The major superoxide dismutase ("slow" electromorph) of the fruit fly, Drosophila melanogaster, has been purified to homogeneity. This enzyme contains 2 Cu\(^{2+}\) and 2 Zn\(^{2+}\)/molecule. The ultraviolet absorption spectrum indicates a lack of tryptophan. This enzyme has a molecular weight of 32,000 and is composed of two subunits of equal size, which are joined by noncovalent interactions. Cyanide at 1 and 3 mM inhibits the activity of superoxide dismutase 92 and 100%, but 5 and 10 mM azide caused 15 and 30% inhibition. The isoelectric point, assessed by isoelectric focusing, is 5.3. Amino acid analyses, as well as the spectral and catalytic properties, are reported. The D. melanogaster superoxide dismutase does not cross-react with antibodies to bovine erythrocyte Cu-Zn-containing superoxide dismutase nor to Escherichia coli manganese- and iron-containing superoxide dismutases.

Superoxide dismutases, which catalytically scaveng the O\(_2^-\) and appear to be essential components of the biological defense against oxygen toxicity (1-3). These enzymes are neither a part of structural proteins nor involved in intermediate metabolism, providing a unique situation to be studied by population geneticists. Genetic polymorphism of superoxide dismutase has already been discovered in diverse living organisms including humans and the fruit fly, Drosophila (4-10). Little is known, however, about the structural basis of such polymorphisms.

Superoxide dismutases have been isolated from several species, thus far, only three grossly dissimilar kinds have been found. The structural and functional relationships of these classes of superoxide dismutases have raised interesting and unresolved questions about their evolution. Copper- and zinc-containing superoxide dismutases have been isolated from various species (11-19) and considered to be characteristic of the cytosol of eukaryotic cells (20), but a similar enzyme has been found in a prokaryote, Photobacterium leiognathi (20). Manganese-containing superoxide dismutases have been isolated from several prokaryotes (21-23) and from the mitochondria of chicken liver (14) and of yeast (24). Structural analyses have demonstrated a close relationship between the bacterial and the mitochondrial enzymes (25, 26), supporting the hypothesis of a symbiotic origin of mitochondria (25-27).

It was believed that the cytosol superoxide dismutase in eukaryotes would contain copper-zinc, while the mitochondria would contain manganese. However, the luminous fungus, Pleurotus olearius, has been shown to contain two superoxide dismutases, both of which contain manganese (28). Furthermore, substantial quantities of manganese enzyme have been found in the cytosol of chicken liver and of baboon liver (29). Superoxide dismutase isolated from the cytosol of unicellular red alga, Porphyridium cruentum, which is considered to be perhaps the most primitive eukaryote, contain manganese (30). However, blue-green algae, which are considered to be the most advanced prokaryotes, have an iron-containing superoxide dismutase (31, 32). Iron-containing enzymes have also been found in several bacteria (33-35). A survey of progressively more advanced plants has failed to find copper-zinc superoxide dismutase in marine plants, but has found it in land plants such as mosses and ferns (36). Thus, the facts are not easily arranged into a coherent theory of descent. There have been several reports indicating that superoxide dismutase protects against ionizing radiation damage to DNA, viruses, bacteria, mammalian cells in culture, and even whole animals (37-43). Since insects have been shown to be more resistant to ionizing radiation than mammals, Drosophila are reported to survive radiation exposure of 64,000 rads (44), and because a superoxide dismutase has not been isolated from an insect, it seemed important to purify and characterize this enzyme from Drosophila melanogaster. Here we report the thorough purification and characterization of one of two electrophoretically detectable algae of superoxide dismutase from D. melanogaster with the expectation that this will relate to the radioresistance of the organism and will also bring us a step closer to disentangling the complex evolutionary history of these enzymes.

**MATERIALS AND METHODS AND RESULTS**

**DISCUSSION**

Cell-free extracts of the fruit fly, D. melanogaster, contain two superoxide dismutases which are separable by polyacrylamide gel electrophoresis. The major superoxide dismutase activity was inhibited by cyanide. This enzyme, like the bovine erythrocyte superoxide dismutase, survived an unusual purification step which included use of chloroform-ethanol to denature extraneous proteins. However, unlike other Cu-Zn superoxide dismutases, the D. melanogaster enzyme would contain manganese.
superoxide dismutases, the *Drosophila* enzyme ("slow" electrophilic) quickly lost its activity when salted out of an ethanol-rich phase with K$_2$HPO$_4$.

