Oxidation of 3-Hydroxyanthranilic Acid to the Phenoxazinone Cinnabarinic Acid by Peroxyl Radicals and by Compound I of Peroxidases or Catalase†

Stephan Christen,‡ Peter T. Southwell-Keely,§ and Roland Stocker*,‡

Biochemistry Group, The Heart Research Institute, 145 Missenden Road, Camperdown, NSW 2050, Australia, and Department of Organic Chemistry, University of New South Wales, Kensington, NSW 2033, Australia

Received February 17, 1992; Revised Manuscript Received May 11, 1992

ABSTRACT: Since 3-hydroxyanthranilic acid (3HAA), an oxidation product of tryptophan metabolism, is a powerful radical scavenger [Christen, S., Peterhans, E., & Stocker, R. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 2506], its reaction with peroxyl radicals was investigated further. Exposure to aqueous peroxyl radicals generated at constant rate under air from the thermolabile radical initiator 2,2′-azobis[2-amidinopropane] hydrochloride (AAPH) resulted in rapid consumption of 3HAA with initial accumulation of its cyclic dimer, cinnabarinic acid (CA). The initial rate of formation of the phenoxazinone CA accounted for ≈75% of the initial rate of oxidation of 3HAA, taking into account that 2 mol of 3HAA are required to form 1 mol of CA. Consumption of 3HAA under anaerobic conditions (where alkyl radicals are produced from AAPH) was considerably slower and did not result in detectable formation of CA. Addition of superoxide dismutase enhanced autoxidation of 3HAA as well as the initial rates of peroxyl radical-induced oxidation of 3HAA and formation of CA by ≈40–50%, whereas inclusion of xanthine/xanthine oxidase decreased the rate of oxidation of 3HAA by ≈50% and inhibited formation of CA almost completely, suggesting that superoxide anion radical ($O_2^{\bullet -}$) was formed and reacted with reaction intermediate(s) to curtail formation of CA. Formation of CA was also observed when 3HAA was added to preformed compound I of horseradish peroxidase (HRPO) or catalytic amounts of either HRPO, myeloperoxidase, or bovine liver catalase together with glucose/glucose oxidase. In the case of preformed HRPO compound I, oxidation of 3HAA into CA proceeded to the same extent under both aerobic and anaerobic conditions, indicating that it did not require molecular oxygen per se. The results suggest that oxidative dimerization of 3HAA into CA can be obtained by initial and successive one-electron oxidation reactions mediated by either free radicals or compound I of peroxidases or catalase.

3-Hydroxyanthranilic acid (3HAA)[1] is an intermediate formed during oxidative tryptophan metabolism along the kynurenine pathway. In most tissues, the initial and rate-limiting step of this pathway is catalyzed by the highly inducible indoleamine 2,3-dioxygenase (EC 1.13.11.17). In addition to serving as precursors in the biosynthesis of NAD^+ (Nishizuka & Hayaishi, 1963), metabolites of the kynurenine pathway are also known to be involved in the formation of the ommochromes, a class of mold and insect pigments (Gripenberg et al., 1957; Summers et al., 1982). In particular, 3-hydroxykynurenine and 3HAA are precursors for xanthommatin and cinnabarinic acid (CA), respectively (Butenandt & Neubert, 1955; Ogawa et al., 1983). Both of these heterocyclic compounds contain the phenoxazinone chromophore, which they have in common with actinomycins, a family of potent antimicrobial and antineoplastic compounds produced by certain strains of *Streptomyces*.

Biosynthesis of actinomycins involves the oxidative dimerization of substituted *o*-aminophenols derived from 3HAA, a reaction catalyzed by the bacterial phenoxazinone synthase (Katz & Weissbach, 1962). The enzyme contains copper at the active site and is believed to catalyze two-electron oxidation reactions (Barry et al., 1989). The *o*-quinone imine has been proposed as intermediate (Scheme I) with some of the subsequent reactions possibly being nonenzymatic (Barry et al., 1989). The oxidation of *o*-aminophenols such as 3HAA to phenoxazinones is also catalyzed by tyrosinase (EC 1.14.18.1) from *Neurospora crassa*, another copper-containing enzyme, and again, a reactive *o*-quinone imine intermediate has been proposed (Toussaint & Lerch, 1987). Tyrosinase normally catalyzes the hydroxylation of monophenols to *o*-quinols which, in turn, are oxidized to *o*-quinones, which are intermediates in the formation of melanins (Lerch, 1981).

Oxidative dimerization of *o*-aminophenols is also catalyzed by cytochrome *c* and cytochrome oxidase (Nagasawa et al., 1959), as well as nonenzymatically by various transition metals, especially Mn^{2+} (Prinz & Savage, 1977). These observations show that formation of phenoxazinones can be mediated by many different enzymatic and nonenzymatic systems. Curiously, a "specific phenoxazinone synthase" could not be identified in a number of tissues of higher organisms that did convert *o*-aminophenols into phenoxazinones. Instead, the hemoprotein catalase copurified with the "activity" responsible for chromophore formation (Savage & Prinz, 1977; Ogawa et al., 1983). While this "activity" was greatly enhanced in the presence of manganese ions, the potential involvement of catalase in the reaction mechanism remained obscure.

The primary biological function of catalase is the removal of H_2O_2 performed in two sequential reactions (eqs 1 and 2) (Chance et al., 1979). In addition to this "catalatic" activity,

† This work was supported in part by an Overseas Postgraduate Research Scholarship from the Department of Employment, Education and Training of Australia and a Sydney University Postgraduate Research Award (S.C.), and by National Health & Medical Research Council of Australia Grant 920371 (R.S.).

* To whom correspondence should be addressed [phone +61 (2) 550-3560, FAX +61 (2) 550-3302].

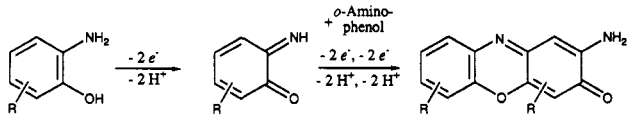
‡ The Heart Research Institute.

§ University of New South Wales.

[1] Abbrevations: AAPH, 2,2′-azobis[2-amidinopropane] hydrochloride; CA, cinnabarinic acid; 3HAA, 3-hydroxyanthranilic acid; HRPO, horseradish peroxidase; MPO, myeloperoxidase; SOD, superoxide dismutase.

