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# Phosphate Inhibition of the Copper- and Zinc-Containing Superoxide Dismutase: A Reexamination†

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ABSTRACT: Phosphate was reported to be an inhibitor of copper- and zinc-containing superoxide dismutase (SOD) [de Freitas, D. M., & Valentine, J. S. (1984) *Biochemistry 23*, 2079–2082]. Thus SOD activity, in 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (pH 7.4), was decreased by approximately 50% when the assay was made 10 mM in phosphate, and the ionic strength was adjusted with sodium fluoride. The inhibitory effect of phosphate was attributed to the neutralization of the positive charge on the guanidino residue of Arg-141. We have reexamined the effects of phosphate inhibition of SOD and found that the enzyme has identical activity in phosphate or HEPES buffer when the ionic strength is adjusted with NaBr. The putative inhibitory effect of phosphate appears to have been due to fluoride inhibition of the superoxide generating system of xanthine/xanthine oxidase. We have confirmed this result by using a photochemical generation of $O_2^-$ in addition to the enzymatic generation of $O_2^-$. Chemical modification of the lysine residues to homoarginines does not affect the activity of the enzyme and does not impart a phosphate sensitivity. Chemical modification with phenylglyoxal caused approximately 80% inactivation of the native enzyme and 90% inactivation of the *O*-methylisourea-modified enzyme. Our results suggest that phosphate does not inhibit the copper- and zinc-containing superoxide dismutase (Cu,Zn-SOD) beyond the expectations of its effect on ionic strength.

The Cu,Zn-SOD[1] is one of a family of SODs that provides a defense against oxygen toxicity by catalyzing the dismutation of $O_2^-$ to $H_2O_2$ plus $O_2$ (Fridovich, 1983, 1986). Electrostatic guidance was proposed as one explanation for the great catalytic efficiency of this enzyme (Koppenol, 1981). Raising the ionic strength depressed the catalytic activity of Cu,Zn-SOD, in accord with the expectations of electrostatic facilitation (Malinowski & Fridovich, 1979; Cudd & Fridovich, 1982a; Rigo et al., 1975). The structure of the bovine enzyme, deduced from X-ray diffraction data, indicated that the cationic residues Arg-141, Lys-120, and Lys-134 are located 5, 12, and 13 Å, respectively, from the active site Cu(II) and suggested that they provide for electrostatic attraction of the anionic substrate (Tainer et al., 1982). Covalent modification of these residues suppresses the catalytic activity of Cu,Zn-SOD (Malinowski & Fridovich, 1979; Cudd & Fridovich, 1982a; Marmocchi et al., 1983; Cocco et al., 1982).

Increasing the concentration of phosphate was seen to decrease the activity of Cu,Zn-SOD, and this was interpreted as an effect due to ionic strength (Cudd & Fridovich, 1982a; Rigo et al., 1975). More recently, de Freitas and Valentine (1984) reported that phosphate inhibited Cu,Zn-SOD, when the ionic strength was held constant, by addition of NaF to the HEPES buffer. These authors concluded that phosphate inhibits Cu,Zn-SOD by binding to Arg-141. In the course of their work de Freitas and Valentine considered several salts that might be used to maintain ionic strength as phosphate was varied. In the first paragraph under Results of their paper they stated, "SOD activities and anion binding affinities were higher when the ionic strength was adjusted with trifluoromethanesulfonate rather than with fluoride, but with the former, little or no effect of added phosphate on the properties of the protein was observed...". This suggested to us that the reported effect of phosphate might well be an artifact derived from the influence of fluoride on the assay system. We have reinvestigated the effects of phosphate and now present results indicating that it does not inhibit, Cu,Zn-SOD beyond the expectations of its contribution to ionic strength.

