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Active Oxygen Species Involved in the Dye-Sensitized Photoinactivation of Mushroom Tyrosinase

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Active oxygen species were responsible for affecting the inactivation of native mushroom tyrosinase at pH 7.0 in photodynamic systems containing rose bengal and riboflavin. The effects of scavengers, quenchers, and D_2O indicated that H_2O_2 and $O_2^{\bullet -}$ were the primary active oxygen species generated by the riboflavin-sensitized reaction, and $^1O_2$ was the primary species generated by the system containing rose bengal. Diazabicyclo[2.2.2]octane (DABCO), Hepes, and Mops accelerated tyrosinase inactivation, and this was attributed to their ability to form secondary radical adducts or generate free radicals in the presence of copper. The effects of $^{\bullet}OH$ scavengers and the chelators, EDTA, phytate, and desferrioxamine mesylate (DFOM), indicated that (1) bulk-phase metal-driven Fenton reactions were not responsible for tyrosinase photoinactivation and (2) site-specific generation of $^{\bullet}OH$ from a copper-driven Fenton reaction played a role in enzyme inactivation.

Enzymes and other biomolecules are sensitive to photochemically induced oxidation (Foote, 1976; Straight and Spikes, 1985). Dye-sensitized photooxidations can be mediated by two processes. Some excited triplet state sensitizers tend to react directly with various substrates and form $O_2^{\bullet -}$ and H_2O_2 upon subsequent autoxidation (type I process), whereas others tend to be quenched by $^3O_2$ and yield $^1O_2$ (type II process). Environmental factors such as the nature and concentration of photosensitizer and substrate, pH, (an)aerobiosis, and the nature of the solvent can markedly influence the mechanism of photochemical damage of a target substrate (Straight and Spikes, 1985). Photochemical damage to biomolecules is believed to be mediated by reactive oxygen intermediates generated by photodynamic systems (Foote, 1976; Straight and Spikes, 1985).

Although photosensitivity of biomolecules can result in deleterious effects to living organisms (Feierabend and Engel, 1986; Ito, 1978), in vitro photooxidations have been used to assist the identification of essential amino acid residues in many enzymes (Westhead, 1972; Aoshima et al., 1977; Pfiffner and Lerch, 1981). Coupled with analyses for amino acid destruction and the pH profile of photosensitivity, many studies have revealed the nature of the active site of specific enzymes (Westhead, 1972; Aoshima et al., 1977), including tyrosinase (Gutteridge et al., 1977; Pfiffner and Lerch, 1981; Fry and Strothkamp, 1983).

Tyrosinase (monophenol, dihydroxyphenylalanine:oxygen oxidoreductase, EC 1.14.18.1) is an enzyme found in microorganisms, plants, and animals (Robb, 1984). It catalyzes the orthohydroxylation of monophenols and the oxidation of *o*-diphenols to their *o*-quinone derivatives. The potentiation of these reactions and attendant melanin formation are considered to be deleterious to the color quality of plant-derived foods. Several investigators have reported the sensitivity of tyrosinase to photodynamic systems containing riboflavin, rose bengal, methylene blue, and citrate (Galston and Baker, 1949; Gutteridge et al., 1977; Pfiffner and Lerch, 1981; Fry and Strothkamp, 1983). However, none of these studies identified the active oxygen species involved in this process.

We have previously described the effect of several experimental parameters on the rate of tyrosinase photoinactivation in photodynamic systems containing rose ben-

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gal and riboflavin (Lowum and Parkin, 1989). This paper reports on the nature of the active oxygen species generated by these photodynamic systems and their involvement in affecting the photoinactivation of tyrosinase. We also provide evidence for a site-specific mechanism for enzyme inactivation.

EXPERIMENTAL PROCEDURES

Tyrosinase from mushroom (2000–4000 units/mg), catalase from bovine liver (purified powder, 2800 units/mg), superoxide dismutase (SOD) from bovine erythrocytes (3000 units/mg), D_2O, riboflavin, rose bengal, chlorogenic acid, Hepes, Mops, sodium formate, mannitol, diazabicyclo[2.2.2]octane (DABCO), histidine, sodium azide, nitrotetrazolium blue (NBT), phytic acid, sodium ethylenediaminetetraacetate (EDTA), and desferrioxamine mesylate (DFOM) were obtained from Sigma Chemical Co. All other chemicals used were of reagent grade. Deionized and distilled water was used to prepare all solutions.