0006-2960/92/0431-8090$03.00/0

Scheme I: Phenoxazinone Synthase- or Tyrosinase-Catalyzed Conversion of *o*-Aminophenols to Phenoxazinones Involving Three Consecutive Two-Electron Oxidation Reactions[a]



o-Aminophenol *o*-Quinone imine Phenoxazinone

[a] As proposed by Barry et al. (1989) and Toussaint and Lerch (1987).

compound I of catalase has also "peroxidatic" activity with either ethanol or methanol (eq 3) or certain one-electron donors (AH_2, eqs 4 and 5), such as aromatic compounds, as substrates (Nicholls & Schonbaum, 1963; Oshino et al., 1973). Reactions 4 and 5 are equivalent to those catalyzed by compounds I and II of various peroxidases (Paul, 1963; Yamazaki & Yokota, 1973).

$$\text{catalase} + H_2O_2 \rightarrow \text{compound I} + H_2O \quad (1)$$

$$\text{compound I} + H_2O_2 \rightarrow \text{catalase} + H_2O + O_2 \quad (2)$$

$$\text{compound I} + RCH_2OH \rightarrow \text{catalase} + RCHO + H_2O \quad (3)$$

$$\text{compound I} + AH_2 \rightarrow \text{compound II} + {}^{\bullet}AH + H_2O \quad (4)$$

$$\text{compound II} + AH_2 \rightarrow \text{catalase} + {}^{\bullet}AH + H_2O \quad (5)$$

Depending on the nature of the substrate and the conditions, the radical(s) formed in reactions 4 and 5 may either dimerize, disproportionate, or reduce molecular oxygen to the superoxide anion radical ($O_2^{\bullet -}$) (eqs 6–8). Phenolic kynurenine

$$2\,{}^{\bullet}AH \rightarrow HAAH \quad (6)$$

$$2\,{}^{\bullet}AH \rightarrow A + AH_2 \quad (7)$$

$${}^{\bullet}AH + O_2 \rightarrow A + O_2^{\bullet -} + H^+ \quad (8)$$

metabolites like 3HAA and 3-hydroxykynurenine are very efficient peroxyl radical scavengers (Christen et al., 1990). Since these metabolites are structurally similar to the reductants reported to be dimerized by the action of compound I (Ruch et al., 1983), we tested whether CA could be formed from 3HAA by compound I of peroxidase and catalase or via nonenzymatic, free radical reactions. We show here that both peroxyl radicals and compound I readily convert 3HAA into CA.

MATERIALS AND METHODS

Materials. Horseradish peroxidase (HRPO) and superoxide dismutase (SOD, bovine erythrocytes) were obtained from Boehringer Mannheim, catalase (bovine liver), glucose oxidase (*Aspergillus niger*), 3HAA (2-amino-3-hydroxybenzoic acid), glutathione peroxidase (bovine erythrocytes), and xanthine oxidase (buttermilk) were from Sigma, human polymorphonuclear leukocyte myeloperoxidase (MPO, A_{430}/A_{280} = 0.74) and B-phycoerythrin (*Porphyridium cruentum*) were from Calbiochem, and 2,2′-azobis[2-amidinopropane] hydrochloride (AAPH) was from Polysciences. Enzyme suspensions were all purified by Sephadex gel filtration (PD-10, Pharmacia), and all aqueous solutions and buffers by treatment with Chelex-100 (Bio-Rad) to remove transition metals. Substituted phenols and aromatic amines were obtained from Aldrich or Fluka and used without further purification. CA, synthesized as described (Manthey et al., 1990), was a generous gift from Dr. R. J. W. Truscott, University of Wollongong, Australia. UV–visible and fluorescence spectroscopy were performed on a Hitachi U-3210 spectrophotometer and a Hitachi F-4010 spectrofluorimeter, respectively. ^{1}H-NMR spectra were obtained in DMSO-d_6 on a Bruker AM 500 spectrometer, and chemical shifts are reported in ppm downfield from the internal standard tetramethylsilane.

Reaction of 3HAA with Peroxyl Radicals. A solution of 3HAA (0.1 mM) in 75 mM sodium phosphate buffer (pH 7.0) was incubated at 37 °C under air in the presence of AAPH (45 mM). Under such conditions, thermal decomposition of the azo compound results in initial formation of alkyl radicals, which then react rapidly with oxygen to form peroxyl radicals at a constant rate (Niki et al., 1986). Reaction mixtures were analyzed at different times for 3HAA and CA by HPLC (Christen & Stocker, 1992). For product identification, 3HAA (55 mg, 0.36 mmol) was added to 6 mL of a stirred aerobic solution of AAPH (0.25 M) in *N*,*N*-dimethylformamide/water (1:1) (Prinz & Savage, 1977) and incubated at 37 °C. After 3 h (i.e., when the absorption at 450 nm of the reaction mixture had reached its maximum), insoluble material was removed from the mixture by centrifugation and filtration, the products were resolved by reversed-phase HPLC (Christen & Stocker, 1992), and interfering substances present in the mobile phase used for HPLC were removed by solid-phase extraction on a C_{18} column. The isolated reaction product was identified as CA (2-amino-3-oxo-3*H*-phenoxazine-1,9-dicarboxylic acid) by comparing it by HPLC and spectroscopically with an authentic standard of CA: UV (DMSO) λ_{max} 457, 432 nm; ^{1}H-NMR (DMSO-d_6) δ 6.60 (s, 1 H), 7.61 (t, 1 H, J = 7.9 Hz), 7.77 (d, 1 H, J = 8.2 Hz), 7.95 (d, 1 H, J = 8.0 Hz), 8.79 (s, 1 H, CO_2H), 9.72 (s, 1 H, CO_2H). These NMR data are in good agreement with those published previously (Toussaint & Lerch, 1987).

Antioxidant Activities of Phenols and Aromatic Amines toward Peroxyl Radicals. The capacity of substituted phenols and aromatic amines to protect phycoerythrin from peroxyl radical-initiated oxidative damage was assessed by measuring their inhibitory activity on the AAPH-mediated loss of protein fluorescence (DeLange & Glazer, 1989). Because phycoerythrin reacts with peroxyl radicals and other oxidants much faster than other proteins, this assay can be used to test for antioxidant activity of a variety of water-soluble compounds (DeLange & Glazer, 1989). Antioxidant activity as defined in this in vitro test requires both efficient scavenging of peroxyl radicals by the compound and a lack of oxidizing activity of the product(s) derived from the compound after its reaction with peroxyl radicals. Product formation was monitored by differential UV–visible spectroscopy (±substrate) in the absence of phycoerythrin.