## Materials and Methods

Bovine liver Cu,Zn-SOD (lyophilized powder) was a generous gift from Diagnostic Data, Inc. (Mountain View, CA). Phenylglyoxal, *O*-methylisourea sulfate salt, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), xanthine, 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris), horse heart cytochrome *c* (type III), 6-hydroxydopamine hydrobromide, methionine, riboflavin, Triton X-100, 2-(*N*-cyclohexylamino)ethanesulfonic acid (CHES), bicine, and nitroblue tetrazolium [2,2′-di-*p*-nitrophenyl-5,5′-diphenyl-3,3′-(3,3′-dimethoxy-4,4′-diphenylene)ditetrazolium chloride (NBT)] were used as received from Sigma. Potassium phosphate dibasic trihydrate and potassium phosphate monobasic were

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[1] Abbreviations: Cu,Zn-SOD, copper- and zinc-containing superoxide dismutase; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; 6-HDA, 6-hydroxydopamine; NBT, nitroblue tetrazolium; CHES, 2-(*N*-cyclohexylamino)ethanesulfonic acid; bicine, *N*,*N*-bis(2-hydroxyethyl)glycine; $KP_i$, potassium phosphate; EDTA, ethylenediaminetetraacetic acid.

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of AR grade from Mallinckrodt; sodium fluoride, sodium bromide, sodium chloride, sodium sulfate, sodium pyrophosphate decahydrate, sodium bicarbonate, sodium nitrate, and sodium perchlorate were of AR grade and were used as received from J. T. Baker Chemical Co. Xanthine oxidase was purified from unpasteurized bovine milk cream by a method that avoids proteolysis (Rajagopalan, 1985) and was a generous gift from K. V. Rajagopalan.

Protein concentrations were estimated by the method of Lowry et al. (1951), with pure bovine serum albumin (Sigma) as a standard. SOD activity was assayed by the inhibitory effect of SOD on the autoxidation of 6-hydroxydopamine (Heikkila & Cabbat, 1976) and on the reduction of cytochrome *c* by the xanthine/xanthine oxidase system (McCord & Fridovich, 1969) and by a modification of the NBT photoreduction assay (Beauchamp & Fridovich, 1971). These activity assays were modified (as described) by using either HEPES/NaOH or Tris-HCl (both at 50 mM with 0.1 mM EDTA) in place of potassium phosphate to investigate the inhibitory effects of phosphate. Absorption changes were recorded at 25 °C by using a Hitachi 100-80 UV–vis spectrophotometer equipped with a thermostated cell compartment. Photoreduction of NBT was induced by placing samples in a foil-lined box equipped with two Sylvania gro-lux fluorescent tubes.

Modification of the arginine residues with phenylglyoxal was performed by exhaustive dialysis of native Cu,Zn-SOD (208 $\mu$M) against 100 mM $Na_4P_2O_7$/100 mM $NaHCO_3$ (pH 9.1) at 4 °C. The dialyzed enzyme solution was then made 20 mM in phenylglyoxal, placed in a constant temperature bath at 30 °C, and allowed to react for 6 h with constant agitation. Excess reactant was removed by dialysis against several 2-L changes of 50 mM potassium phosphate buffer (pH 7.8) containing 0.1 mM EDTA.

*O*-Methylisourea modification of lysine residues was performed by exhaustive dialysis of native enzyme (208 $\mu$M) against 100 mM borate buffer (pH 9.5) containing 0.1 mM EDTA. The enzyme solution was made 170 mM in *O*-methylisourea by addition of a concentrated *O*-methylisourea solution whose pH had been adjusted to 9.5 with NaOH to neutralize the $HSO_4^-$ counterion. The reaction was allowed to proceed for 24 h at 30 °C with constant agitation, followed by dialysis against several 2-L changes of 50 mM potassium phosphate buffer (pH 7.8) containing 0.1 mM EDTA. The homoarginine derivative so generated was further modified with phenylglyoxal by the aforementioned procedure. Native Cu,Zn-SOD was dialyzed extensively against 10 mM NaCl (pH 7.8) to remove sucrose added prior to lyophilization.

