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# Superoxide dismutase activity in the cochlea

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It was found that superoxide dismutase (SOD) exhibits a higher specific activity in a fraction of the cochlea that contains the organ of Corti than in most other neural tissues. SOD may comprise as much as 4.3% of the soluble protein in this fraction. Approximately 74% of this SOD is cyanide-inhibitable, indicating that it is the Cu/Zn isoenzyme usually found in the cytoplasm. SOD activity was also found in a fraction containing the stria vascularis. Two enzymes that regulate peroxide concentrations, catalase and glutathione peroxidase, were also prominent in both of these fractions.

Key words: cochlear biochemistry; superoxide dismutase; glutathione peroxidase; catalase.

## Introduction

Relatively little is known about the biochemistry of the inner ear, largely because of the difficulty in obtaining sufficient quantities of tissue for study. Nonetheless, it is known that aerobic oxidations occur at high rates in hair cells and in the stria vascularis [4,17,18,20,25,34]. For this reason, active oxygen species, such as the superoxide anion ($O_2^-$) or peroxides, may be generated at levels sufficiently high to require detoxification. By extension, events that increase aerobic rates in the hair cells or the stria may cause active oxygen species to reach deleterious concentrations. Such events might include exposure to high noise levels. The present study concerns measurements in the cochlea of enzymes that are known for their ability to detoxify active oxygen species. These enzymes are superoxide dismutase (SOD), glutathione peroxidase and catalase.

Although aerobic oxidation is essential to life, the thermodynamics of the reduction of oxygen to water favors a univalent pathway that involves dangerously reactive species, including the superoxide anion and peroxides [11]. Superoxide free radicals are capable of inactivating viruses, killing cells and damaging membranes [10,22]. Peroxides are generally less reactive, although in the presence of transition metal ions, they can form extremely reactive radicals that can attack both aromatic and aliphatic compounds [16]. It is thought that the greatest danger posed by these

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two species is their mutual interaction [15,22,23,30] to form hydroxyl radicals ($OH^{\cdot}$), which can attack DNA as well as membrane lipids and proteins. It is clear that mechanisms must exist that inactivate or eliminate toxic oxygen products in order that aerobic cells do not literally degrade themselves. In this regard, most oxidative enzymes that have evolved allow the divalent or tetravalent reduction of oxygen, thereby avoiding the release of toxic intermediates. These include flavins and copper-dependent or heme-dependent oxidases. For example, cytochrome oxidase accounts for the majority of oxygen consumed in respiring cells and avoids the release of either $O_2^{\cdot-}$ or $H_2O_2$. Nonetheless, there are biological reactions that unavoidably utilize a univalent pathway of oxygen reduction. These include certain enzymatic pathways (xanthine oxidase, aldehyde dehydrogenase, and dihydroorotic dehydrogenase as well as flavo-enzyme dehydrogenases) and spontaneous autoxidations of hydroquinones, leucoflavins, catecholamines, thiols, and tetrahydropterins. In addition, unspecified autoxidations occur within mitochondria and microsomes, which release $O_2^{\cdot-}$ [9,10,22,26]. The main source of peroxides has been found to be pathways utilizing the amino oxidase enzymes or the reaction of TPNH oxidase with phospholipids [7].

The enzymes superoxide dismutase (EC 1.15.1.1), glutathione peroxidase (EC 1.11.1.19) and catalase (EC 1.11.1.6) are involved in regulating concentrations of $O_2^{\cdot-}$ and peroxides. SOD catalyzes the conversion of $O_2^{\cdot-}$ to oxygen and $H_2O_2$. Catalase removes $H_2O_2$ by degrading it to oxygen and water. Glutathione peroxidase also catalyzes peroxide degradation and may utilize lipid peroxides as substrates. These enzymes are present in many aerobic tissues, and they have been assayed in the central nervous system and eye [6,7,12–14,19,21,23,30,32). However there is a lack of data concerning SOD and peroxidases in the inner ear. The present studies were done to determine the levels of these three enzymes in tissues of the inner ear, and to compare them to levels in the central nervous system, the retina and the lung.