Irradiation experiments were done in a water bath maintained at 25 ± 0.5 °C. The source of illumination was a 100-W incandescent bulb yielding an intensity of 45 000 lx at the sample tube surface. The standard photooxidation mixture included 0.01% dye (riboflavin or rose bengal), 0.7 mg of tyrosinase/mL, and air-equilibrated 10 mM sodium phosphate buffer (pH 7.0), unless otherwise stated, in a total volume of 2 mL. Identical samples wrapped in aluminum foil served as dark controls for all conditions evaluated. The active oxygen scavengers/quenchers used and their final concentrations were catalase (26.6 units/mL); SOD (24.0 units/mL); mannitol, ethanol, and DABCO (100 mM); histidine and sodium formate (50 mM); and sodium azide (10 mM). Phytic acid, EDTA, and DFOM were used at 10 mM, and Mops and Hepes were used at 5 and 10 mM. Stock solutions of components added to the standard photooxidation mixtures were adjusted to pH 7.0 prior to use. None of these components had any direct effect on tyrosinase activity, except sodium azide and DABCO (see Results and Discussion). All dark controls retained 95–100% of their original activity after at least 3 h of illumination.

Denatured catalase was prepared by treatment with 8 M urea for 30 min at room temperature. Untreated catalase and urea-denatured catalase were dialyzed against 10 mM sodium phosphate buffer (pH 7.0) overnight at 5 °C by using 12 000–14 000 M_r cutoff membrane tubing. Activity remained high for the untreated enzyme and was absent in the urea-treated enzyme as measured by evolution of oxygen from H_2O_2 in the same phosphate buffer using an oxygen polarograph (Gilson Medical Electronics, Inc., Model 5/6) fitted with a Clark-type electrode.

The NBT assay was used to detect superoxide generation in illuminated samples (Korycka-Dahl and Richardson, 1978). This assay is based on the reaction of superoxide and NBT to form diformazan, which is detected by absorbance at 560 nm (E_{mM} = 30). The amount of superoxide formed by photodynamic mixtures illuminated for 1.5 h was calculated from the A_{560} of sample mixtures with and without 12 units/mL of SOD.

Tyrosinase activity was determined by assaying for oxygen consumption in 10 mM chlorogenic acid and air-saturated (250 μM O_2) 50 mM sodium phosphate buffer (pH 7.0) at 25 ± 0.1 °C with an oxygen polarograph. Apparent first-order rate constants (k_i) were determined from the slopes of semilog plots of percent residual tyrosinase activity vs time of illumination, after the lag period for inactivation was accounted for (generally 30 min; Lowum and Parkin, 1989). Correlation coefficients (r^2) for all first-order plots were greater than 0.95.

Statistical analysis was done by analysis of variance and Duncan's multiple range test.

RESULTS AND DISCUSSION

Effects of Reactive Oxygen Scavengers/Quenchers. Reactive oxygen scavengers and quenchers affected the rates of photoinactivation of tyrosinase mediated by rose bengal (Table I). All of the $^1O_2$ quenchers had an effect on the photoinactivation of tyrosinase. The presence of 10 mM azide and 100 mM DABCO in the incubation mixture led to 40–50% and 25–35% inhibition of enzyme activity when assayed prior to illumination (in the assay mixture, azide and DABCO were diluted to 0.67 and 6.7 mM, respectively). Therefore, the rates of inactivation relative to the control are based on those taking place only during the illumination period. Histidine and azide inhibited the rate of enzyme inactivation by 75% and 86%, respectively, compared to the control. In contrast to the expected inhibition, DABCO, another $^1O_2$ quencher, accelerated the rate of photoinactivation by 16% in the presence of rose bengal. We have previously shown that rates of tyrosinase inactivation in the presence of this dye are minimal in oxygen-deprived media or in oxygen-saturated media in the absence of illumination (Lowum and Parkin, 1989).