Electrophoresis was performed on polyacrylamide slab gels at 25 mA/slab as described by Davis (1964). Protein bands were visualized by staining with Coomassie Blue R 250 and destaining in methanol/acetic acid/$H_2O$ [3:1:9 (v/v)]. Gels were stained for enzymatic activity according to the method described by Beauchamp and Fridovich (1971). HEPES buffer was titrated to the indicated pH with a standardized NaOH solution, and the sodium ion contribution to ionic strength was included in the final ionic strength calculation.

## RESULTS

*Cytochrome c Reduction Assay.* Cytochrome *c* is cationic in the neutral pH range, and its interaction with anionic reductants occurs with electrostatic facilitation (Cudd & Fridovich, 1982b). Cu,Zn-SOD is anionic in the neutral pH range (Marmocchi et al., 1983), but a cluster of cationic residues in the active site region nevertheless provides for electrostatic facilitation of its interaction with its anionic

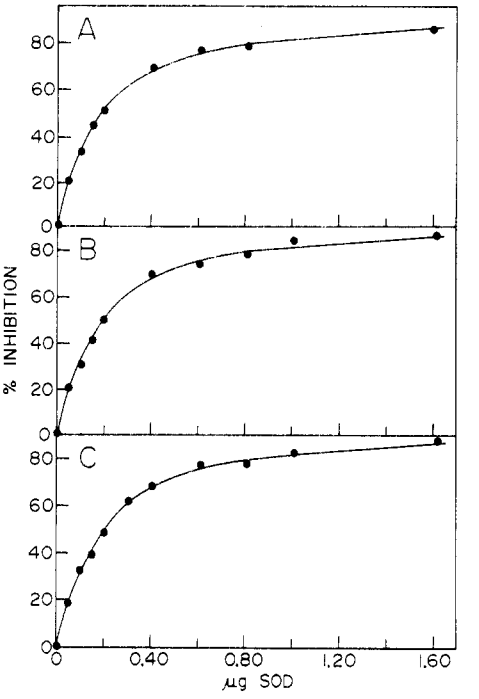


FIGURE 1: Inhibition of cytochrome *c* reduction by native Cu,Zn-SOD. $O_2^-$ was generated by the xanthine/xanthine oxidase system. (A) Assay was done in 50 mM potassium phosphate (pH 7.8). (B) Assay was done in 50 mM HEPES/NaOH (pH 7.4); no adjustment was made to ionic strength. (C) Assay was done in 50 mM HEPES/NaOH (pH 7.4) with ionic strength adjusted to 0.13 with NaBr. All buffers contained 0.1 mM EDTA.

substrate (Cudd & Fridovich, 1982a; Malinowski & Fridovich, 1979; Tainer et al., 1982). In the cytochrome *c* reduction assay, SOD and cytochrome *c* compete for a flux of $O_2^-$ provided by the xanthine oxidase reaction. Since the reactions of $O_2^-$ with both cytochrome *c* and Cu,Zn-SOD are similarly affected by ionic strength, we expect no significant overall effect of ionic strength on this assay (Cudd & Fridovich, 1982b). Inhibition of cytochrome *c* reduction by Cu,Zn-SOD was examined in three buffer systems: (a) 50 mM potassium phosphate (pH 7.8), (b) 50 mM HEPES (pH 7.4), and (c) 50 mM HEPES plus 0.1 M NaBr (pH 7.4), all containing 0.1 mM EDTA at 25 °C. As shown in Figure 1, Cu,Zn-SOD exhibited identical activity in all three buffers, causing 50% inhibition of cytochrome *c* reduction at a concentration of 0.066 $\mu$g/mL. These results establish that neither phosphate nor bromide inhibits Cu,Zn-SOD relative to HEPES.