Isolation of the major superoxide dismutase of *D. melanogaster* revealed that the molecular properties of this enzyme appear to have been rigidly preserved during the evolution of eukaryotes. Thus, the enzyme is similar to the cytoplasmic isozyme of *Drosophila* and Drosophila which could be homologous by the criteria of polyacrylamide gel electrophoresis and sodium dodecyl sulfate gel electrophoresis. Since a 245-fold purification from the cell-free extract was achieved, it seems reasonable to believe that the high concentrations of highly active superoxide dismutase detected in *Drosophila* could be contributing to the higher resistance of these flies to ionizing radiation. The evolutionary relationships among superoxide dismutases are obviously of great interest. The structural basis of genetic polymorphisms of this enzyme noticed in *Drosophila* (9) needs to be explored.

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**REFERENCES**

1. McCord, J. M., Keele, B. B., Jr., and Fridovich, I. (1971) *Proc. Natl. Acad. Sci. U. S. A.* 68, 1024-1027
2. Fridovich, I. (1975) *Annu. Rev. Biochem.* 44, 147-159
3. Hassan, H. M., and Fridovich, I. (1977) *J. Bacteriol.* 129, 1574-1583
4. Kirk, R. L., Keats, B., Blake, N. M., Mc Dermid, E. M., Alia, F., Karimi, N., Nickbin, B., Shinabah, H., and Kmet, J. (1977) *Am. J. Phys. Anthropol.* 46, 377-390
5. Beams, G., Beckman, L., and Nilsson, L. O. (1973) *Hereditas* 75, 138
6. Marklund, S., Beckman, G., and Stigbrand, T. (1976) *Eur. J. Biochem.* 65, 415-421
7. Crosti, N., Serra, A., Cagiano-Malbeizzi, D., and Tagliaferri, G. (1976) *Ann. Hum. Biol.* 3, 343-350
8. Feaster, W. W., Kwok, L. W., and Epstein, C. J. (1977) *Am. J. Hum. Genet.* 29, 563-570
9. Richardson, R. C., and Powell, J. R. (1976) *Procr. Natl. Acad. Sci. U. S. A.* 67, 1264-1267
10. Ayala, F. J., Powell, J. R., and Dobzhansky, T. (1971) *Proc. Natl. Acad. Sci. U. S. A.* 68, 2490-2493
11. McCord, J. M., and Fridovich, I. (1969) *J. Biol. Chem.* 244, 6049-6055
12. Misra, H. P., and Fridovich, I. (1972) *J. Biol. Chem.* 247, 3410-3411
13. Keeler, B. B., Jr., McCord, J. M., and Friisovich, I. (1971) *J. Biol. Chem.* 246, 2875-2888
14. Węsiger, R. A., and Fridovich, I. (1973) *J. Biol. Chem.* 248, 3982-3992
15. Banister, W. H., Dalgleish, D. G., Banister, J. V., and Wood, E. J. (1972) *Int. J. Biochem.* 3, 560-568
16. Beauchamp, C. O., and Fridovich, I. (1973) *Biochim. Biophys. Acta* 317, 50-64
17. Asada, K., Urano, M., and Takehashi, M. (1973) *Eur. J. Biochem.* 36, 257-266
18. Goscin, S. A., and Fridovich, I. (1972) *Biochim. Biophys. Acta* 270, 276-283
19. Sawada, Y., Ohyama, T., and Yamazaki, I. (1972) *Biochim. Biophys. Acta* 268, 305-312
20. Puget, K., and Michelson, A. M. (1974) *Biochem. Biophys. Res. Commun.* 58, 830-838
21. Keele, B. Jr., McCord, J. M., and Fridovich, I. (1970) *J. Biol. Chem.* 245, 6175-6181
22. Vance, P. G., Keele, B. Jr., and Rajagopalan, K. V. (1972) *J. Biol. Chem.* 247, 4782-4786
23. Avron, G. P., Henry, L., Palmer, J. M., and Hall, D. O. (1976) *Biochem. Soc. Trans.* 4, 618-620
24. Ravindrannath, S. D., and Fridovich, I. (1975) *J. Biol. Chem.* 250, 6107-6112