Reaction of 3HAA with Compound I. In some experiments, various concentrations of compound I of HRPO (2.5–20 μM) were prepared at 4 °C by the addition of equimolar amounts of H_2O_2 to the enzyme dissolved in 75 mM sodium phosphate buffer (pH 7.0), and its formation was verified spectrophotometrically as described (Job & Dunford, 1976). Oxidation of 3HAA (0.1 mM) by preformed HRPO compound I was performed at 4 °C for 15 s before the mixture was subjected to HPLC analysis for 3HAA and CA.

Alternatively, compound I of either HRPO, MPO, or catalase was prepared by incubating these enzymes at 37 °C under air in 0.1 M sodium acetate buffer (pH 5.0–6.0)

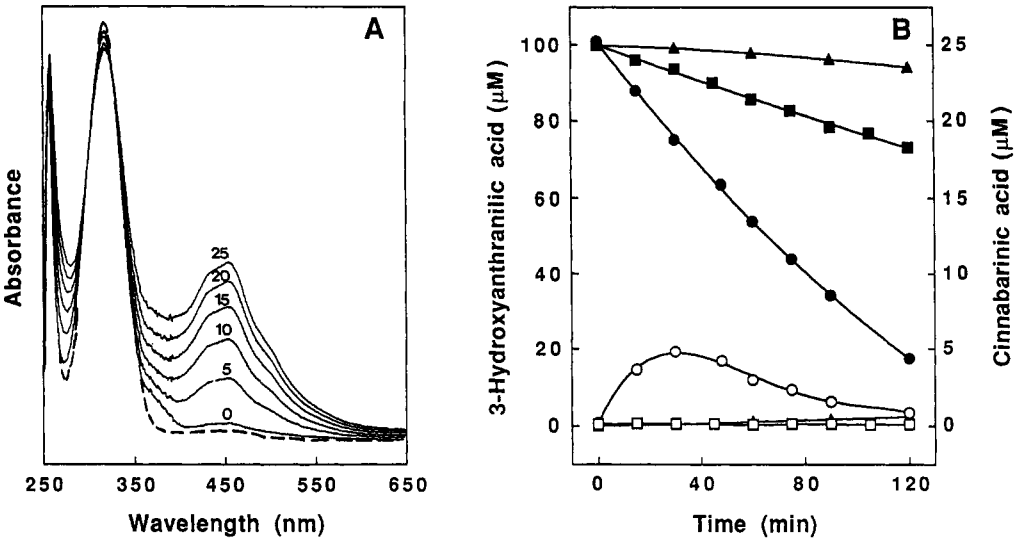


FIGURE 1: UV–visible spectra (A) and HPLC analysis (B) of the oxidation of 3HAA (100 μM) mediated by peroxyl radicals generated by the thermal decomposition of the azo compound AAPH (45 mM) at 37 °C. (A) Spectra were obtained under aerobic conditions (solid lines), with the numbers indicating the reaction time in minutes. The broken line represents the spectraum of the reaction mixture taken after 25 min of incubation in the absence of air. The scan speed was 5 nm s^{-1}. (B) Reaction carried out in aerobic (circles) and anaerobic (squares) 75 mM sodium phosphate buffer (pH 7.0) at 37 °C in the presence of radical initiator (45 mM). The control experiment (absence of AAPH) was performed under air (triangles). Aliquots of the reaction mixture were removed at different times and analyzed for 3HAA (closed symbols) and CA (open symbols) by HPLC (Christen & Stocker, 1992).

containing D–glucose (10 mM), glucose oxidase (≈0.8 nM), and 3HAA (0.1 mM). Under these conditions glucose oxidase produced 0.6 ± 0.1 μM H_2O_2/min (pH 5.0) (Root et al., 1975). At different times aliquots of the reaction mixture were removed and analyzed for 3HAA and CA.

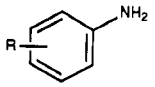
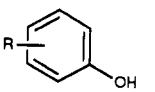
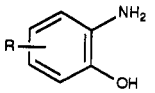
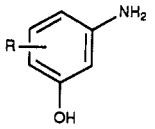
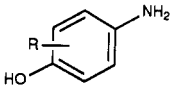
RESULTS

Peroxyl Radical-Initiated Oxidation of 3HAA. Incubation of 3HAA with the radical initiator AAPH in the presence of air resulted in an immediate and time-dependent decrease in absorbance of the reaction mixture at 314 nm with a concomitant increase at 451 nm (Figure 1A). In contrast, when the experiment was carried out under anaerobic conditions, no increase in the 451-nm absorbance could be observed even after prolonged periods of incubation (Figure 1A, broken line). In a separate experiment using dimethylformamide/water (1:1) as the solvent to improve solubility of 3HAA, the product responsible for the observed increase at 451 nm was isolated and identified by HPCL, UV–visible, and ^{1}H-NMR spectroscopy as CA (see Materials and Methods). To obtain kinetic information on the peroxyl radical-mediated oxidation of 3HAA, aerobic reaction mixtures (in phosphate buffer, pH 7.0) were analyzed at various times for 3HAA and CA by HPLC (Figure 1B). In the absence of AAPH, only about 6% of 3HAA disappeared within the first 120 min, forming very small amounts of CA, indicating that autoxidation of 3HAA is of minor importance under these conditions. In the presence of AAPH, where peroxyl radicals were generated at a constant rate of 3.3 ± 0.2 μM min^{-1} (n = 5),[2] 3HAA was consumed initially at linear rate with concomitant formation of CA. The initial rate of formation of CA (0.32 μM min^{-1}) accounted for ≈75% of the initial rate of disappearance of 3HAA (0.86 μM min^{-1}), taking into account that 2 mol of 3HAA is required to form 1 mol of CA. Accumulation of CA was maximal after ≈30 min of reaction, subsequent to which CA was consumed (Figure 1B). Separate experiments under similar oxidizing conditions showed that, upon exposure to peroxyl radicals, CA disappeared rapidly at rates that were proportional to the initial concentration of CA but were apparently unaffected by the presence of 3HAA (not shown). This can at least partly explain why substantial amounts of CA did not accumulate at later stages of the reaction (Figure 1B). The extent of reaction of 3HAA with peroxyl radicals was pH-dependent as initial rates of consumption of 3HAA increased by lowering the pH from 7.0 to 5.0 and then remained constant down to pH 3.0 (not shown). This was not reflected in corresponding changes in the rate of formation of CA, as the latter decreased sigmoidally to values close to zero when the pH was lowered from 7.0 to 3.0 (not shown). pH values >7.0 were not used since base-catalyzed autoxidation of 3HAA proceeds very rapidly at alkaline pH (Dykens et al., 1987). In agreement with the spectral data (Figure 1A), formation of CA was not detected when 3HAA was exposed to AAPH in the absence of air (Figure 1B), which results in formation of alkyl radicals only (Niki et al., 1986). While 3HAA was consumed under these conditions, its rate of oxidation was considerably slower when compared to that observed with peroxyl radicals. Increasing the rate of alkyl radical formation increased the rate of consumption of 3HAA but still failed to result in detectable formation of CA (not shown).