## Materials and Methods

Animals were killed by ether anesthesia. Dissections of the guinea pig nervous system and peripheral auditory system were repeated five separate times. Twenty animals were used for each repetition. Each dissection was performed as follows. The bony wall of the cochlea was fractured away from the spiral organ with fine iridectomy scissors. The bone fragments were ground by hand with a glass pestle in order to free tissues adhering to this bony shell. Material prepared by this procedure is referred to as the lateral fraction. This fraction may include some bone contamination, and includes the spiral ligament and stria vascularis. Watchmaker's forceps were used to extract the stump of the auditory nerve from the internal meatus. The spiral organ was delicately separated from the temporal bone (modiolus) before homogenization. This fraction is referred to as the medial fraction. The medial fraction was modestly contaminated with bone from the modiolos and presumably unmyelinated portions of the auditory nerve. The medial fraction included the organ

of Corti, the basilar membrane and associated blood vessels. Excised tissues were placed in 0.05 M sodium phosphate buffer (pH 7.8) in an ice bath prior to fine dissection. This procedure assured a diffusion of blood out of the tissues. A lack of hemoglobin in assayed samples could be spectrophotometrically confirmed. Lungs were flushed with about 10 ml of saline by injection into the right ventricle of the heart after severing the descending aorta. This removed most red blood cells from the lungs. Tissue homogenates were prepared by grinding samples in glass homogenizers or with a Brinkman Instruments Polytron. Cochlear and retinal homogenates were prepared at 1:2 (w/v) ratios in 0.05 M sodium phosphate buffer (pH 7.8). Lung and brain homogenates were prepared at 1:10 (w/v) ratios. Tissue homogenates were centrifuged at 0–4°C for 15 min at $5000 \times g_{max}$. Supernatant fluids were assayed immediately for catalase or glutathione peroxidase. Superoxide dismutase assays were done on fluids dialyzed 12–16 h against 0.05 M sodium phosphate buffer (pH 7.8).

All spectrophotometric assays were done with a Varian model 219 spectrophotometer at 25°C. Glutathione peroxidase was assayed by the method of Paglia and Valentine [27], and reaction mixtures were stirred continuously. Catalase was assayed by the method of Beer and Sizer [2]. Superoxide dismutase was assayed by the method of Sun and Zigman [31]. Dialyzed supernatant fluids were assayed or diluted for assay as required. The SOD reaction mixture was stirred continuously, and the reaction rate was determined 5–6 min after the addition of epinephrine by measuring the rate of change in optical density at 320 nm. Using linear regression analysis, double-reciprocal plots were generated for percent inhibition of rate of change in optical density at 320 nm as a function of supernatant volume added per ml of reaction mixture. One unit of enzyme activity was defined as the supernatant volume that reduced by 50% the change in optical density at 320 nm. The assay for determining whether the SOD was cyanide-inhibited was by the method of Weisiger and Fridovich [33], using cytochrome *c* reduction by $O_2^-$ as a basis. Protein was assayed by the method of Bradford [3], using bovine plasma gamma-globulin as a standard (Bio-Rad Laboratories).

Polyacrylamide gel electrophoresis of samples was done using the method of Davis [5]. Protein bands in gels were visualized by staining with Coomassie brilliant blue. SOD bands in gels were visualized by the staining techniques of Beauchamp and Fridovich [1]. Destained gels were scanned using a gel scanner accessory attached to a Varian model 219 spectrophotometer.

## Results

The specific activity of SOD was determined in several gross neural and sensory tissues and in one nonneural tissue (the lung) of the guinea pig. The rank ordering of these activities was always as shown in Table I, with brainstem > cochlea > cerebellum > retina > cortex > lung. The specific activities of SOD within these tissues were comparable, whether albino or pigmented guinea pigs were used. Column 2 of Table I shows the relative SOD percentages based on the tissue having

TABLE I

SPECIFIC ACTIVITY OF SUPEROXIDE DISMUTASE (SOD) IN GUINEA PIG TISSUES AND ITS COMPARISON TO REGIONAL DISTRIBUTION IN RAT BRAIN

| Tissue | SOD specific activity * | % SOD specific activity re. | |
|---|---|---|---|
| | | guinea pig brainstem | rat medulla ** |
| Brainstem | 10.02 ± 0.79 | 100 | 100 |
| Cochlea | 8.78 ± 0.29 | 88 | – |
| Cerebellum | 8.60 ± 0.19 | 86 | 72 |
| Retina | 6.27 ± 0.64 | 63 | – |
| Cortex | 5.94 ± 0.41 | 59 | 52 |
| Lung | 2.93 ± 0.48 | 29 | – |

* Means and S.E. from duplicate determinations (usually, $n = 5$). One unit SOD activity is that quantity of enzyme which yields 50% inhibition of the epinephrine autoxidation reaction.