25. Steinman, H. M., and Hill, R. L. (1973) *Proc. Natl. Acad. Sci. U. S. A.* 70, 3725-3729
26. Bridge, J. S., Harris, J. A., and Northrop, F. (1975) *FEBS Lett.* 49, 392-395
27. Fridovich, I. (1974) *Life Sci.* 14, 819-826
28. Lavelle, F., and Michelson, A. M. (1975) *Biochimie* 57, 375-381
29. McCord, J. M. (1976) *Adv. Exp. Med. Biol.* 74, 540-560
30. Misra, H. P., and Fridovich, I. (1977) *J. Biol. Chem.* 252, 6421-6423
31. Misra, H. P., and Keeler, B. B., Jr. (1975) *Biochim. Biophys. Acta* 379, 418-425
32. Asada, K., Yoshikawa, K., Takehashi, M., Maeda, Y., and Enmanji, K. (1975) *J. Biol. Chem.* 250, 2981-2987
33. Yoo, F. J., Jr., and Fridovich, I. (1973) *J. Biol. Chem.* 248, 4905-4909
34. Yamakura, F. (1976) *Biochim. Biophys. Acta* 422, 280-294
35. Puget, K., and Michelson, A. M. (1974) *Biochimie* 56, 1255-1267
36. Asada, K., Kanematsu, S., Takehashi, M., and Kona, Y. (1976) *Adv. Exp. Med. Biol.* 74, 551-564
37. Michelson, A. M., and Buckingham, M. E. (1974) *Biochim. Biophys. Res. Commun.* 58, 1079-1086
38. Petkau, A., and Chelack, W. S. (1974) *Int. J. Radiat. Biol.* 26, 421-426
39. Misra, H. P., and Fridovich, I. (1976) *Arch. Biochem. Biophys.* 176, 577-581
40. Oberley, L. W., Lindgren, A. L., Baker, S. A., and Stevens, R. H. (1976) *Radiat. Res.* 68, 292-298
41. Petkau, A., Kelly, K., Chelack, W. S., Pieskach, S. D., Barefoot, C., and Meeker, B. E. (1975) *Biochim. Biophys. Res. Commun.* 67, 1167-1174
42. Petkau, A., Kelly, K., Chelack, W. S., and Barefoot, C. (1976) *Biochim. Biophys. Res. Commun.* 70, 452-458
43. Petkau, A., Chelack, W. S., and Pieskach, S. D. (1976) *Int. J. Radiat. Biol.* 29, 297-299
44. Casarett, A. P. (1968) *Radiation Biology*, Chap. 10, p. 219, Prentice-Hall, Inc., Englewood Cliffs, NJ
45. McDonald, J. F., and Ayala, F. J. (1978) *Genetics* 89, 371-388
46. Davis, B. J. (1964) *Annu. N. Y. Acad. Sci.* 121, 404-427
47. Beauchamp, C., and Fridovich, I. (1971) *Anal. Biochem.* 44, 276-287
48. Bailey, J. L. (1967) *Techniques in Protein Chemistry*, pp. 340-341, Elsevier, NY
49. Moore, S. and Stein, W. H. (1963) *Methods Enzymol.* 6, 819-831
50. Moore, S. (1963) *J. Biol. Chem.* 238, 235-237
51. Matsuura, H., and Sasaki, R. (1969) *Biochem. Biophys. Res. Commun.* 35, 175-181
52. Weber, K., and Osborn, M. (1967) *J. Biol. Chem.* 242, 4406-4412
53. Benacerraf, B., and Unanue, E. R. (1979) *Textbook of Immunology*, Williams and Wilkins, Baltimore
54. Rotilio, G., Bray, R. C., and Fielden, E. M. (1972) *Biochim. Biophys. Acta* 268, 605-609
55. Misra, H. P., and Fridovich, I. (1978) *Arch. Biochem. Biophys.* 189, 317-322
Superoxide Dismutase from Drosophila

Materials and Methods

Juvenile female flies were made isogenic for the whole third chromosome by crossing flies collected in Tumaco, Aragua, with the balancer stocks 76B as described (48). One of the isogenic lines was multiplied and collected at 24°-26° C. These flies were frozen and stored at -20°C until used. Cytocentrifuge (type III), centrifuge and sonicator cells were products of Sipex. Microcrystalline diethylaminoethyl cellulose and superoxide dismutase 0.75 were obtained from IIIB and Pharmacia, respectively. Antigens were purchased from IIB.