de Freitas and Valentine (1984) chose NaF for the maintenance of ionic strength. This was an unfortunate choice for at least two reasons. Fluoride has been reported to inhibit Cu,Zn-SOD (Rigo et al., 1977; Bertini et al., 1981; Viglino et al., 1981). Moreover, fluoride causes a progressive diminution in the activity of xanthine oxidase, whether assayed in terms of the conversion of xanthine to urate (Figure 2) or in terms of the reduction of cytochrome *c* by $O_2^-$ (Figure 3). The inhibitory effect of fluoride was not reduced by raising the concentration of EDTA from 0.1 to 2.5 mM and was therefore judged not to depend upon trace metal contaminants. Neither HEPES nor bromide exerted this effect. This effect of fluoride sets the stage for an erroneous overestimation of the activity of SOD. Thus the first 30 s of cytochrome *c* reduction appears to be linear (Figure 3) and could be taken as the uninhibited rate. Addition of SOD would then sharply decrease the rate of reduction for two reasons: the first being due to the scavenging of $O_2^-$ by SOD and the second being due to the progressive inhibition by fluoride. The curvature

Table I: Effect of Various Salts on the Rate of Uric Acid Production and Cytochrome *c* Reduction in HEPES Buffer[a]

| | xanthine oxidase rate | | cytochrome *c* reduction rate | |
|---|---|---|---|---|
| added salt | $\Delta A_{293}$/min | % | $\Delta A_{550}$/min | % |
| none | 32 | 100 | 27 | 100 |
| NaF | nl[b] | | nl[b] | |
| NaCl | 36.5 | 114.0 | 29.2 | 109.5 |
| $NaClO_4$ | 10.5 | 32.8 | 10.7 | 40.3 |
| NaBr | 36 | 112.5 | 27.2 | 102.0 |
| $KP_i$ | 38.5 | 120.3 | 30.5 | 114.2 |
| $N_2SO_4$ | 36 | 112.5 | 32 | 119.8 |
| $NaNO_3$ | 28.5 | 89.1 | 21 | 78.6 |

[a] Reactions were done in 50 mM HEPES/NaOH (pH 7.4) containing 0.1 mM EDTA, 50 $\mu$M xanthine, and 10 $\mu$M cytochrome *c* (type III). A total of 7.5 $\mu$L of a $5 \times 10^{-6}$ M xanthine oxidase solution was diluted into a reaction volume of 3.0 mL equilibrated at 25 °C, and the absorbance change at the appropriate wavelength was recorded as a function of time. Salts were added to a final concentration of 0.10 M. Data are the mean of four measurements. [b] nl = nonliinear rate of reaction.

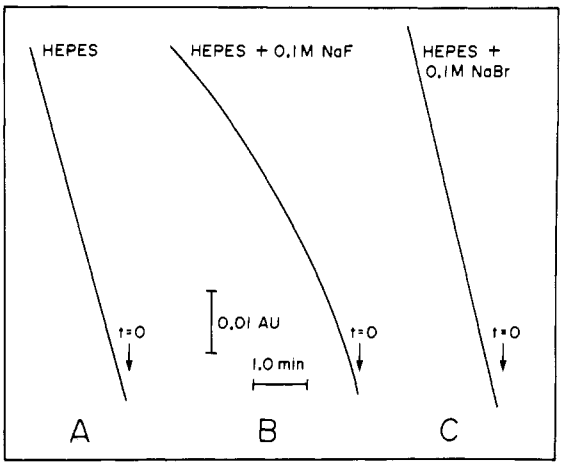


FIGURE 2: Effect of NaF and NaBr (0.1 M) on urate production by xanthine oxidase on xanthine. Reaction was performed in 50 mM HEPES/NaOH (pH 7.4) containing 0.1 mM EDTA and 50 $\mu$M xanthine. (A) A total of 7.5 $\mu$L of a $5 \times 10^{-6}$ M solution of xanthine oxidase (in HEPES buffer) was diluted into a reaction volume of 3.0 mL equilibrated at 25 °C. The change in absorbance at 293 nm was recorded as a function of time. (B) Conditions as in (A) except the ionic strength was adjusted to 0.13 with NaF. (C) Conditions as in (A) except the ionic strength was adjusted to 0.13 with NaBr.