The antioxidant activity of 3HAA and various substituted phenols and aromatic amines was tested by the phycoerythrin assay (Table I) to assess the structural requirement for the reaction of 3HAA with this type of radical. Only the *o*- and *p*-aminophenols inhibited peroxyl radical-induced loss of phycoerythrin fluorescence substantially (82–90%). Depletion of the antioxidant was indicated by a break point, after which the loss of fluorescence proceeded at a rate similar to that of unprotected phycoerythrin. The *o*- and *p*-aminophenols scavenged between 2.5 and 3 radicals per molecule as judged by comparing the length of time they protected phycoerythrin with that of ascorbate (cf. footnotes to Table I). Upon oxidation, all of the *o*-aminophenols formed colored products with absorbance maxima between 425 and 455 nm and spectra similar to that of CA. The remaining compounds provided only poor

[2] Calculated from the duration of complete inhibition of AAPH-initiated fluorescence loss of phycoerythrin by known concentrations of ascorbate (cf. footnotes to Table I).

Table I: Peroxyl Radical-Scavenging Activity of 3-Hydroxyanthranilic Acid and Structure-Related Compounds[a]

structure	compound	% inhibition[b]	n value[c]	colored product[d]
	2-aminobenzoic acid	<0		ND
	3-aminobenzoic acid	<0		ND
	4-aminobenzoic acid	<0		ND
	2-hydroxybenzoic acid	20		ND
	3-hydroxybenzoic acid	20		ND
	4-hydroxybenzoic acid	20		ND
	2-aminophenol	88	2.5	yes
	2-amino-3-hydroxybenzoic acid	87	2.7	yes
	3-amino-2-hydroxybenzoic acid	82	2.6	yes
	3-amino-4-hydroxybenzoic acid	90	2.9	yes
	3-aminophenol	62		no
	4-amino-2-hydroxybenzoic acid	57		no
	4-aminophenol	83	2.7	no[e]
	2-amino-5-hydroxybenzoic acid	85	3.0	no
	5-amino-2-hydroxybenzoic acid	90	2.7	no

[a] Reaction mixture, consisting of 75 mM sodium phosphate buffer (pH 7.0), 17 nM B-phycoerythrin, 4 mM AAPH, and one of the various compounds at 5 μM, was incubated at 37 °C under air. [b] Calculated from the initial linear rates of fluorescence loss in the absence (control) and presence of a compound. [c] Calculated by comparing the duration of initial protection of phycoerythrin by the various compounds with that of ascorbate, which scavenges two peroxyl radicals per molecule under these conditions (DeLange & Glazer, 1989). [d] Product formation in the reaction mixture in the absence of phycoerythrin was monitored time-dependently by scanning between 250 and 650 nm. Formation of a "colored product" was defined as such if the reaction mixture had an absorbance maximum between 425 and 455 nm with spectral features similar to those of CA. [e] An increase in absorbance at 482 nm was observed. Data represent means of values obtained in two to four independent experiments. ND, not determined.

inhibition, and in addition, no break point in the rate of fluorescence loss was observed. While CA rapidly reacted with peroxyl radicals (see above), its presence failed to significantly inhibit AAPH-mediated loss of phycoerythrin fluorescence and peroxidation of linoleic acid (not shown), suggesting that the oxidation product(s) formed from CA by peroxyl radicals retain oxidizing potential.

Addition of SOD to the aerobic reaction mixture in the absence of AAPH increased the extent of autoxidation of 3HAA (cf. Figures 1B and 2A). In the presence of AAPH, the initial rates of oxidation of 3HAA and formation of CA also increased, by ≈40 and 50%, respectively (Figure 2A). Under these conditions, the initial rate of formation of CA accounted for ≈90% of the initial rate of consumption of 3HAA. Inclusion of the $O_2^{\bullet -}$-generating xanthine/xanthine oxidase (≈1.3 μM $O_2^{\bullet -}$ min^{-1}) decreased the rate of AAPH-induced oxidation of 3HAA by ≈50% and inhibited formation of CA almost completely (Figure 2B). In the combined presence of SOD and xanthine/xanthine oxidase, the rate of AAPH-induced oxidation of 3HAA was similar to that obtained in the absence of enzymes whereas formation of CA remained strongly inhibited (not shown). In contrast, inclusion of catalase did not influence peroxyl radical-mediated conversion of 3HAA into CA (not shown).

Oxidation of 3HAA by Compound I of Peroxidases and Catalase. Compound I of peroxidases is relatively stable in the absence of substituted phenols, aromatic amines, or other reductants (e.g., ascorbate) (Paul, 1963; Job & Dunford, 1976). In the presence of these reductants, compound I initially catalyzes a one-electron oxidation reaction with formation of a substrate-derived free radical intermediate (eq 4) (Yamazaki et al., 1960; Shiga & Imaizumi, 1975; Josephy et al., 1983). Indeed, at 4 °C preformed HRPO compound I reacted very rapidly and almost quantitatively with 3HAA, and this was accompanied by instantaneous formation of CA with a conversion corresponding to ≈45% of 3HAA used (Figure 3). Unlike the reaction with AAPH however, compound I-mediated formation of CA proceeded to the same extent under aerobic and anaerobic conditions, demonstrating that molecular oxygen per se is not required for the formation of CA from 3HAA.

In an attempt to study the time-dependent conversion of 3HAA into CA by compound I, 3HAA was incubated at 37 °C with the H_2O_2-generating glucose/glucose oxidase in the absence or presence of small amounts of either HRPO, MPO, or catalase. In the absence of peroxidases or catalase the extent of oxidation of 3HAA did not exceed that of autoxidation, indicating that 3HAA is not readily oxidized by H_2O_2 (cf. Figures 2 and 4). In the presence of the peroxidases, 3HAA was oxidized at the same rate at which H_2O_2 was formed by glucose/glucose oxidase (0.6 μM min^{-1}) and the initial rate of formation of CA accounted for ≈40% of the initial rate of consumption of 3HAA (not shown). Formation of CA was reduced significantly and concentration-dependently, though not completely, when glutathione/glutathione peroxidase (10–250 milliunits/mL) were added. The inhibition of compound I-mediated formation of CA from 3HAA by glutathione alone was observed although it was always smaller than that in the presence of glutathione and glutathione peroxidase. This indicates that both removal of H_2O_2 by glutathione/glutathione peroxidase and glutathione acting as substrate for compound I contributed to the observed inhibition.