Superoxide dismutase was assayed and units were defined as previously described (49). Electrophoresis on polyacrylamide gels was performed according to Davis (46). Zones of protein were localized by staining with amido black (48), while zones of superoxide dismutase were negatively stained by the photometric procedure previously described (47). Electrophoretic assays were performed at 20° in a Shifted model 201 gel electrophorimeter. The gel was stained with Coomassie blue. Protein was measured by the spectrophotometric procedure of Lowry (48) with recrystallized bovine serum albumin as the standard.

Amino acid analysis was performed on a Durrum model 5500 amino acid analyzer using a single-column, three-buffer elution program. Protein samples were hydrolyzed for 24, 48 and 72 hr at 110° in vacuo in the presence of a small crystal of phenol (46). Half system was denatured as cysteic acid after oxidation of the protein samples with performic acid (50). For the determination of tryptophan and tyrosine, samples were hydrolyzed in 0.7 N HCl -15% mercaptoacetic acid (51). Molecular weight was determined by gel filtration on a Sephadex G-75 superfine column (1.6 x 400 cm). Sedimentation was estimated by ultracentrifugal gel electrophoresis in the presence of linear dodecyl sulfate (52). The samples were performed by atomic absorption spectrophotometry with a Perkin-Elmer model 350.

Antibody was prepared by injecting rabbits with 5 booster doses of purified superoxide dismutase, purified from bovine erythrocytes and this enzyme (91), in Freund's adjuvant (33). The rabbit serum was collected and then injected at 5% until the antibody titer was dissolved in water, dialyzed in 2M Tris, pH 7.6, and frozen in aliquots at -20°C (2.0 x 10 ml). Concentrated on a PM 10 Amicon membrane and then stored at -20°C.

Results

Purification of the Enzyme. Frozen flies were homogenized in a Biorad blender with four volumes of 5M potassium phosphate, 7.5 M, pH 7.2, to 1.0. The homogenate was clarified by centrifugation at 25,000 x g for one hour at 4°C. After passing the supernatant through a G-10 column, the column was washed with 2 M potassium phosphate buffer, pH 7.5, to 1.0. The precipitate, collected by centrifugation, was suspended in a minimal volume of chilled 5M potassium phosphat, 2.5 M, pH 7.2, and was dialyzed against several changes of the same buffer. The dialyzed solution was then treated with 0.25 volume of ethanol and 0.05 volume of chloroform. Although the superoxide dismutase without chloroform-ethanol treatment, it quickly lost its activity when sonicated by lyophilization procedure, effectively retarded for C57B16 dismutase [Fig. 1]. Therefore, the following modification was adopted to carry out the chromatography from Drosophila most effective. After centrifugation, the supernatant was centrifuged at 13,000 x g for 4°C, was removed by centrifugation and the supernatant (1.0 ml) was concentrated by lyophilization. The concentrated sample (1.2 ml) was dialyzed against water and several changes of 2.5 M potassium phosphate, 0.1 M, to 1.0, and the dialyzed solution was then sonicated for 1.5 ml. The activity of the sonicated solution was measured at 2.5°C in a Hitachi, model 800 spectrophotometer. The fractions were collected by centrifugation at 30°C and 30 ml of each fraction was assayed at 2.5°C in a Hitachi, model 800 spectrophotometer. The fractions were collected by centrifugation at 30°C and 30 ml of each fraction was assayed at 2.5°C in a Hitachi, model 800 spectrophotometer. The fractions were collected by centrifugation at 30°C and 30 ml of each fraction was assayed at 2.5°C in a Hitachi, model 800 spectrophotometer. 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Figure 2. Chromatography of SOD on Sephadex G-75 superfine column, as described in the text. The flow rate of about 40 ml per hour was obtained by using a peristaltic pump. Dashed and solid lines show protein elution profile and superoxide dismutase activity respectively.

preparation. Final purification of superoxide dismutase was accomplished by preparative electrophoresis on Sephadex G-75 superfine gel using a pH range of 3.5-6.8 amphotile. The enzyme was freed from amphotile by gel exclusion chromatography using a Sephadex G-75 column (2 x 55 cm). The results of this purification procedure are summarized in Table I.