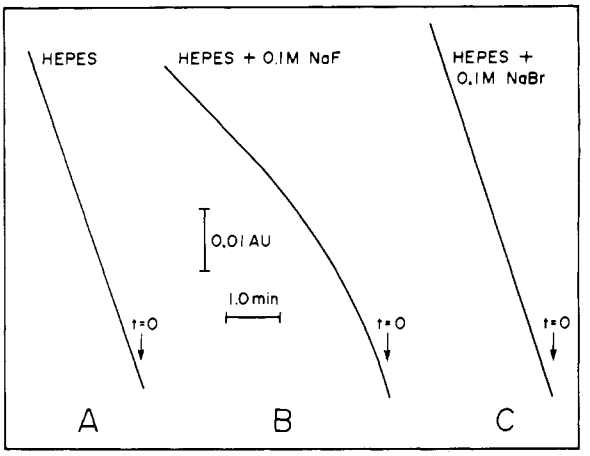


FIGURE 3: Effect of NaF and NaBr (0.10 M) on cytochrome *c* reduction due to $O_2^-$ generated by xanthine/xanthine oxidase reaction. Reaction was performed in 50 mM HEPES/NaOH (pH 7.4) containing 0.1 mM EDTA, 50 $\mu$M xanthine, and 10 $\mu$M horse heart cytochrome *c* (III). (A) A total of 7.5 $\mu$L of a $5 \times 10^{-6}$ M solution of xanthine oxidase (in HEPES buffer) was diluted into a reaction volume of 3.0 mL equilibrated at 25 °C. The increase in absorbance due to production of ferrocytochrome *c* was monitored at 550 nm as a function of time. (B) Conditions as in (A) except the ionic strength was adjusted to 0.13 with NaF. (C) Conditions as in (A) except the ionic strength was adjusted to 0.13 with NaBr.

so apparent in Figure 3 might be overlooked after the rate had been reduced, due to the effect of SOD. Since one unit of SOD activity is defined as the amount needed to halve the rate of reduction of cytochrome *c* (McCord & Fridovich, 1969), the progressive inhibition of xanthine oxidase by NaF would lead to an overestimation of the activity of SOD. While de Freitas and Valentine (1984) did not describe their methods in sufficient detail to allow certainty concerning the basis of their overestimation of SOD activity, the explanation offered above seems likely in view of their use of xanthine oxidase/cytochrome *c* method. We examined the effects of several salts on the xanthine oxidase/cytochrome *c* reaction. The results in Table I show that sodium fluoride was the only one, of seven salts tested, to cause a progressive decline in the rate of cytochrome *c* reduction. Salts of $Cl^-$, $Br^-$, phosphate, or $SO_4^{2-}$ were without effect and could have been safely used to maintain ionic strength.

Since Arg-141 was supposed (de Freitas & Valentine, 1984) to be the site of phosphate binding, we used group-sepecific modification to explore the importance of arginine. *O*-Methylisourea was employed to convert lysine to homoarginine residues, and phenylglyoxal was used to derivatize both arginine and homoarginine residues. As shown in Table II, treatment of Cu,Zn-SOD with *O*-methylisourea did not affect the enzymic activity of Cu,Zn-SOD and did not make make it susceptible to inhibition by phosphate. Phenylglyoxal caused ~80% inactivation when applied to native Cu,Zn-SOD and caused ~90% inactivation when applied to the *O*-methylisourea-treated enzyme. In no case were the residual activities inhibited by addition of phosphate or of bromide to the HEPES buffer.

Polyacrylamide gel electrophoresis, followed by staining for protein and for SOD activity, was used to verify the effects of these group-modifying reagents, as shown in Figure 4. Elimination of the positive charge on arginine residues with phenylglyoxal should increase anodic mobility. This was the case, as can be seen by comparing lanes 3 and 4 with lanes