Bovine liver catalase in the presence of H_2O_2 generated continuously by glucose/glucose oxidase also catalyzed the conversion of 3HAA into CA (Figure 4). In this case, the initial rate of oxidation of 3HAA (0.41 ± 0.09 μM min^{-1}, n = 3) corresponded to only ≈60% of the H_2O_2 produced, while the initial rate of formation of CA accounted for ≈60% of the initial rate of consumption of 3HAA. 3-Amino-1,2,4-triazole is known to react with compound I to form an enzymatically inactive complex (Darr & Fridovich, 1986). When this compound (1 mM) was added to the catalase system, consumption of 3HAA and formation of CA were inhibited

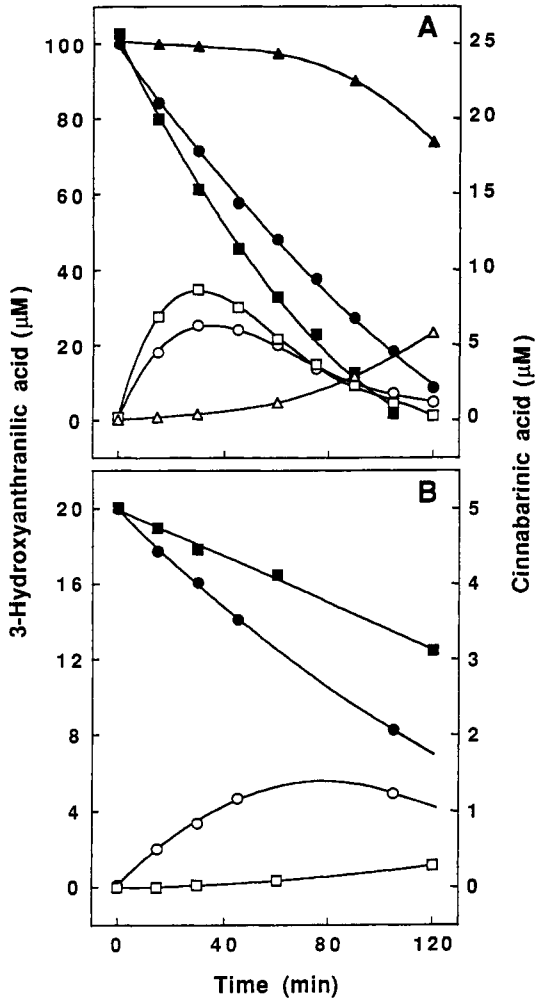


FIGURE 2: Peroxyl radical-mediated conversion of 3HAA into CA. 3HAA was exposed to peroxyl radicals generated at a constant rate by thermal decomposition of AAPH at 37 °C under air in the absence (circles) or presence (squares) of either (A) SOD (0.1 mg/mL) or (B) xanthine (≈1 mM) and xanthine oxidase (10 milliunits/mL). Analysis of the reaction mixture for 3HAA (closed symbols) and CA (open symbols) was performed as described in the legend to Figure 1. Conditions: 75 mM sodium phosphate buffer (pH 7.0) in (A) in (B); 100 and 20 μM 3HAA; 45 and 4 mM AAPH, respectively. Triangles in (A) represent incubation of 3HAA with SOD in the absence of AAPH.

initially by ≈30 and ≈50%, respectively (Figure 4). Inhibition increased further to ≈50 and ≈75%, respectively, when catalase was preincubated with aminotriazole and glucose/glucose oxidase for 60 min before addition of 3HAA, a treatment that decreased the "catalatic" activity by ≈40% (not shown). Similar to the HRPO experiments, catalase-mediated oxidation of 3HAA and formation of CA were inhibited by glutathione/glutathione peroxidase (10 milliunits/mL) and, to a lesser extent, by glutathione alone. In contrast, the reaction was not affected by SOD (0.1 mg/mL) (not shown).

DISCUSSION

A variety of different enzymes, including phenoxazinone synthase and tyrosinase, have been reported to catalyze the oxidative dimerization of 3HAA to CA. These enzymatic conversions are proposed to proceed in a sequence of three successive two-electron oxidation reactions (Scheme I) (Barry et al., 1989; Toussaint & Lerch, 1987). The results obtained in this study indicate an alternative and also biologically feasible pathway for dimerization of 3HAA into CA, i.e., one initiated nonenzymatically by peroxyl radicals or mediated

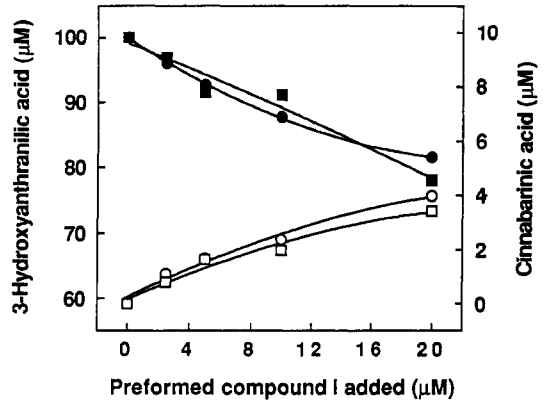


FIGURE 3: Reaction of 3HAA with preformed HRPO compound I. Various concentrations of preformed HRPO compound I were mixed with ice-cold solutions of 3HAA (100 μM) in 75 mM sodium phosphate buffer (pH 7.0) under aerobic (circles) or anaerobic (squares) conditions and analyzed immediately for 3HAA (closed symbols) and CA (open symbols) by HPLC as described under Materials and Methods.

