyzes a reaction with MCD by use of peracetic acid as the oxidant of bromide under conditions of high pH and low concentrations of bromide and peracetic acid (e.g., 0.1 M phosphate, pH 8.5, 2 mM bromide, and 1 mM peracetic acid). Figure 5 shows a comparison of the V-BrPO-catalyzed (Figure 5a) and uncatalyzed (Figure 5b) reaction with MCD observed spectrophotometrically, which clearly shows V-BrPO catalysis. Peracetic acid also reacts with MCD directly, causing an absorbance decrease in both the presence (Figure 5c) and absence (data not shown) of V-BrPO. The rate of this decomposition is not affected by the presence of V-BrPO. As established above, V-BrPO catalyzes the oxidation of bromide by peracetic acid, forming a mixture of oxidized bromine species. Similarly, the uncatalyzed reaction of bromide and peracetic acid also produces the oxidized bromine species. Thus, the absorbance decrease in Figure 5a,b is reasonably MCD bromination, although the brominated product has not been isolated.

Comparison of the rate of MCD bromination using peracetic acid and hydrogen peroxide was investigated under the conditions which showed catalysis with peracetic acid (i.e., 0.1 M phosphate, pH 8.5, 2 mM bromide, 1 mM peracid, 30 nM V-BrPO). The rate of bromination of MCD is significantly faster with peracetic acid (Figure 6A) than with hydrogen peroxide (Figure 6B). The curve in Figure 6A was obtained by subtracting the uncatalyzed rate of reaction of peracetic acid with MCD (Figure 5b) from the V-BrPO-catalyzed reaction (Figure 5a).

Preferential Bromination. It is not possible to establish whether amines are preferentially brominated over cytosine or MCD. If bromination of MCD is carried out with peracetic acid as the source of peroxide, and in the presence of 3 mM Tris, brominated Tris is only observed after the MCD is completely reacted. This does not mean that MCD is brominated preferentially, because the brominated amines are very effective brominating agents. *N*-Bromo-Tris brominates MCD within the time of rapid hand mixing. Moreover, the rates of the enzymatic MCD bromination by peracetic acid and bromide in various buffers (e.g., Tris, bicarbonate, phosphate, and Capso) at pH 8.5 are identical. Bromo-Capso, bromotaurine, and *N*-bromosuccinamide, like bromo-Tris, are also efficient brominating agents. Thus, the mechanisms of bromination via the intermediate pathway of amine-buffer bromination versus direct bromination of MCD cannot be distinguished.

Other Peroxides. V-BrPO uses other acyl peroxides to catalyze the bromination reactions under conditions of 0.1 M phosphate buffer, pH 8.5, 1–2 mM bromide, and 1–2 mM acyl peroxide. Phenylperacetic acid is used by V-BrPO to catalyze the formation of Br-Tris and $HOBr/Br_3^-$ which were followed spectrophotometrically at 290 and 267 nm, respectively, as well as the bromination of MCD (data not shown). With *p*-nitroperoxybenzoic acid and *m*-chloroperoxybenzoic acid, catalysis of MCD bromination by V-BrPO was observed; however, following the reaction by observation of the absor-

bance increase at 267 nm, due to the oxidized bromine species, was not possible due to the interference from the absorbance of the corresponding acetate derivatives. Like peracetic acid, however, none of these acyl peracids catalyze the formation of dioxygen. We have also found that the alkyl hydroperoxides ethyl hydroperoxide, cuminyl hydroperoxide, and *tert*-butyl hydroperoxide do not support bromination or dioxygen formation catalyzed by V-BrPO.

Discussion and Conclusions

The brown algae *F. distichus* and *M. pyrifera* contain vanadium bromoperoxidase. These enzymes are very similar to V-BrPO from *A. nodosum* and *L. saccharina* in physical properties and reactivity, except that the specific activity of MCD bromination of V-BrPO from *Fucus* (i.e., 1580 units/mg at pH 6.5) and *Macrocystis* (i.e., 1730 units/mg at pH 6.0) is significantly higher than that of V-BrPO from *A. nodosum* (i.e., 170 units/mg at pH 6.5). In the absence of a halogen acceptor, V-BrPO catalyzes the formation of dioxygen. However, unlike iron heme bromoperoxidase (Manthey & Hager, 1981) and iron heme chloroperoxidase (Thomas et al., 1970), which catalyze the direct disproportionation of hydrogen peroxide, V-BrPO requires bromide for dioxygen formation. The iron heme haloperoxidases also have a halide-assisted pathway of hydrogen peroxide disproportionation (Manthey & Hager, 1981).

