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 scavenge O$_2^-$, 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 and 3% 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 have been isolated from several organisms; thus far, only three grossly dissimilar kinds have been found. The structural and functional relationships of these three classes of superoxide dismutase 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 dismutases 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 in 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*. We here report the thorough purification and characterization of one of two electrophoretically detectable allozymes of superoxide dismutase from *D. melanogaster* with the expectation that this will relate to the radioreistance 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 on 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, it is not inhibited by azide.

\[1\]
Portions of this paper (including "Materials and Methods," "Results," Figs. 1-4, and Tables 1 and 2) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 81M-717, cite authors, and include a check or money order for $4.00 per set of photocopies. Full size photo copies are also included in the microfilm edition of the Journal that is available from Waverly Press.
Superoxide Dismutase from Drosophila

8507

Superoxide dismutases, the Drosophila enzyme ("slow" electron donor) 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 enzymes of other eukaryotes (11–19) with respect to molecular weight, quaternary structure, metal prosthetic groups, and ultraviolet spectrum, but it does not cross-react with a rabbit antibody to the bovine erythrocyte enzyme. The D. melanogaster enzyme was stable to freezing and thawing and was homogeneous by the criteria of polyacrylamide gel electrophoresis and sodium dodecyl sulfate gel electrophoresis.

Since a 245-fold purification from the cell-free extract was homogeneous, and since the net recovery was 8%, we can estimate that this superoxide dismutase constituted 0.4% of the protein of the crude soluble extract. Drosophila superoxide dismutase is at least 1.5 times more active than the bovine erythrocyte enzyme. The evolutionary relationships among superoxide dismutases are obviously of great interest. The structural and functional differences among superoxide dismutases are obvious. Weisiger, R. A., and Fridovich, I. (1972) Biochemistry 11, 1264-1267.


Superoxide Dismutase from Drosophila

Materials and Methods

...
Superoxide Dismutase from Drosophila

preparation. Final purification of superoxide dismutase was accomplished by preparative electrofocusing on Sephacryl G-75 superfine gel using a pH range of 3.6–6.8 ampholine. The enzyme was free from ampholine by gel exclusion chromatography using a Sephacryl G-75 column (2 x 55 cm). The results of this purification procedure are summarized in Table 1.

Table 1. Purification of Superoxide Dismutase from D. melanogaster

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume (mL)</th>
<th>Total Protein (mg)</th>
<th>Total Units</th>
<th>Specific Activity (units/mg)</th>
<th>Yield (%)</th>
<th>Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soluble extract*</td>
<td>1,020</td>
<td>6,120</td>
<td>250,000</td>
<td>40.8</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>35% MgCl₂/treatment</td>
<td>1,100</td>
<td>6,400</td>
<td>250,000</td>
<td>54.8</td>
<td>100</td>
<td>1.4</td>
</tr>
<tr>
<td>HSS (MgCl₂) precipitation</td>
<td>130</td>
<td>2,500</td>
<td>230,000</td>
<td>92.2</td>
<td>92</td>
<td>2</td>
</tr>
<tr>
<td>DEAE column</td>
<td>15</td>
<td>1,100</td>
<td>230,000</td>
<td>92.2</td>
<td>92</td>
<td>5</td>
</tr>
<tr>
<td>Ethanol-chloroform</td>
<td>90</td>
<td>1,100</td>
<td>230,000</td>
<td>92.2</td>
<td>92</td>
<td>5</td>
</tr>
<tr>
<td>DEAE column</td>
<td>40</td>
<td>1,100</td>
<td>230,000</td>
<td>92.2</td>
<td>92</td>
<td>5</td>
</tr>
<tr>
<td>Sephacryl G-75 column</td>
<td>120</td>
<td>60</td>
<td>80,000</td>
<td>1,170</td>
<td>32</td>
<td>29</td>
</tr>
<tr>
<td>Isoelectrofocusing</td>
<td>1</td>
<td>2</td>
<td>20,000</td>
<td>10,000**</td>
<td>32</td>
<td>29</td>
</tr>
</tbody>
</table>

*The specific activity of each fraction was estimated against 0.5% potassium phosphate, pH 7.4, in an exhaustive way. The samples were then centrifuged to remove precipitates. After normalizing the sample volume, the assay for superoxide dismutase was undertaken.

**When based on absorbance at 200-225 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 1.5 times more than the bovine erythrocyte superoxide dismutase.