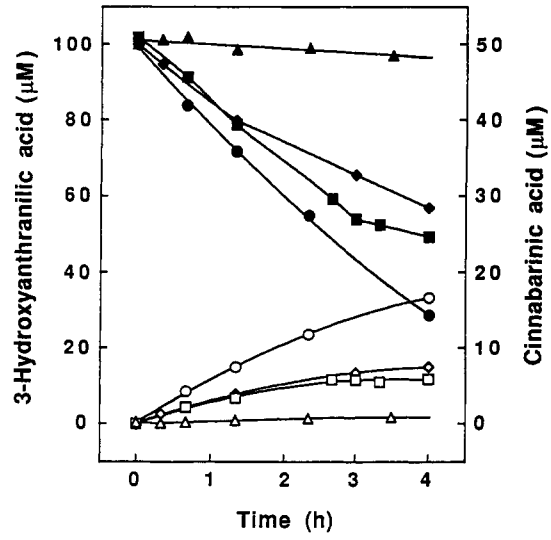
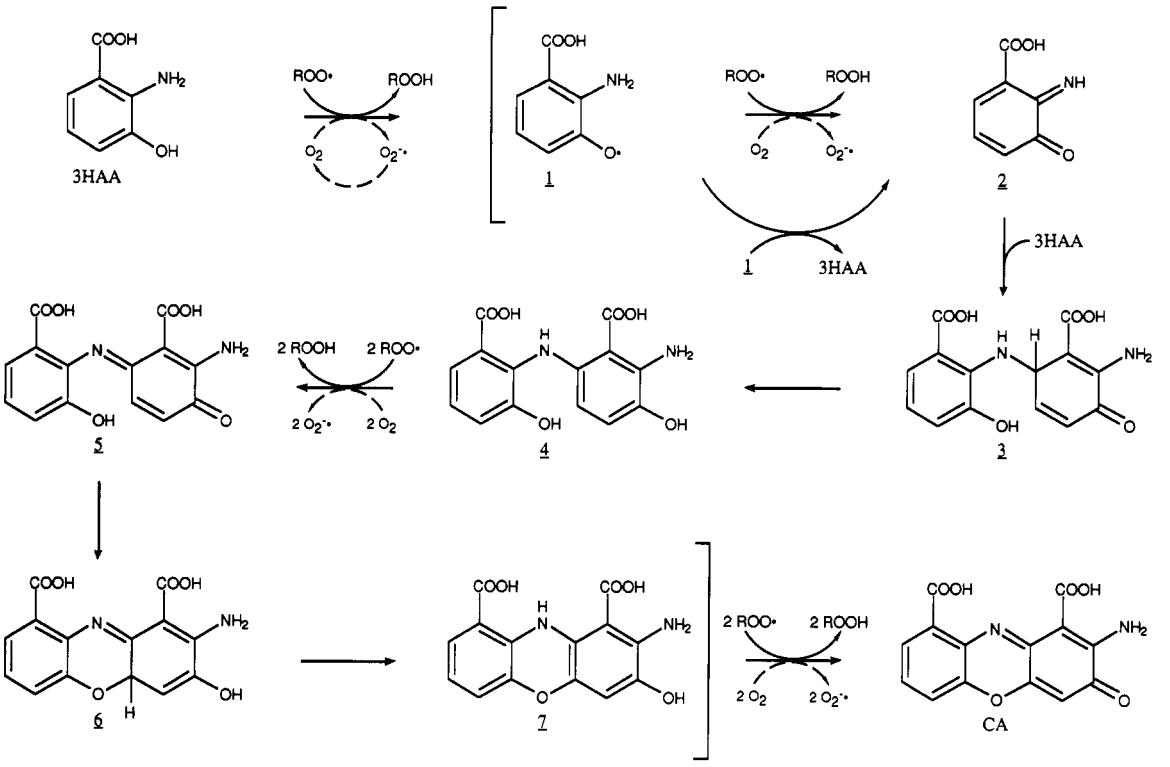


FIGURE 4: Catalase compound I-mediated oxidation of 3HAA. 3HAA was incubated in glucose and glucose oxidase-containing acetate buffer (pH 5.0) at 37 °C in the presence (circles) or absence (triangles) of bovine liver catalase (0.1 mg/mL). For some experiments, the catalase-containing reaction mixtures were supplemented with 3-amino-1,2,4-triazole (1 mM) (squares) or with reduced glutathione (0.5 mM) and glutathione peroxidase (10 milliunits/mL) (diamonds). The reaction was analyzed for 3HAA (closed symbols) and CA (open symbols) by HPLC as described in the legend to Figure 1.

by compound I derived from catalase or peroxidases. A common feature of the oxidants in this alternative pathway is that they initiate one-electron oxidation reactions. Formation of the (amino)phenoxyl radical from 3HAA is most likely the initial and rate-determining step in this pathway (Scheme II), in analogy to the (dimerization) reaction of phenolic substances with HRPO compound I (Yamazaki et al., 1960; Shiga & Imaizumi, 1975; Job & Dunford, 1976; Josephy et al., 1983) or peroxyl radicals (Burton et al., 1985).

Free Radical-Initiated Oxidation of Phenols and Aromatic Amines. Oxidative dimerization of 3HAA to CA requires the loss of 3 e^- and 3 H^+ per molecule of 3HAA. Considering its chemical complexity, a surprising finding was that the rather nonselective peroxyl radical initially converted ≈75% of 3HAA into CA, as judged by the initial rates of disappearance of 3HAA and formation of CA. This, together with the fact that each mole of 3HAA scavenges 2.7 mol of peroxyl

Scheme II: Proposed Reaction Mechanism of Peroxyl Radical-Mediated Conversion of 3HAA into CA[a]



[a] Formation of the quinone imine (**2**) is obtained either by reaction of a (second) peroxyl radical with the anthranilyl radical (**1**) or by disproportionation of **1**. Reduction of molecular oxygen to the superoxide anion radical ($O_2^{\bullet -}$) by 3HAA, **1**, **4**, and/or **7** may occur as side reaction(s) (broken arrows). The nucleophilic addition reactions involved in formation of **3** and **6** are analogous to those reported previously (Barry et al., 1989). This scheme may be extended to compound I-mediated conversion of 3HAA into CA where ROO• would be replaced with compound I/compound II. No attempts were made to draw the various structures in their actual ionization state under the experimental conditions.

radicals (Table I), suggests that the latter participate in all oxidation reactions required for the formation of CA (Scheme II).