V-BrPO uses peracetic acid to catalyze the oxidation of bromide. In the absence of a halogen acceptor, a mixture of oxidized bromine species (e.g., HOBr, Br_2, and Br_3^-) is formed. In the presence of certain amine compounds (e.g., taurine, Tris, and Capso) the corresponding monobromamine derivatives are formed. Catalysis of cytosine bromination by V-BrPO and peracetic acid was observed. The formation of the mixture of $HOBr/Br_3^-$ does not necessarily mean that these are the active brominating species produced by V-BrPO. The equilibria among these oxidized species including bromamines are established very rapidly. Formation of bromine and tribromide from HOBr or *N*-bromosuccinimide is particularly enhanced at low pH and high bromide concentration.

Unlike the ability of V-BrPO to catalyze the bromide-assisted disproportionation of hydrogen peroxide, V-BrPO does not catalyze the formation of dioxygen when peracetic acid is used as an oxidant of bromide. In addition, V-BrPO does not use alkyl peroxides to catalyze bromination or dioxygen formation. Peracetic acid and ethyl hydroperoxide oxidize iron heme BrPO by two electrons, forming compound 1, although dioxygen is not formed in the presence or absence of a halogen acceptor. By contrast, iron heme chloroperoxidase catalyzes the formation of dioxygen from peracetic acid and ethyl hydroperoxide, even in the absence of chloride (Thomas et al., 1970). The main difference between V-BrPO and iron heme BrPO lies in the reactivity in the absence of bromide. Iron heme BrPO catalyzes the oxidation of *o*-dianisidine, guaiacol, and pyrogallol in the absence of bromide, whereas V-BrPO is completely inactive in the absence of bromide. The difference reflects the reactivity of the native enzyme with peroxide. Fe(III) heme BrPO is oxidized by peroxides to the compound I state, which can oxidize the organic compounds (Manthey & Hager, 1985). V^V-BrPO may bind peroxide; however, vanadium(V) cannot be oxidized further. The V^V peroxide may oxidize bromide but not the organic compounds.

The myeloperoxidase/hydrogen peroxide/chloride system catalyzes the chlorination of endogenous amines (Grisham et al., 1984; Test et al., 1984). The buildup of chloramine occurs because excess hydrogen peroxide does not reduce the chloramine to dioxygen and chloride (Kanofsky, 1989c). In contrast, hydrogen peroxide readily reduces bromamines, thus precluding observation of bromamine formation. In fact, Kanofsky (1989c) has recently reported that singlet oxygen is produced in the reaction of many brominated amino acids and derivatives (e.g., glycine, taurine, serine, phenylalanine, lysine, etc.) with hydrogen peroxide. The use of peracetic acid has allowed us to show the first evidence of enzymatic formation of *N*-bromamines.

Nieder and Hager (1985) and Kanofsky (1989) have suggested that haloperoxidase-catalyzed halogenation reactions could proceed by formation of halogenated amine intermediates. Such intermediates could implicate the formation of an enzyme-bound bromonium ion as an intermediate in the enzyme-catalyzed halogenation reactions. One factor affecting the stability of bromamines is the concentration of excess bromide. Bromonium ions are readily displaced by bromide, forming bromine or tribromide, particularly at lower pH. An attractive feature of an enzyme-bound bromamine, or other enzyme-bound brominated moiety, is the possible generation of chiral brominated compounds. Many chiral halogenated terpenes have been isolated from marine algae [see, for example, Neidleman and Geigert (1986)]. Of course the critical factor would be the reactivity of the putative enzyme-bound brominated intermediate toward bromination, hydrolysis, or displacement by excess bromide.

Acknowledgments

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Registry No. BrPO, 69279-19-2; Capso, 73463-39-5; Tris, 77-86-1; Br^-, 24959-67-9; H_2O_2, 7722-84-1; $(CH_3CO)_2O$, 108-24-7; CH_3CO-OOH, 79-21-0; monochlorodimedone, 7298-89-7; taurine, 107-35-7; cytosine, 71-30-7; phenylperacetic acid, 19910-09-9; *m*-chloroperoxybenzoic acid, 937-14-4; *p*-nitroperoxybenzoic acid, 943-39-5; 5-bromocytosine, 2240-25-7; bromotaurine, 52316-57-1; bromo-Tris, 128271-42-1; bromo-Capso, 128271-43-2.

References

Cotton, M. L., & Dunford, H. B. (1973) *Can. J. Chem. 51*, 582–587.

de Boer, E., & Wever, R. (1988) *J. Biol. Chem. 263*, 12326–12332.

de Boer, E., van Kooyk, Y., Tromp, M. G. M., Plat, H., & Wever, R. (1986a) *Biochim. Biophys. Acta 869*, 48–53.

de Boer, E., Tromp, M. G. M., Plat, H., Krenn, G. E., & Wever, R. (1986b) *Biochim. Biophys. Acta 872*, 104–115.

Dubray, G., & Bezard, G. (1982) *Anal. Biochem. 119*, 325–329.

Ellman, G. L. (1959) *Arch. Biochem. Biophys. 82*, 70–77.