** From Thomas et al. [32].

the highest specific activity. These values are presented in comparison with the ranking of activities in the rat (column 3) as measured by Thomas et al. [32]. The relative activities for the three tissues that were assayed in common are quite similar. A single assay for SOD done with rat tissues correlated well with the results of Thomas et al. [32]. SOD specific activities in rat tissues were about 60% of those in analogous guinea pig tissues. However, the SOD specific activity in the rat's cochlea was comparable in our hands to the mean activity in the guinea pig's cochlea. This preliminary result suggests a disproportionately high SOD activity (by a factor of 2) in the rat's cochlea relative to other rat neural tissues.

Since SOD was present in the cochlear fraction, further localization of enzyme activity in, or associated with, particular inner ear structures was attempted. SOD specific activity in different tissues associated with the peripheral auditory system is shown in Table II. Since the temporal bone fraction has about half the SOD activity, and whereas SOD was not measurable in the auditory nerve fraction, any contamination of the medial fraction by these two tissues could have resulted only in the artifactual suppression (not enhancement) of its specific activity. These results indicate that the cochlea is prominent in its SOD specific activity (Table I) and that a considerable proportion of this enzymatic activity is derived from the medial fraction (Table II). The hypothesis that high levels of SOD may be present in the hair cells of the organ of Corti was particularly attractive since these cells are very active metabolically. An indirect test of this idea was made by assaying SOD levels in the organ of Corti fraction from 20 animals that had been treated with 200 mg/kg neomycin sulfate for 2 weeks and later demonstrated no Pryor reflex. Neomycin specifically destroys hair cells in the organ of Corti, and we felt that a sizable drop in SOD activity in that fraction could indicate high enzyme concentrations in these cells. SOD specific activity in extracts prepared from neomycin-treated animals was 18% lower than activities from control animals. Although these results suggest higher levels of SOD in hair cells than in other elements present in the medial fraction, the

TABLE II

REGIONAL DISTRIBUTION OF SUPEROXIDE DISMUTASE (SOD) WITHIN CERTAIN AUDITORY STRUCTURES IN GUINEA PIGS

| Region * | SOD specific activity ** |
|---|---|
| Medial fraction | 9.8 |
| Lateral fraction | n.m. |
| Auditory nerve | n.m. |
| Temporal bone | 5.0 |
| Cochlear nucleus | 6.7 |

n.m., none measurable.

* $n=3$.

** One unit of SOD activity is defined as that quantity of enzyme which yields 50% inhibition of the epinephrine autoxidation reaction.

magnitude of the depression was marginally significant, in view of a lack of supporting histological data.

A second confirmatory assay for SOD in the organ of Corti was done by using the polyacrylamide gel electrophoresis technique developed by Beauchamp and Fridovich [1]. Duplicate gels loaded with bovine SOD and homogenates prepared from the organ of Corti were electrophoresed and stained either with Coomassie brilliant blue to identify protein bands or with nitro blue tetrazolium to locate SOD regions. An SOD band found in gels containing organ of Corti samples matched the known SOD band found in gels loaded with purified bovine SOD ($M_r = 31\,000$). Moreover, gels loaded with extracts from the organ of Corti had two prominent protein bands, one of which matched the location of SOD on bovine SOD-loaded and electrophoresed gels (Fig. 1). The integrated area of the SOD peak on the gel scan shown in Fig. 1 indicates that about 15% of the soluble protein in the homogenate is SOD. A second estimate was made by extracting the Coomassie stain from the SOD band (in *n*-butanol) and photometrically determining (at 590 nm) that about 4.3% of the total dye in the gel was located in the SOD location. This roughly indicates that 4.3% of the soluble protein in the medial fraction was in the SOD location. Although this is less than suggested by the first prediction, it is nonetheless an extraordinary proportion.