Table I. Effects of Active Oxygen Scavengers and Quenchers on Rates of Tyrosinase Photoinactivation

components	no. of trials	rel rates of inact[a] ± SD
rose bengal (control A)	12	100 ± 6[a,f]
control A + formate, mannitol, or ethanol	3 each	100 ± 10[a,f]
control A + histidine	3	30 ± 5[b]
control A + azide	2	13 ± 2[c]
control A + DABCO	6	117 ± 6[d]
control A + D_2O	2	148 ± 14[e]
control A + D_2O + DABCO	2	138 ± 12[e]
control A + catalase	3	93 ± 5[f]
control A + SOD	3	112 ± 6[a,d]
riboflavin (control B)	13	100 ± 7[a,c]
control B + formate, mannitol, or ethanol	3 each	97 ± 8[a,c]
control B + histidine	3	100 ± 8[a,c]
control B + azide	2	0 ± 0[b]
control B + DABCO	6	114 ± 12[a]
control B + D_2O	2	89 ± 10[c]
control B + D_2O + DABCO	2	91 ± 9[c]
control B + catalase	3	27 ± 6[d]
control B + SOD	3	131 ± 8[e]

[a] Values with the same superscript are not significantly different at the $P < 0.05$ level. Values for k_i ranged from 0.92 to 1.20 h^{-1} for control A and from 0.41 to 0.54 h^{-1} for control B. Controls contained 0.70 mg of tyrosinase/mL, 10 mM sodium phosphate, pH 7.0, and 0.01% dye. Mannitol, ethanol, and DABCO were used at 100 mM; histidine and formate were used at 50 mM; azide was used at 10 mM; catalase was used at 26.6 units/mL; SOD was used at 24.0 units/mL; D_2O was used at 40% (v/v).

Rose bengal is known to be an efficient generator of $^1O_2$ due to its low triplet-state energy and propensity to react by type II processes (Houba-Herin et al., 1982; Kutsuki et al., 1983). Although histidine and azide had similar effects on the rates of photoinactivation caused by rose bengal, their mechanisms of action may differ. Histidine would be expected to be present in the bulk phase of the reaction medium, whereas azide, being an inhibitor of tyrosinase (K_i of 1.76 mM for the broad bean enzyme; Robb et al., 1966), would be expected to partition between the active site and the bulk phase. This may explain the added protection offered by azide.

The presence and damaging effect of $^1O_2$ in the rose bengal system was also confirmed by the increased rate of enzyme inactivation in mixtures containing D_2O, with and without DABCO (Table I). The effect of DABCO, although unusual, is not without precedent. An accelerating effect of substrate photooxidations by DABCO in systems where $^1O_2$ is formed has been previously observed for the photoinactivation of liver microsomal benzo[α]pyrene hydroxylase (Rahimtula et al., 1978), *Saccharomyces cerevisiae* cells (Ito, 1978), and isolated *Escherichia coli* ribosomes (Singh and Vadasz, 1977), and the photooxidation of *o*-dianisidine (Frenkel, 1983). In the

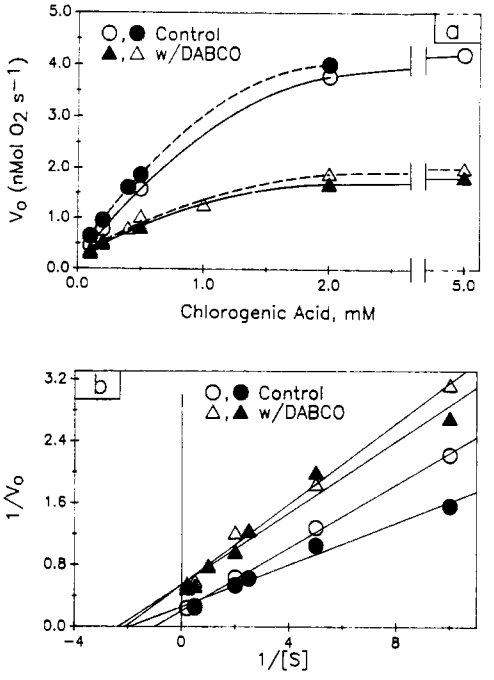

Figure 1. (a) Effect of DABCO on tyrosinase activity. Enzyme (0.067 mg/mL) was assayed by oxygen uptake in the presence of 0.10–5.0 mM chlorogenic acid in the presence and absence of 20 mM DABCO. (b) Transformation of data to a double-reciprocal plot. The data are from two separate experiments.