The fact that inclusion of SOD and xanthine/xanthine oxidase into the reaction mixture resulted in increased and decreased formation of CA, respectively (Figure 2), indicates that $O_2^{\bullet -}$ is formed during and participates to some extent in the peroxyl radical-induced conversion of 3HAA into CA. Univalent reduction of molecular oxygen to $O_2^{\bullet -}$ has been proposed to be associated with the initial formation of the anthranilyl radical (**1**) as a result of autoxidation of 3HAA (Dykens et al., 1987; Manthey et al., 1988). As this reaction is of minor importance under our conditions (Figure 1B), it is more likely that reaction of **1** (Dykens et al., 1987; Manthey et al., 1988) or proposed subsequent intermediates (**4** or **7**) reduces molecular oxygen to $O_2^{\bullet -}$ (Scheme II). In analogy with the chromanoxyl radical (Cadenas et al., 1989) a reduction of **1** to 3HAA by $O_2^{\bullet -}$ may explain the observed stimulatory effect of SOD on consumption of 3HAA (Figure 2A, Scheme II). This is in line with the observation that in the presence of xanthine/xanthine oxidase the rate of peroxyl radical-mediated oxidation of 3HAA was inhibited (Figure 2B). SOD also increased the initial rate of formation of CA, a finding that could be explained by displacement of the equilibrium of reactions where $O_2^{\bullet -}$ is involved (Scheme II). Direct decomposition of CA by $O_2^{\bullet -}$ seems unlikely to contribute significantly, as CA does not react efficiently with this radical.[3] Since quinones are good substrates of xanthine oxidase (Winterbourn, 1981), it can be expected that formation of CA is inhibited in the presence of xanthine oxidase.

o- and *p*-aminophenols but not *m*-aminophenols and hydroxy/aminobenzoic acids efficiently protected the protein phycoerythrin from peroxyl radical-mediated oxidative damage (Table I), in agreement with their one-electron redox potential (Suatoni et al., 1961). Similarly, the rate constants reported for the reaction of phenols and aromatic amines with HRPO compound I are dependent on their one-electron redox potential with decreasing reactivity in the order aminophenols > phenols > aromatic amines, each rate being at least an order of magnitude different (Job & Dunford, 1976). Notably, all of the *o*-aminophenols reacted with peroxyl radicals, forming colored products with UV–visible spectra very similar to that of CA (Butenandt et al., 1957). It is therefore conceivable that all of the *o*-aminophenols formed phenoxazinones or phenoxazinone-like molecules, analogous to the substrate-unspecific reaction catalyzed by phenoxazinone synthase (Katz & Weissbach, 1962; Barry et al., 1989). Oxidation of *p*-aminophenols cannot lead to formation of cyclic dimers (cf. Table I) due to steric reasons.

Compound I-Mediated Oxidation of 3HAA. The results in Figure 3 show that preformed compound I of HRPO can dimerize 3HAA into CA, in a rapid process [cf. Job and Dunford (1976)] that is clearly independent of the presence of molecular oxygen. Where H_2O_2 was produced continuously, catalase as well as MPO and HRPO also converted 3HAA into CA readily. The fact that catalytic amounts of the peroxidases were sufficient for this reaction indicates that the normal peroxidase cycle (eqs 4 and 5) was operative. Evidence for the participation of compound I in the catalase-mediated dimerization reaction is indicated by the inhibitory effect of

[3] Complete depletion of **2** (50 μM) in DMSO in the presence of potassium superoxide (15 mM) required ≈30 min of incubation at room temperature.

3-amino-1,2,4-triazole (Figure 4). In addition, inclusion of glutathione/glutathione peroxidase to the HRPO or catalase system also inhibited oxidative dimerization, indicating the requirement of H_2O_2 in the reaction.

The observed "peroxidatic" activity of bovine catalase compound I toward 3HAA (Figure 4) is in agreement with earlier reports using other aromatic substrates (George, 1952; Nicholls & Schonbaum, 1963; Awasthi et al., 1977; Srivastava & Ansari, 1980). A partial denaturation or modification of the protein (Sichak & Dounce, 1986; Sichak et al., 1990) is unlikely to explain our observation since "peroxidatic" activity of catalase compound I toward ethanol (Oshino et al., 1973) or various aromatic substances (Christen and Stocker, unpublished) is favored under our conditions of a low ratio of the rate of H_2O_2 generation to catalase concentration.

Enzymatic dimerization of 3HAA into CA has been shown to occur in extracts prepared from different tissues of various animals (Morgan et al., 1965; Nair, 1972; Feinstein, 1978), and a partially purified preparation from a rat liver nuclear fraction showed some specificity toward 3HAA (Subba Rao & Vaidyanathan, 1966). CA formed by such a nuclear fraction was associated with DNA and inhibited RNA synthesis in isolated nuclei (Nair, 1972). Recently we have confirmed the presence of "cinnabarinic acid synthase" activity in a nuclear fraction of mouse liver (Christen & Stocker, 1992), and this activity was inhibited partially by aminotriazole (unpublished data). The nuclear fraction, like other isolated cellular compartments, spontaneously forms and releases small amounts of H_2O_2 (Chance et al., 1979). Thus, it seems plausible that some of the tissue "cinnabarinic acid synthase" activities reported were actually mediated by compound I of catalase or peroxidase. This might explain, at least partly, why attempts to isolate the "synthase" from a number of different tissues resulted in purification of catalase (Savage & Prinz, 1977; Ogawa et al., 1983).

The results presented in this study indicate that formation of CA from 3HAA can occur under certain biological conditions, where both 3HAA and free radicals/H_2O_2 are produced at elevated rates. For example, interferon-γ-treated mononuclear phagocytes rapidly degrade tryptophan, resulting in extracellular accumulation of 3HAA (Werner-Felmayer et al., 1989), and, upon stimulation, produce large amounts of H_2O_2, processes that have both been associated separately with antimicrobial and/or antitumor activity (Nathan et al., 1983; Carlin et al., 1989). As phenoxazinones other than actinomycins also possess antigrowth activity (Gerber & Lechevalier, 1964), such activity may extend to CA. It therefore seems feasible that oxidative dimerization of 3HAA into CA, if occurring, may contribute to the inhibition of microbial and tumor cell growth mediated by interferon-γ-stimulated cells or interferon-γ-itself (Takikawa et al., 1988, 1990; Carlin et al., 1989).

ACKNOWLEDGMENT

We thank Dr. R. J. W. Truscott (University of Wollongong, New South Wales) for providing us with an authentic sample of CA, Dr. V. W. Bowry for his participation in many helpful discussions, and Dr. A. J. Kettle for critically reading the manuscript. We also acknowledge the technical assistance of Mrs. H. Stender (University of New South Wales) for running the NMR spectra.

REFERENCES

Awasthi, Y. C., Srivastava, S. K., Snyder, L. M., Edelstein, L., & Fortier, N. L. (1977) *J. Lab. Clin. Med. 89*, 763–769.

Barry, C. E., Nayar, P. G., & Begley, T. P. (1989) *Biochemistry 28*, 6323–6333. *Ref. Data 14*, 1041–1100.

Burton, G. W., Doba, T., Gabe, E. J., Hughes, L., Lee, F. L., Prasad, L., & Ingold, K. U. (1985) *J. Am. Chem. Soc. 107*, 7053–7065.