A further examination of SOD in the extract from the medial fraction was made to determine what percentage of the activity was cyanide-inhibitable. The Cu/Zn cytoplasmic isoenzyme is inhibited by cyanide whereas the mitochondrial isoenzyme SOD is not [22,23]. The assay used was the acetylated cytochrome C reduction reaction (see Materials and Methods). About 74% of the SOD activity in the medial fraction was inhibited by 1 mM KCN. This percentage is consistent with the subcellular distribution of cytoplasmic versus mitochondrial SOD in the rat brain [32]. In addition, all SOD activity was abolished by placing samples in a boiling water bath for 30 min.

In view of the reportedly high rates of metabolic activity in the stria vascularis, it had seemed unusual that SOD activity was not measurable in the lateral fraction. In

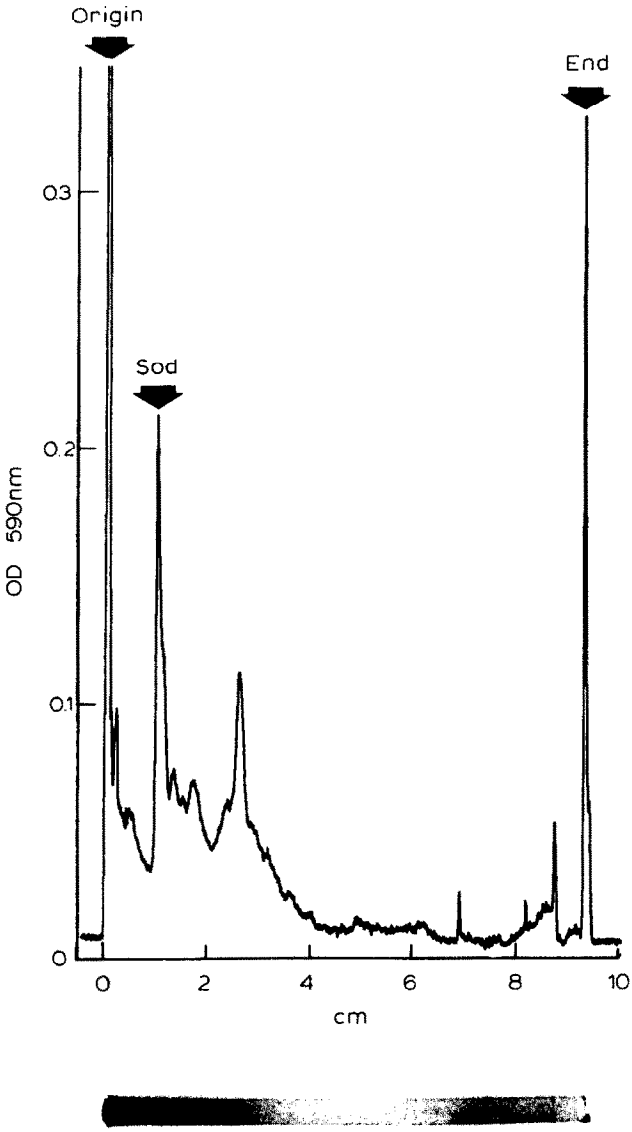


Fig. 1. Optical density scan of destained electrophoretic gel loaded with medial fraction extract.

order to examine whether the low protein content in the lateral fraction (averaging 0.52 mg/ml) was accountable, the number of cochleas was doubled for one assay. This strategy increased the protein concentration while maintaining the supernatant volume required by the assay. In this manner an SOD-specific activity in the lateral fraction was finally obtained and was found to be 22.2 units per mg protein. Parenthetically, it is noted that a reliable SOD activity was never obtainable in the

eighth nerve fraction, even after concentrating the sample by this method. It is apparent from examining Tables I and II that the measurement of activity in the lateral fraction reported above is more than twice the activity found in any other tissue, and it is seven times higher than the activity measured in the lung. While Table II possibly should have been modified accordingly, the data regarding lateral fractions were too limited to justify the change. However, Fig. 2 was modified in keeping with this one assay. We have restricted all data in tables in this paper to experiments using 20 animals, but Fig. 2 reflects SOD activity in the lateral fraction when 40 animals were used.