presence of $^1O_2$, DABCO may produce other active oxygen species through secondary reactions (Kanofsky, 1986), increase the lifetime of $^1O_2$ (Deneke and Krinsky, 1977), or cause the sensitization of substrate after quenching $^1O_2$ (Singh and Vadasz, 1977). DABCO is also believed to form nitrogen-centered radicals and mediate the destruction of DNA guanine residues in systems containing copper and H_2O_2 (Yamamoto and Kawanishi, 1989).

Due to the unusual effect of DABCO (Table I) and its direct inhibitory effect on tyrosinase activity, we evaluated the nature of its interaction with the enzyme (Figure 1). Inhibition of tyrosinase activity by DABCO was noncompetitive with a K_i of about 18 mM (13 and 23 mM in two trials). Therefore, the acceleration of photoinactivation of tyrosinase by DABCO in the presence of rose bengal may be mediated by its ability to directly interact with the enzyme and either enhance the lifetime of $^1O_2$ or produce secondary radical adducts.

Catalase and SOD had little effect on the rate of enzyme photoinactivation in the presence of rose bengal (Table I), suggesting that neither H_2O_2 nor $O_2^{\bullet-}$ is produced in significant quantities in this photodynamic system. The amount of $O_2^{\bullet-}$ formed in this system as determined by the NBT assay was 6.4 nmol in 1.5 h of illumination. The presence of NBT also inhibited enzyme inactivation by 93 ± 6% (three trials), probably due to its ability to scavenge other active oxygen species.

Active oxygen quenchers and scavengers also affected the rates of tyrosinase photoinactivation caused by illuminated mixtures containing riboflavin (Table I). Catalase inhibited the photoinactivation of tyrosinase by 66%, indicating that H_2O_2 was a primary oxidizing species generated by this photodynamic system. Since catalase is also susceptible to photoinactivation (Feierabend and Engel, 1986), we tested whether the protective effect of catalase was due to its ability to decompose H_2O_2 or to its simply being another oxidizable substrate. The rate of tyrosinase photoinactivation in the absence of catalase or in the presence of urea-denatured catalase was the same (k_i = 0.34 h^{-1}), whereas in the presence of dialyzed catalase the rate was inhibited 53%. Therefore, the ability of native catalase to inhibit tyrosinase inactivation was due to its H_2O_2-decomposing activity.

The presence of SOD accelerated the rate of tyrosinase inactivation by 31% (Table I). This indicates that $O_2^{\bullet-}$ is generated during the photosensitization of riboflavin and suggests again that H_2O_2 is more damaging to tyrosinase than $O_2^{\bullet-}$. Illuminated mixtures of riboflavin and tyrosinase formed 47 nmol of $O_2^{\bullet-}$ in 1.5 h, as indicated by the NBT assay, and NBT inhibited enzyme inactivation by 83 ± 10% (five trials).

Photosensitized flavins are converted into a high-energy triplet state that favors type I processes and the production of free radicals (Kutsuki et al., 1983; Korycka-Dahl and Richardson, 1978). Therefore, these photodynamic systems are weak $^1O_2$ generators and, instead, tend to form H_2O_2 and $O_2^{\bullet-}$. Triplet-state riboflavin can also be damaging to tyrosinase in this photodynamic system. However, the rate of enzyme photoinactivation in the presence of riboflavin in oxygen-deprived media is only about 25% of that occurring in oxygen-saturated media (Lowum and Parkin, 1989), indicating that active oxygen is primarily responsible for enzyme inactivation in this photodynamic system.