Molecular weight and quaternary structure - Molecular weight was determined by gel filtration on a Sephadex G-75 superfine column (1.5 x 60 cm). The column was equilibrated with 0.05 M sodium chloride (0.002 M EDTA, pH 7.0) at 7°C. The exclusion weight standards: bovine serum albumin, 67,000; lactalbumin, 44,000; cytochrome a, 25,000; and catalase, 15,700. The molecular weight of the purified superoxide dismutase was found to be 32,000 by this method. Subunit molecular weight was determined by sodium dodecyl sulfate gel electrophoresis as previously described (32). The standard used to make a plot of log molecular weight versus mobility of protein band were: phosphorylase b (100,000), bovine serum albumin (67,000), ovalbumin (44,000), carbonic anhydrase (30,000), trypsin inhibitor (21,000) and lysozyme (14,300). The superoxide dismutase exhibited mobility consistent with a molecular weight of 18,000 to the absence of amperometrically overtlying bands on SDS gel electrophoresis. The subunit molecular weight of 18,000, which is in agreement with other workers (33) suggested that D. melanogaster is composed of two subunits of equal size held together by noncovalent interaction. No other bands were visible in these gels (see Fig. 3).

Figure 3. SDS gel electrophoresis. The gel in left has standard proteins as described in the text; the gel in right has purified Drosophila superoxide dismutase.

Table I. Purification of Superoxide Dismutase from D. melanogaster

| Fraction | Volume (ml) | Total Protein (mg) | Total Units | Specific Activity (units/mg) | Yield (%) | Purification |
|----------|-------------|--------------------|-------------|-----------------------------|-----------|-------------|
| Soluble extract* | 1,020 | 6,120 | 250,000 | 40.8 | 100 | 1 |
| 3x (NH4)2SO4 supernatant | 1,100 | 4,400 | 250,000 | 56.8 | 100 | 1.4 |
| 8x (NH4)2SO4 precipitation | 130 | 2,560 | 230,000 | 92 | 92 | 2 |
| treatment | | | | | | |
| DEAE column | 56 | 1,100 | 230,000 | 209 | 92 | 5 |
| Ethanol-chloroform* | | | | | | |
| Sephadex G-75 column | 120 | 68 | 80,000 | 1,170 | 32 | 29 |
| Isoelectrofocusing | 1 | 2 | 20,000 | 10,000* | 10 | 240 |

*The activity of each fraction was assayed against 50 μM potassium phosphate - 0.1 mM EDTA, pH 7.0, in an exhaustive way. The samples were then centrifuged to remove precipitants. After normalizing the sample volume, the assay for SOD was undertaken.

**When based upon absorbance at 245-254 nm, this specific activity was 4,000. In parallel studies when protein and activity of both Drosophila enzyme and bovine erythrocyte enzyme were measured, the specific activity of Drosophila superoxide dismutase was 1.3 times that of the bovine erythrocyte superoxide dismutase.

Table II. Amino Acid Analyses

| Amino Acid | Residue/10,000 | με |
|------------|----------------|----|
| Ala | 15.16 | - |
| Thr | 7.34 | 6.25 |
| Ser | 9.28 | 0.70 |
| His | 5.84 | - |
| Gly | 22 | - |
| Arg | 10.21 | 14.02 |
| Val | 13.13 | - |
| Ile | 4.22 | - |
| Met | 7.17 | - |
| Leu | 6.34 | 7.86 |
| Lys | 10 | 5.16 |
| Tyr | 3.16 | 7.28 |
| Phe | 4.95 | 0.88 |
| His | 2.96 | - |
| Lys | 4.95 | - |
| Arg | 3.05 | - |

A. Values shown are average of two independent analyses.
B. Values obtained from 24 hydrolyzates.
C. Values obtained from 24, 48 and 72 hr hydrolyzates and corrected for time-dependent losses by extrapolating to zero time.
D. The relative number of residues for each amino acid is reported per subunit molecule was calculated by assigning a residue value to give the best fit for an enzyme of 18,000.

Figure 4. Absorption spectrum of superoxide dismutase in the ultraviolet. The enzyme was at 450 μg per ml in 0.05 M potassium phosphate at pH 7.0.

Polyacrylamide gel electrophoresis - The crude soluble extract of D. melanogaster was analyzed by gel electrophoresis (40). As was the purified superoxide dismutase. Protein was visualized by staining with amperometric quenching, whereas superoxide dismutase activity was negatively stained by a photographic procedure as described (41). The crude extract of D. melanogaster color distained bands, but only one band of quench-sensitive superoxide dismutase activity was immediately evident. However, a separation of two enzyme bands (Lot. 3,3) of enzyme activity did appear in the gel by using of the brown colored proteins which were overlapping in the second, minor (15%) band of superoxide dismutase in the gels. The purified enzyme gave only one discernible band of protein, which coincided with the zone of enzymic activity.

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