Both catalase and glutathione peroxidase regulate the concentration of a second

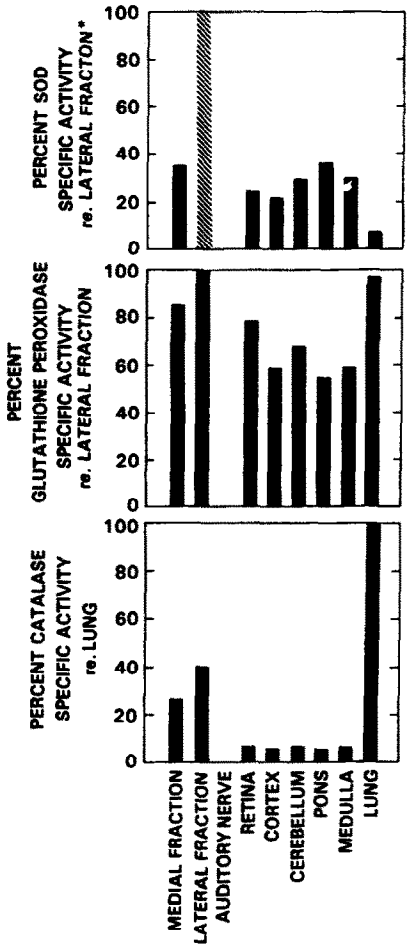


Fig. 2. Preponderant distribution of superoxide dismutase (SOD), glutathione peroxidase, and catalase in some cochlear, neural and lung tissues of guinea pig.

* SOD specific activity for the lateral fraction was assayed under conditions different from those normally used (see text).

TABLE III

COMPARISON OF TISSUE DISTRIBUTION OF SUPEROXIDE DISMUTASE (SOD), GLUTATHIONE PEROXIDASE AND CATALASE IN GUINEA PIG, WITH SPECIAL EMPHASIS ON DISCRETE COCHLEAR FRACTIONS

| Tissue | SOD specific activity * | Glutathione peroxidase specific activity ** | Catalase specific activity *** |
|---|---|---|---|
| Medial fraction | 8.0 (1) | 18.8 (1) | 12.7 (2) |
| Lateral fraction | c.n.m. (3) | 21.9 (1) | 19.5 (2) |
| Auditory nerve | c.n.m. (3) | c.n.m. (1) | c.n.m. (1) |
| Retina | 5.6 (1) | 17.2 (3) | 3.1 (2) |
| Cortex | 4.8 (1) | 13.0 (1) | 2.9 (1) |
| Cerebellum | 7.5 (1) | 15.0 (1) | 3.4 (1) |
| Pons | 8.2 (1) | 12.0 (1) | 3.0 (1) |
| Medulla | 7.1 (1) | 13.0 (1) | 3.4 (1) |
| Lung | 2.0 (1) | 21.3 (2) | 47.3 (2) |

c.n.m., could not be measured.

* One unit of SOD activity is that quantity of enzyme which yields 50% inhibition of the epinephrine autoxidation reaction.

** One unit of glutathione peroxidase activity is that quantity of enzyme which catalyzes the breakdown of 1 nmol $H_2O_2$ per minute.

*** One unit of catalase activity is that quantity of enzyme which catalyzes the breakdown of 1 $\mu$mol $H_2O_2$ per minute.

active oxygen species, hydrogen peroxide. In view of the relatively high SOD-specific activity in the cochlea, it seemed appropriate to also assay levels of these two enzymes. Homogenates prepared from several tissues were assayed for SOD, catalase and glutathione peroxidase. The results are presented in Table III. The parenthesized numbers in Table III indicate the number of assays performed in generating each value shown. When $n > 1$, the specific activity shown is an average. Although, as we have pointed out, protein levels in the lateral and auditory nerve fractions were too low to permit SOD assays, the catalase and glutathione peroxidase activities were always measurable. Indeed, the glutathione peroxidase specific activity was higher in the lateral extract than in any other tissues examined. Moreover, while catalase activity is so low in neural tissue that for years it was considered to be absent in the brain, it is clearly a significant component of both strial and organ of Corti extracts. These relationships and that of SOD activity are shown in Fig. 2. SOD activity in the lateral fraction shown in Fig. 2 was derived from the single assay using tissue from 80 cochleas.