Under some conditions, photosensitized riboflavin can produce sufficient levels of $^1O_2$ to enhance the rate of some photooxidations (Frenkel, 1983). The effects of the $^1O_2$ quenchers on the riboflavin-sensitized photoinactivation of tyrosinase were somewhat conflicting (Table I). Histidine and DABCO had little effect on enzyme inactivation, whereas azide was completely inhibitory. The difference between these effects may be due to the ability of azide to bind to the active site of tyrosinase, whereas histidine and DABCO would be expected to reside primarily in the bulk phase. Therefore, azide might afford protection from any oxidizing species, not only $^1O_2$. Ligands bound to the active site of γ-aminolevulinic acid dehydratase (Rao et al., 1987) and lipoxygenase (Aoshima et al., 1977) offer protection from photoinactivation. That very little $^1O_2$ is generated by photosensitized riboflavin was also indicated by the lack of effect on the rate of enzyme inactivation in mixtures containing D_2O, relative to the control.

Enzyme photoinactivation by both of these dyes appeared to not involve $^{\bullet}OH$ generated in the bulk phase as formate, mannitol, and ethanol had no effect on the rates of inactivation (Table I). The absence of any effect by the presence of these scavengers cannot be taken as an absolute indication that $^{\bullet}OH$ is not involved in the photoinactivation of tyrosinase. These scavengers would be expected to reside in the bulk phase of the illuminated mixtures, and there is increasing evidence that bulk-phase scavengers are not effective in quenching site-specific generation of $^{\bullet}OH$ (Que et al., 1980; Rowley and Halliwell, 1983).

We suggest that active oxygen species generated by the riboflavin photodynamic system were damaging, in part, due to Fenton-type reactions with copper at sites vital to enzyme activity. Copper salts are efficient generators of $^{\bullet}OH$ from H_2O_2 and $O_2^{\bullet-}$ via Fenton-type reactions (Que et al., 1980; Rowley and Halliwell, 1983). Copper bound to bacteria and enzymes is known to cause site-specific damage in the presence of H_2O_2 or $O_2^{\bullet-}$, presumably through the generation of $^{\bullet}OH$, which is not inhibited by free radical scavengers (Gutteridge and Wilkins, 1983). H_2O_2 is also known to bind at the active site of tyrosinase (Robb, 1984; Andrawis and Kahn, 1985). A study on the inactivation of tyrosinase by added H_2O_2

Table II. Effects of Metal Ion Chelators on Rates of Tyrosinase Photoinactivation

components	rel rates of inact[a] ± SD
rose bengal (control A)	100 ± 9[a]
control A + EDTA	73 ± 5[b]
control A + phytate	127 ± 5[c]
control A + DFOM	20 ± 2[d]
riboflavin (control B)	100 ± 8[a]
control B + EDTA:[b] first phase	58 ± 7[b]
second phase	16 ± 4[c]
control B + phytate	114 ± 7[a]
control B + DFOM	104 ± 10[a]

[a] Legend is the same as in Table I, except each condition was evaluated in triplicate. Values for k_i ranged from 1.03 to 1.14 h^{-1} for control A and from 0.37 to 0.42 h^{-1} for control B. All chelators were used at 10 mM. [b] See Results and Discussion for explanation.

revealed that (1) inactivation required active site bound copper, (2) azide protected the enzyme from inactivation, and (3) •OH scavengers offered no protection of the enzyme from inactivation by H_2O_2 (Andrawis and Kahn, 1985). *Neurospora crassa* tyrosinase is completely protected from methylene blue sensitized photoinactivation when cobalt is substituted for copper at the active site (Pfiffner and Lerch, 1981). Therefore, we conclude that riboflavin-sensitized photogeneration of H_2O_2 and $O_2^{\bullet-}$ leads to Fenton-type reactions with copper to yield •OH at sites vital to the activity of tyrosinase, leading to enzyme inactivation. Our observations on the effects of chelators (see below) also support this conclusion.

Site-specific damage to tyrosinase may also take place in the rose bengal photodynamic system since low levels (6.4 nmol/1.5 h) of $O_2^{\bullet-}$ were produced. Oxidation of some substrates by $^1O_2$, the primary active oxygen species generated by photosensitized rose bengal, can also yield $O_2^{\bullet-}$ (Saito and Matsuura, 1984).

Effects of Metal Ion Chelators. The presence of trace levels of metals has a marked impact on the interconversion of active oxygen species in biological systems (Halliwell and Gutteridge, 1984). Iron was determined to be about 30 μM in our experimental mixtures (analyzed by atomic absorption spectroscopy; L. Jackson, personal communication).