Table II: Effect of Different Buffers and Ionic Strength upon SOD Activity of Native and Modified Cu,Zn-SOD[a]

| | | specific activity (units/mg)[c] | | | |
|---|---|---|---|---|---|
| buffer[b] | ionic strength | native Cu,Zn-SOD | *O*-methylisourea Cu,Zn-SOD | phenylglyoxal Cu,Zn-SOD | *O*-methylisourea/ phenylglycoxal Cu,Zn-SOD |
| phosphate | 0.13 | $4.76 \times 10^3$ (100) | $4.74 \times 10^3$ (99.6) | 899.6 (18.9) | 451.9 (9.5) |
| HEPES | 0.03 | $4.27 \times 10^3$ (89.7) | $4.74 \times 10^3$ (99.6) | 415.2 (8.7) | 201.1 (4.2) |
| HEPES + 16.4 mM $KP_i$ | 0.07 | $4.40 \times 10^3$ (92.4) | $4.74 \times 10^3$ (99.6) | 732.6 (15.4) | 305.0 (6.4) |
| HEPES + 0.10 M NaBr | 0.13 | $4.61 \times 10^3$ (96.8) | $5.14 \times 10^3$ (107.9) | 650.8 (13.7) | 308.4 (6.5) |

[a] SOD activity was determined by using the xanthine/xanthine oxidase, cytochrome *c* assay. [b] All buffers were at 50 mM and contained 0.1 mM EDTA. Phosphate buffers were at pH 7.8; HEPES buffers were at pH 7.4. [c] Values in parentheses indicate the percentage of activity based on native Cu,Zn-SOD in phosphate buffer as 100% activity. Error in measurements is estimated to be ±5%.

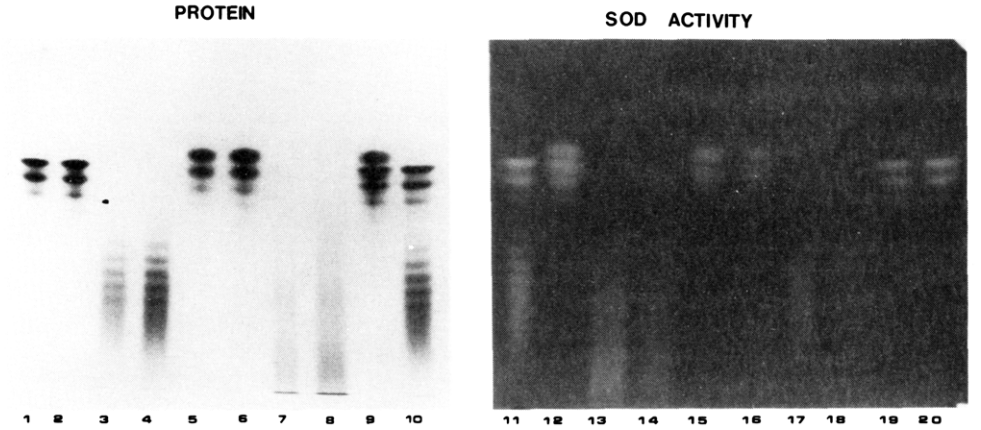


FIGURE 4: Native acrylamide gels (7.5%) of native and modified Cu,Zn-SODs. Samples were electrophoresed in 7.5% acrylamide gels at 25 mA/gel until the tracking dye bromphenol blue was ca. 1–2 cm from the bottom of the gel. Lanes 1–10 were stained for protein with Coomassie Blue R-250, while lanes 11–20 were stained for SOD activity according to the method described by Beauchamp and Fridovich (1971). Lanes 1 and 2, native Cu,Zn-SOD (9.1, 18.2 μg); lanes 3 and 4, phenylglyoxal-modified Cu,Zn-SOD (10, 20 μg); lanes 5 and 6, *O*-methylisourea-modified Cu,Zn-SOD (8.6, 17.2 μg); lanes 7 and 8, *O*-methylisourea/phenylglyoxal-modified Cu,Zn-SOD (100, 200 μg); lane 9, native Cu,Zn-SOD (10 μg) + *O*-methylisourea Cu,Zn-SOD (12 μg); lane 10, native Cu,Zn-SOD (12 μg) + phenylglyoxal Cu,Zn-SOD (20 μg); lane 11, native Cu,Zn-SOD (2.0 units) + phenylglyoxal Cu,Zn-SOD (2.0 units); lane 12, native Cu,Zn-SOD (2 units) + *O*-methylisourea Cu,Zn-SOD (2.0 units); lanes 13 and 14, *O*-methylisourea/phenylglyoxal-modified Cu,Zn-SOD (4 units, 2 units); lanes 15 and 16, *O*-methylisourea Cu,Zn-SOD (4.0 units, 2.0 units); lanes 17 and 18, phenylglyoxal-modified Cu,Zn-SOD (4.0 units, 2.0 units); lanes 19 and 20, native Cu,Zn-SOD (4.0 units, 2.0 units).