Butenandt, A., & Neubert, G. (1955) *Biol. Chem. Hoppe Seyler 301*, 109–114.

Butenandt, A., Keck, J., & Neubert, G. (1957) *Liebigs Ann. Chem. 602*, 61–72.

Cadenas, E., Merényi, G., & Lind, J. (1989) *FEBS Lett. 253*, 235–238.

Carlin, J. M., Ozaki, Y., Byrne, G. I., Brown, R. R., & Borden, E. C. (1989) *Experientia 45*, 535–541.

Chance, B., Sies, H., & Boveris, A. (1979) *Physiol. Rev. 59*, 527–605.

Christen, S., & Stocker, R. (1992) *Anal. Biochem. 200*, 273–279.

Christen, S., Peterhans, E., & Stocker, R. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 2506–2510.

Darr, D., & Fridovich, I. (1986) *Biochem. Pharmacol. 35*, 3642.

DeLange, R. J., & Glazer, A. N. (1989) *Anal. Biochem. 177*, 300–306.

Dykens, J. A., Sullivan, S. G., & Stern, A. (1987) *Biochem. Pharmacol. 36*, 211–217.

Feinstein, R. N. (1978) *Proc. Soc. Exp. Biol. Med. 158*, 398–401.

George, P. (1952) *Biochem. J. 52*, xix.

Gerber, N. N., & Lechevalier, M. P. (1964) *Biochemistry 3*, 598–602.

Gripenberg, J., Honkanen, E., & Patoharju, O. (1957) *Acta Chem. Scand. 11*, 1485–1492.

Ishii, T., Iwahashi, H., Sugata, R., Kido, R., & Fridovich, I. (1990) *Arch. Biochem. Biophys. 276*, 248–250.

Job, D., & Dunford, H. B. (1976) *Eur. J. Biochem. 66*, 607–614.

Josephy, P. D., Eling, T. E., & Mason, R. P. (1983) *Mol. Pharmacol. 23*, 461–466.

Katz, E., & Weissbach, H. (1962) *J. Biol. Chem. 237*, 882–886.

Lerch, K. (1981) *Met. Ions Biol. Syst. 13*, 143–186.

Manthey, M. K., Pyne, S. G., & Truscott, R. J. W. (1988) *J. Org. Chem. 53*, 1486–1488.

Manthey, M. K., Pyne, S. G., & Truscott, R. J. W. (1990) *Biochem. Biophys. Acta 1034*, 207–212.

Morgan, L. R., Weimorts, D. M., & Aubert, C. C. (1965) *Biochem. Biophys. Acta 100*, 393–402.

Nagasawa, H. T., Gutmann, H. R., & Morgan, M. A. (1959) *J. Biol. Chem. 234*, 1600–1604.

Nair, P. M. (1972) *Arch. Biochem. Biophys. 153*, 139–146.

Nathan, C. F., Murray, H. W., Wiebe, M. E., & Rubin, B. Y., (1983) *J. Exp. Med. 158*, 670–689.

Nicholls, P., & Schonbaum, G. R. (1963) in *The Enzymes* (Boyer, P. D., Lardy, H., & Myrbäck, K., Eds.) pp 147–225, Academic, New York.

Niki, E., Saito, M., Yoshikawa, Y., Yamamoto, Y., & Kamiya, Y. (1986) *Bull. Chem. Soc. Jpn. 59*, 471–477.

Nishizuka, Y., & Hayaishi, O. (1963) *J. Biol. Chem. 238*, 3369–3377.

Ogawa, H., Nagamura, Y., & Ishiguro, I. (1983) *Biol. Chem. Hoppe Seyler 364*, 1059–1066.

Oshino, N., Oshino, R., & Chance, B. (1973) *Biochem. J. 131*, 555–567.

Packer, J. E., Willson, R. L., Bahnemann, D., & Asmus, K.-D. (1980) *J. Chem. Soc., Perkin Trans. 2*, 296–299.

Paul, K. G. (1963) in *The Enzymes* (Boyer, P. D., Lardy, H., & Myrbäck, K., Eds.) pp 227–274, Academic, New York.

Prinz, W., & Savage, N. (1977) *Biol. Chem. Hoppe Seyler 358*, 1161–1163.

Root, R. K., Metcalf, J., Oshino, N., & Chance, B. (1975) *J. Clin. Invest. 55*, 945–955.

Ruch, W., Cooper, P. H. & Baggiolini, M. (1983) *J. Immunol. Methods 63*, 347–357.

Savage, N., & Prinz, W. (1977) *Biochem. J. 161*, 551–554.
Shiga, T., & Imaizumi, K. (1975) *Arch. Biochem. Biophys. 167*, 469–479.
Sichak, S. P., & Dounce, A. L. (1986) *Arch. Biochem. Biophys. 249*, 286–295.
Sichak, S. P., Basu, D., & Turner, C. (1990) *J. Toxicol. Environ. Health 31*, 227–233.
Simic, M. G., & Jovanovic, S. V. (1989) *J. Am. Chem. Soc. 111*, 5778–5782.
Srivastava, S. K., & Ansari, N. H. (1980) *Biochim. Biophys. Acta 633*, 317–322.
Suatoni, J. C., Synder, R. E., & Clark, R. O. (1961) *Anal. Chem. 33*, 1894–1897.
Subba Rao, P. V. & Vaidyanathan, C. S. (1966) *Biochem. J. 99*, 317–322.
Summers, K. M., Howells, A. J., & Pyliotys, N. A. (1982) *Adv. Insect Physiol. 16*, 119–166.
Takikawa, O., Kuroiwa, T., Yamazaki, F., & Kido, R. (1988) *J. Biol. Chem. 263*, 2041–2048.
Takikawa, O., Habara-Ohkubo, A., & Yoshida, R. (1990) *J. Immunol. 145*, 1246–1250.
Toussaint, O., & Lerch, K. (1987) *Biochemistry 26*, 8567–8571.
Werner-Felmayer, G., Werner, E. R., Fuchs, D., Hausen, A., Reibnegger, G., & Wachter, H. (1989) *Biochim. Biophys. Acta 1012*, 140–147.
Winterbourn, C. C. (1981) *Arch. Biochem. Biophys. 209*, 159–167.
Yamazaki, I., & Yokota, K. (1973) *Mol. Cell. Biochem. 2*, 39–52.
Yamazaki, I., Mason, H. S., & Piette, L. (1960) *J. Biol. Chem. 235*, 2444–2449.