The effects of metal chelators on dye-sensitized photoinactivation of tyrosinase are summarized in Table II. Phytate had little effect on the rate of enzyme inactivation in the riboflavin system and slightly accelerated enzyme inactivation in the rose bengal system. Although the latter observation is difficult to explain, these data suggest that iron-driven Fenton-type reactions in the bulk phase were not responsible for tyrosinase inactivation, since phytate coordinates with iron in a manner that prevents Fenton reactions (Graf et al., 1984).

EDTA inhibited the rate of tyrosinase photoinactivation in both photodynamic systems (Table II). Enzyme inactivation in the presence of riboflavin and EDTA did not follow first-order kinetics, but could be fitted to two first-order plots, representing two phases of tyrosinase photoinactivation (Figure 2). EDTA inhibited the rate of enzyme inactivation during both phases compared to the control.

The inhibitory effect of EDTA in both photodynamic systems may be due to its ability to chelate copper, which is liberated from the active site of tyrosinase after photoinactivation by methylene blue or citrate (Pfiffner and Lerch, 1981; Fry and Strothkamp, 1983) or after reaction with H_2O_2 (Andrawis and Kahn, 1985). EDTA is an efficient chelator of copper (log K of 15.5 at pH 7.0; Dawson et al., 1986) and prevents copper-driven Fenton

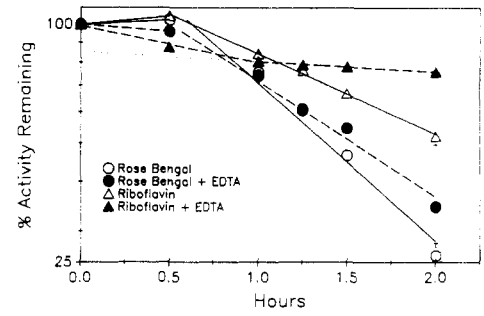

Figure 2. Effect of EDTA on rates of tyrosinase photoinactivation. Mixtures contained 0.70 mg of tyrosinase/mL, 10 mM sodium phosphate, pH 7.0, and 0.01% dye. EDTA was 10 mM, and the results are from two separate experiments (bars denote range of values observed). Dotted line represents extension of first-order plot of second phase of enzyme inactivation in the presence of EDTA and riboflavin.

reactions (Halliwell and Gutteridge, 1984). The chelating ability of EDTA may prevent the released copper from binding to remaining active enzyme and causing site-specific Fenton reactions. EDTA inhibits the degradation of DNA caused by an intercalated copper–1,10-phenanthroline complex by competing for copper (Que et al., 1980).

The inhibitory effect of EDTA on enzyme inactivation in the riboflavin photodynamic system may also be due to the acceleration of iron-driven Fenton reactions with H_2O_2 in the bulk phase, thus limiting the amount of H_2O_2 available to react with the active site of the enzyme. Iron–EDTA complexes are promoters of the Fenton reaction, often being more catalytic than uncomplexed iron (Halliwell and Gutteridge, 1986).

DFOM inhibited the rate of enzyme inactivation caused by rose bengal and had no effect on the rate of the riboflavin-sensitized reaction (Table II). DFOM is an excellent chelator of iron (log K = 30; Neilands, 1967) and, as does phytate, completely prevents iron-driven Fenton production of •OH from H_2O_2 (Graf et al., 1984). DFOM has little influence on the copper-driven Fenton reaction (Rowley and Halliwell, 1983). Since bulk-phase iron-driven Fenton-type reactions have been ruled out as being damaging to tyrosinase in these photodynamic systems as a result of the effects of phytate, the effect of DFOM must be accounted for by some mechanism(s) other than trace iron chelation. In addition to its iron-chelating ability, DFOM can scavenge •OH (Halliwell and Gutteridge, 1986) and $O_2^{\bullet-}$ (Davies et al., 1987). DFOM can also interact with $^1O_2$ generated by illuminated mixtures of rose bengal (Anson et al., 1989). This may account for its ability to inhibit the photoinactivation of tyrosinase in the presence of rose bengal, as did the $^1O_2$ quenchers histidine and azide (Table I). The absence of any effect of DFOM on the riboflavin-sensitized inactivation of tyrosinase may indicate the inability of DFOM to scavenge the primary active oxygen species, H_2O_2, generated by this photodynamic system. Tyrosinase activity at 0.1–5.0 mM chlorogenic acid was unaffected by the presence of DFOM (data not shown), indicating DFOM did not directly interact with the enzyme.