## Discussion

The present data indicate that three enzyme systems that specifically inactivate toxic oxygen species are prominent in the cochlea, the medial fraction containing the organ of Corti and in the lateral fraction containing the stria vascularis. SOD activity in the medial fraction was generally higher than in other neural or sensory

tissues examined (with exception of the pons). And, as we have indicated, SOD activity in the lateral fraction may be twice as high as in other neural tissues, including the organ of Corti. Even more remarkable, perhaps, were the findings regarding the peroxide-reducing enzymes. First, catalase levels in the medial fraction and the lateral fraction were about four and seven times more active, respectively, than in other neural or sensory tissues (although less than half as active as in the lung). Second, glutathione peroxidase levels in both the medial fraction and the lateral fraction were higher than in other neural tissues, whereas the activity in the lateral fraction was the highest measured in any tissue including the lung (although other investigators have referred to the lung levels as being only 'intermediate' [24] when compared to other extraneural tissues). Although we have concentrated on SOD in this paper, particularly within the medial fraction (due to the greater availability of tissue and protein in this fraction), it is clear that the lateral fraction, perhaps to an even greater extent, is remarkably equipped to deal with active oxygen species.

Soluble SOD in the cochlea is heat labile (indicating that the exhibited activity is an enzymatic function), 74% inhibitable by cyanide, and migrates in an electrophoresed disc gel to the same position as purified bovine erythrocyte SOD having a molecular weight of 31000. These observations reflect the general characteristics of the cytoplasmically located Cu/Zn isoenzyme of SOD. An interesting observation was that 4.3% of the soluble proteins in the organ of Corti extracts were in the SOD location on our disc gels. There may be additional proteins that migrate to the same position as SOD in polyacrylamide gels. Indeed, SOD enzyme activity was mistakenly ascribed to carbonic anhydrase due to contamination of the anhydrase by SOD [21]. The two proteins have similar molecular weights, and about 1% of soluble protein in the stria vascularis is carbonic anhydrase. Although the organ of Corti appears to lack carbonic anhydrase [8], our medial fraction included other elements. Thus either carbonic anhydrase or some other soluble proteins could be present in the prominent SOD band in our gels. Therefore, the 4.3% value must be viewed as a maximum SOD content in the soluble protein fraction.

It is striking that the medial fraction containing the organ of Corti and the lateral fraction containing the stria vascularis are especially rich in these three enzymes that detoxify active oxygen species. By inference, the data suggest that these tissues, especially the lateral fraction, generate toxic oxygen species in their metabolic reactions. Nonetheless, the source of these species must be sought in fairly unusual reactions since most aerobic pathways avoid the univalent reduction of oxygen. Those reactions that may be significant sources of the superoxide radical or $H_2O_2$ were discussed in the introduction. Unfortunately the predominance of such reactions has never been specifically examined in cochlear tissues. It is important to note that those neuronal molecules generally held to be the most susceptible to superoxide anion attack are the sulfhydryl-containing enzymes or the sulfhydryl-dependent enzymes (e.g., acetylcholinesterase, adenyl cyclase, (Na + K)-ATPase, or glutamic acid decarboxylase) as well as catecholamines and phospholipids in general. Peroxidative attack is generally targeted on phospholipids.

It has recently been shown [29] that a known free radical scavenger, WR 2721.

acts prophylactically against the ototoxic effects of the antibiotic kanamycin. That finding suggested that kanamycin ototoxicity occurs either through the inactivation of an endogenous free radical trap or that kanamycin is metabolized by the inner ear in a manner that generates free radicals. Although we are unable at present to support either of these hypotheses, it is reasonable to believe that WR 2721 acted in either an additive or synergistic fashion to enhance the considerable enzymatic protection against free radicals and active oxygen available in the cochlea.

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