An amino acid composition and serological cross-reactivity (Table 2) presents the amino acid composition of D. melanogaster superoxide dismutase. In comparison with the amino acid compositions reported for several superoxide dismutase enzymes, D. melanogaster superoxide dismutase exhibits notable differences which suggest differences in structure which might be related to protein specificity. Therefore, the cross-reactivity of D. melanogaster superoxide dismutase was tested by a microcrystalline precipitin method (33) with rabbit antibodies to bovine erythrocyte superoxide dismutase. As expected, no reaction occurred with specific antibodies, yet none of them cross-reacted with the D. melanogaster enzyme.

Materials and methods - Atomic absorption spectrophotometry indicated 2.11 g/mL of Ca** and 0.5 g/mL of Mg** per 20,000 g of this superoxide dismutase. As described previously, the Ca**2+ superoxide dismutase in general were more sensitive to cyanide (55) and were less sensitive to arsine (51) than the corresponding iron or Mn superoxide dismutases. The D. melanogaster enzyme behaved like the other Ca**2+ superoxide dismutases in this respect. Thus, the cyanide inhibited 25% of this superoxide dismutase activity when measured by cyanide inhibition of respiratory chain-linked superoxide generation (51). Similarly, it is possible to block the oxidation of cytochrome c by CN- or CO-generating buffer (47). Arseni of 0.01 M inhibited the D. melanogaster enzyme by 15 and 35%, respectively.

[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.]

[Figure 4: Absorption spectrum of superoxide dismutase in the ultraviolet. The enzyme was at 450 μg of protein in 0.5% potassium phosphate at pH 8.0.]

Table 2. Amino Acid Analysis

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>α-Residue/10,000</th>
<th>μg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ala</td>
<td>15.11</td>
<td>5</td>
</tr>
<tr>
<td>Thr</td>
<td>7.94</td>
<td>5</td>
</tr>
<tr>
<td>Gly</td>
<td>9.26</td>
<td>5</td>
</tr>
<tr>
<td>Ser</td>
<td>5.42</td>
<td>5</td>
</tr>
<tr>
<td>Val</td>
<td>15.11</td>
<td>5</td>
</tr>
<tr>
<td>Ile</td>
<td>22</td>
<td>5</td>
</tr>
<tr>
<td>Leu</td>
<td>10.21</td>
<td>5</td>
</tr>
<tr>
<td>Lys</td>
<td>13.13</td>
<td>5</td>
</tr>
<tr>
<td>Phe</td>
<td>4.72</td>
<td>5</td>
</tr>
<tr>
<td>Met</td>
<td>7.17</td>
<td>5</td>
</tr>
<tr>
<td>Trp</td>
<td>3.64</td>
<td>5</td>
</tr>
<tr>
<td>Tyr</td>
<td>1.18</td>
<td>5</td>
</tr>
<tr>
<td>His</td>
<td>7.26</td>
<td>5</td>
</tr>
<tr>
<td>Asn</td>
<td>9.45</td>
<td>5</td>
</tr>
<tr>
<td>Arg</td>
<td>3.05</td>
<td>5</td>
</tr>
</tbody>
</table>

a. Values shown are average of two independent analyses.
b. Values obtained from 24 hydrolyzate.
c. Values obtained from 24, 48 and 72 hr hydrolyzate, and corrected for time-dependent losses by extrapolating to zero time.
d. The relative number of residues for each amino acid per subunit molecule was calculated by assigning a. The number of residues that gave the best fit for an enzyme of 18,000.

[Figure 5: Absorption spectrum of superoxide dismutase. The enzyme was at 450 μg per ml in 0.05% potassium phosphate at pH 8.0.]

**Sodium dodecyl sulfate gel electrophoresis - The crude soluble extract of D. melanogaster was analyzed by gel electrophoresis (46). As was the purified superoxide dismutase, protein was visualized by staining with Coomassie brilliant blue, whereas superoxide dismutase activity was negatively stained by a photochemical procedure as described (47). The crude extract of D. melanogaster color displayed several bands, but only one band of cyanide-sensitive superoxide dismutase activity was immediately evident. However, a simple in immunoelectrophoretic assay (22) of this crude enzyme disclosed other bands which were also cyanide-sensitive superoxide dismutase activity. In order to give the best fit for the enzyme of 18,000.