Effects of Buffering Agents. For the riboflavin-sensitized reaction, replacing phosphate with Mops or Hepes (all at 10 mM) accelerated the rate of photoinactivation (Table III). Equimolar mixtures (5 mM each) of phosphate and Mops had an effect similar to 10 mM phosphate alone (control), whereas equimolar mixtures of phosphate and Hepes had an effect similar to 10 mM Hepes alone. An equimolar mixture of Mops and Hepes behaved similarly to the system containing 10 mM Mops.

Table III. Effects of Buffer Composition on Rates of Tyrosinase Photoinactivation

components	rel rates of inact[a] ± SD
rose bengal, phosphate (control A)	100 ± 12[a]
rose bengal, Mops	144 ± 15[b]
rose bengal, phosphate, Mops	121 ± 23[a,b]
rose bengal, Hepes	158 ± 14[b]
rose bengal, phosphate, Hepes	128 ± 20 [a,b]
rose bengal, Mops, Hepes	144 ± 12[b]
riboflavin, phosphate (control B)	100 ± 11[a]
riboflavin, Mops	184 ± 16[b,c]
riboflavin, phosphate, Mops	105 ± 14[a]
riboflavin, Hepes	228 ± 37[b]
riboflavin, phosphate, Hepes	239 ± 30[b]
riboflavin, Mops, Hepes	150 ± 18[c]

[a] Legend is same as in Table I, except for the buffer composition, and all conditions were evaluated four times. All buffers were used at 10 mM alone or at 5 mM each in combination. Values of k_i ranged from 1.01 to 1.23 h^{-1} for control A and from 0.40 to 0.50 h^{-1} for control B.

The effects of these buffer substitutions on the rates of tyrosinase inactivation caused by rose bengal were similar to those caused by riboflavin, except that the mixture containing both phosphate and Hepes behaved similarly to the phosphate-buffered mixture (control).

One explanation for the effects of replacing phosphate with either Mops or Hepes may be the changes in ionic strength accompanying these substitutions. The rate of tyrosinase photoinactivation is decreased by increasing ionic strength (0.02–0.20) of sodium phosphate, and phosphate appears to have a specific inhibitory effect on the riboflavin-mediated reaction at an ionic strength of 0.20 (Lowum and Parkin, 1989). For both of these photodynamic systems the effect of Mops (I = 0.007 at 10 mM) is attenuated by the copresence of phosphate (I = 0.014 for 5 mM each). However, the effect of Hepes (I = 0.004 at 10 mM) is unaffected by the copresence of phosphate (I = 0.012 at 5 mM each) in both photodynamic systems. Although the change in ionic strength accompanying these buffer substitutions can account for some of these observations, a specific effect of Hepes is apparent.

Tris, Hepes, and Tricine have recently been shown to be efficient $^{\bullet}OH$ scavengers (Hicks and Gebicki, 1986). Most "good" buffers are able to scavenge $^{\bullet}OH$ (Halliwell et al., 1987), and Hepes may also scavenge other active oxygen species (Puppo and Halliwell, 1988). However, Hepes can react with $^{\bullet}OH$ and H_2O_2 and form a nitroxide radical (Grady et al., 1988). Hepes and Mops also stimulate free-radical formation in the presence of copper and H_2O_2, which can lead to an enhanced rate of substrate oxidations (Simpson et al., 1988). We conclude that Hepes and Mops stimulate tyrosinase inactivation in these photodynamic systems by forming secondary nitrogen-centered radical adducts or free radicals following their coordination with copper liberated from inactivated enzyme. Regardless of the nature of the effects of Mops and Hepes, our results indicate that studies of free-radical damage in biological systems where good buffers are used require careful interpretation, as others have noted (Hicks and Gebicki, 1986; Simpson et al., 1988).

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