1 and 2. When native and phenylglyoxal-treated enzymes were mixed, the expected pattern emerged (lanes 10 and 11). The multiplicity of rapidly migrating bands seen with the phenylglyoxal-treated enzyme undoubtedly reflects different degrees of modification of the enzyme. There are thus two arginine residues per subunit (four per molecule), and each of the native electromorphs could give rise to four phenylglyoxal-modified products. It must be noted that no residual native electromorphs remained in the phenylglyoxal-modified enzyme and that the activity which remained after this covalent modification was associated with the newly generated rapidly migrating bands.

Conversion of lysine to homoarginine residues should increase the positive charge at the pH of the gels (pH 8.7) and thus decrease anodic mobility, and this was observed (compare lanes 5 and 6 with lanes 1 and 2). When native enzyme was mixed with *O*-methylisourea-modified enzyme, the expected mixed pattern was observed (lane 12). Treatment with *O*-methylisourea followed by phenylglyoxal generated a virtual continuum of rapidly migrating species, as expected.

*Nitroblue Tetrazolium Photoreduction Assay.* Unlike the cytochrome *c* reduction assay of SOD, wherein the like effects of ionic strength on the reactions of $O_2^-$ with the enzyme and with the cytochrome *c* are mutually canceling, the NBT reduction assay is responsive to changes in ionic strength (Cudd & Fridovich, 1982a). We modified the procedure originally described by Beauchamp and Fridovich (1971) by adding Triton X-100 to 0.025% (w/v) to prevent aggregation of the insoluble formazan product of NBT reduction (Nishikimi, 1975). Since one unit of SOD activity is properly defined as that amount causing half-maximal inhibition of NBT photoreduction (Beauchamp & Fridovich, 1971), we examined the effects of salts on the maximum extent to which SOD could inhibit this photoreduction. The results shown in Table III demonstrate that only ~50% of the photoreduction of NBT is inhibitable by SOD in HEPES, whereas ~72% is inhibitable in potassium phosphate. Tris buffer also provided ~70% SOD-inhibitable NBT reduction and was therefore chosen as the buffer in which to examine the effects of phosphate and of ionic strength on SOD activity.

Cu,Zn-SOD activity, assessed by inhibition of NBT photoreduction, was 40% greater in 1.0 mM than in 50 mM potassium phosphate. However, when the ionic strength of these two phosphate buffers was equalized by addition of NaCl to the more dilute buffer, the difference in SOD activity disappeared. Virtually identical results were seen when NaCl was added to Tris-buffered assay mixtures (Table IV). It is clear that the effect of phosphate on the NBT photoreduction assay of Cu,Zn-SOD activity was no greater than the effect of NaCl and can be accounted for as an effect of ionic strength.

*6-Hydroxydopamine Autoxidation Assay.* de Freitas and Valentine (1984) reported that the inhibitory effect of phosphate on Cu,Zn-SOD could be seen in both cytochrome *c* reduction and 6-HDA autoxidation assays, but they did not

Table III: Differences of Various Buffers on the Maximum Amount of NBT Photoreduction due to $O_2^-$ [a]

| buffer[b] | $\Delta A_{560}$/7 min: no SOD | $\Delta A_{560}$/7 min: 60.5 μg of SOD | max inhibition by SOD (%) |
|---|---|---|---|
| potassium phosphate | 0.436 | 0.116 | 72.8 |
| HEPES[c] (pH 7.4) | 0.530 | 0.268 | 49.4 |
| HEPES[c] (pH 7.8) | 0.528 | 0.242 | 54.2 |
| CHES[c] | 0.439 | 0.136 | 69.1 |
| bicine[c] | 0.700 | 0.250 | 64.3 |
| Tris[d] | 0.403 | 0.120 | 70.2 |