Everett, R. R., & Butler, A. (1989) *Inorg. Chem. 28*, 393–395.

Everett, R. R., Kanofsky, J. R., & Butler, A. (1990a) *J. Biol. Chem. 265*, 4908–4914.

Everett, R. R., Soedjak, H. S., & Butler, A. (1990b) *J. Biol. Chem.* (in press).

Galal-Gorchev, H., & Morris, J. C. (1965) *Inorg. Chem. 4*, 899–905.

Gander, J. E. (1984) *Methods Enzymol. 104*, 447–451.

Grishman, M. B., Jefferson, M. M., Melton, D. F., & Thomas, E. L. (1984) *J. Biol. Chem. 259*, 10404–10413.

Gschwend, P. M., MacFarlane, J. K., & Newman, K. A. (1985) *Science 227*, 1033–1035.

Hager, L. P., Morris, D. R., Brown, F. S., & Eberwein, H. (1966) *J. Biol. Chem. 241*, 1769–1777.

Hewson, W. D., & Hager, L. P. (1980) *J. Phycol. 16*, 340–345.
Itoh, H., Izumi, Y., & Yamada, H. (1986) *J. Biol. Chem. 261*, 5194–5200.
Itoh, N., Izumi, Y., & Yamada, H. (1987) *Biochemistry 26*, 282–289.
Johannesson, J. K. (1985) *Chem. Ind.* 97–98.
Kanofsky, J. R. (1984) *J. Biol. Chem. 259*, 5596–5600.
Kanofsky, J. R. (1989a) *Chem.-Biol. Interact. 70*, 1–28.
Kanofsky, J. R. (1989b) in *Oxygen Radicals in Biology and Medicine* (Simic, M. G., Taylor, K. A., Ward, J. E., & von Sonntag, C., Eds.) pp 211–218, Plenum, New York.
Kanofsky, J. R. (1989c) *Arch. Biochem. Biophys. 274*, 229–234.
Krenn, B. E., Plat, H., & Wever, R. (1987) *Biochim. Biophys. Acta 912*, 287–291.
Krenn, B. E., Tromp, M. G. M., & Wever, R. (1989a) *J. Biol. Chem. 264*, 19287–19292.
Krenn, B. E., Izumi, Y., Yamada, H., & Wever, R. (1989b) *Biochim. Biophys. Acta 998*, 63–68.
Laemli, U. K. (1970) *Nature 227*, 680–685.
Manthey, J. A., & Hager, L. P. (1981) *J. Biol. Chem. 256*, 11232–11238.
Manthey, J. A., & Hager, L. P. (1985) *J. Biol. Chem. 260*, 9654–9659.
Manthey, J. A., & Hager, L. P. (1989) *Biochemistry 28*, 3052–3057.
Neidleman, S. L., & Geigert, J. (1986) *Biohalogenation*, p 203, Ellis Horwood, Chichester, U.K.
Nieder, M., & Hager, L. (1985) *Arch. Biochem. Biophys. 240*, 121–127.
Plat, H., Krenn, B. E., & Wever, R. (1987) *Biochem. J. 248*, 277–279.
Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Garther, F. H., & Provenzano, M. D. (1985) *Anal. Biochem. 150*, 76–85.
Stelmaszynska, T., & Zgliczynski, J. M. (1978) *Eur. J. Biochem. 92*, 301–308.
Swern, D. (1949) *Chem. Rev. 45*, 1–68.
Swern, D. (1970) *Organic Peroxides*, Vol. 1, Wiley, New York.
Test, S. T., Lampert, M. K., Ossanna, P. J., Thoene, J. G., & Weiss, S. J. (1984) *J. Clin. Invest. 74*, 1341–1349.
Thomas, E. L. (1979) *Infect. Immun. 23*, 522–531.
Thomas, J. A., Morris, D. R., & Hager, L. P. (1970) *J. Biol. Chem. 245*, 3129–3134.
Thompson, R. C. (1986) *Advances in Inorganic and Bioinorganic Mechanisms*, Vol. 4, pp 65–106, Academic Press, London.
Vilter, H. (1984) *Phytochemistry 23*, 1387–1390.
Vilter, H., & Glombitza, K.-W. (1983) *Bot. Mar. 26*, 341–344.
Wajon, J. E., & Morris, J. C. (1982) *Inorg. Chem. 21*, 4258–4263.
Wardi, A. H., & Michos, G. A. (1972) *Anal. Biochem. 49*, 607–609.
Wever, R., Plat, H., & de Boer, E. (1985) *Biochim. Biophys. Acta 830*, 181–186.
Yamada, H., Itoh, N., Murakami, S., & Izumi, Y. (1985) *Agric. Biol. Chem. 49*, 2961–2967.
Yazdanbakhsh, M., Eckmann, C. M., & Roos, D. (1987) *Am. J. Trop. Med. Hyg. 37*, 106–110.