[a] Bovine Cu,Zn-SOD was dialyzed against 10 mM NaCl (pH 7.8), and 10 μL of a stock solution, 6.05 mg/mL, was added to a total reaction volume of 1.0 mL. (Total added SOD was 60.5 μg.) [b] All buffers were present at 50 mM and contained 0.025% (w/v) Triton X-100 and 0.1 mM EDTA. The pH of each buffer was 7.8 unless noted otherwise. [c] pH adjusted with NaOH. [d] pH adjusted with HCl.

Table IV: Activity of Native Cu,Zn-SOD in Various Buffer Systems As Determined by $O_2^-$-Dependent Photoreduction of NBT

| buffer[a] | ionic strength | % max inhibition by SOD | μg of SOD needed for half-max inhibition |
|---|---|---|---|
| 50 mM $KP_i$ | 0.13 | 69 | 0.26 |
| 1 mM $KP_i$ | 0.003 | 67 | 0.190 |
| 1 mM $KP_i$ + 0.13 M NaCl | 0.13 | 75 | 0.26 |
| 50 mM Tris[b] | 0.03 | 73 | 0.18 |
| 50 mM Tris[b] + 0.1 M NaCl | 0.13 | 73 | 0.25 |

[a] All buffers were at pH 7.8 and contained 0.1 mM EDTA and 0.025% (w/v) Triton X-100. [b] HCl was used to adjust pH.

provide any data derived from the latter assay. We prepared a 10 mM stock solution of 6-HDA in cold, anaerobic, glass-distilled water by working in an anaerobic glovebox. Aliquots (30 μL) of this solution were withdrawn with a hypodermic syringe through a rubber septum seal and were delivered into 3.0 mL of aerated buffer at 25 °C to initiate autoxidation, which was followed at 490 nm. A linear rate was maintained for no longer than 12 s under these conditions. Moreover, the rate of autoxidation of 6-HDA was 25% faster in HEPES than in phosphate buffer, both at pH 7.8. We conclude that the 6-HDA autoxidation assay is not useful for precise assay of SOD activity where comparisons in different buffers are essential. This is not surprising since autoxidations proceeding by free radical chain reactions are very sensitive to catalysis by a variety of initiators and to inhibition by chain breakers.

## Discussion

The Cu,Zn-SOD, isolated from bovine erythrocytes, exhibits identical activity in the xanthine oxidase/cytochrome *c* reduction assay (McCord & Fridovich, 1969) in 50 mM potassium phosphate or HEPES buffers. Phosphate is thus not an inhibitor of this SOD. The same result was obtained when the enzyme activity was measured in the NBT photoreduction assay. The earlier paper by de Freitas and Valentine (1984), which reported inhibition of Cu,Zn-SOD by phosphate in the xanthine oxidase/cytochrome *c* assay, was flawed by the presence of sodium fluoride, which progressively inhibits xanthine oxidase and causes an overestimation of SOD activity. In fact, we can reproduce their results by substitution of NaF for NaBr in the cytochrome *c* assay system. We find that NaBr and several other salts may be used to adjust ionic strength without interfering with the assay.

Conversion of lysine to homoarginine residues by treatment of Cu,Zn-SOD with *O*-methylisourea did not change the activity of this enzyme and did not impose a sensitivity to phosphate. Derivatization of arginine and of homoarginine residues with phenylglyoxal did cause 80–90% loss of activity, as we have previously reported. This cannot, however, be taken as an indication of the essentiality of Arg-141 for the enzymatic activity, since this modification introduces a bulky substituent into the active site region, which might diminish catalytic activity for purely steric reasons. Replacement of this Arg-141 by site-specific mutagenesis would provide a valid test for the importance of this residue. We have undertaken such studies.

**Registry No.** SOD, 9054-89-1; L-Arg, 74-79-3; $PO_4^{3-}$, 14265-44-2; phenylglyoxal, 1074-12-0.

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