The activities of glutathione peroxidase (GSH-Px) and glutathione-S-transferase (GSH-S-tase) were investigated in adult and premature human retinas. The measurements were done in the vascular and avascular regions of premature retinas at gestational age of 22–33 weeks and in the central, mid-peripheral, and far peripheral regions of mature retinas from the age of 1 month to 73 years. Among these, reduced glutathione (GSH), together with glutathione peroxidase (GSH-Px; E.C. 1.11.1.9), plays an important role by removing both hydrogen peroxide (H₂O₂) and possibly lipid hydroperoxides. Furthermore, GSH neutralizes electrophilic sites of several compounds which might attack cell macromolecules through covalent binding or by initiating lipid peroxidation. Such conjugation reactions are catalyzed by glutathione-S-transferase (GSH-S-tase; E.C.2.5.1.18).¹ The present study was undertaken to (1) compare the level of GSH-Px and GSH-S-tase from the vascular and avascular regions of premature retina to the central and peripheral regions of adult retina; (2) determine the variation of GSH-Px and GSH-S-tase activities with age in premature and mature retinas; and (3) establish the regional distribution of these enzymes in the retina.

Vitamin E is an efficient free radical terminator and is found in relatively large quantities in adult human retinas.² However, as demonstrated by Nielsen et al (1987), premature human retinas have about 10% of the adult levels of vitamin E. Several enzymes and other non-enzymatic constituents are involved in maintaining a reducing environment in the cell. Among these, reduced glutathione (GSH), together with glutathione peroxidase (GSH-Px; E.C.1.11.1.9), plays an important role by removing both hydrogen peroxide (H₂O₂) and possibly lipid hydroperoxides. Furthermore, GSH neutralizes electrophilic sites of several compounds which might attack cell macromolecules through covalent binding or by initiating lipid peroxidation. Such conjugation reactions are catalyzed by glutathione-S-transferase (GSH-S-tase; E.C.2.5.1.18).³ The present study was undertaken to (1) compare the level of GSH-Px and GSH-S-tase from the vascular and avascular regions of premature retina to the central and peripheral regions of adult retina; (2) determine the variation of GSH-Px and GSH-S-tase activities with age in premature and mature retinas; and (3) establish the regional distribution of these enzymes in the retina.

Materials and Methods. Retinas were obtained from premature infants who had been treated with the routine preterm protocol used in level III neonatal intensive care units at Baylor College of Medicine-affiliated hospitals. Seven eyes came from premature infants maintained in an atmosphere of elevated oxygen are at high risk for developing retinopathy of prematurity (ROP).¹ Since oxidation of retinal lipids leads to retinal degeneration,² it is likely that ROP may result from an oxidative insult to the developing retina.³ The living cell has developed several defense mechanisms against oxidative attacks.
mature infants (22–33 weeks gestational age) who survived 12 hr or less and were not supplemented with vitamin E. Seven eyes (22–32 weeks gestational age) came from premature infants who survived 2.5–42 days and were supplemented with a standard protocol of vitamin E and oxygen whenever needed. Supplemented infants received 1 IM injections of 15, 10, 10, and 10 mg/kg of dl-α-tocopherol (Ephynal, Hoffmann-La Roche, Des Plaines, IL) on days 1, 2, 4, and 6 of life or concomitantly with oral dl-α-tocopheryl acetate (Aquasol E, USV Pharmaceuticals, now Armour Pharmaceuticals, Tarrytown, NY) in medium chain triglyceride (100 mg/kg/day). Sixteen mature retinas (age 1 month to 73 years) were obtained from eyes donated to the Lions Eyes of Texas and 6 of life or concomitantly with oral dl-α-tocopherol (Aquasol E, USV Pharmaceuticals, now Armour Pharmaceuticals, Tarrytown, NY) in medium chain triglyceride (100 mg/kg/day). Sixteen mature retinas (age 1 month to 73 years) were obtained from eyes donated to the Lions Eyes of Texas Eye Bank. Eyes were removed within 3 hr postmortem, transported to the lab on ice, and dissected immediately. After removing the vitreous, retinas were teased away from the eye cup under the following medium: 140 mM NaCl, 2.7 mM KCl, 11 mM dextrose, 0.05 mM diethylenetriamine pentaacetic acid (DTPA), and 10 mM HEPES, pH 7.4. In order to prevent oxidation, all dissections and enzyme preparations were conducted under a stream of argon, and all solutions and solvents were purged with argon.

Portions of retina were taken from the central (vascular) and far peripheral (avascular) regions of the premature infant retina. Portions were also taken from the posterior pole (central), the equatorial area (mid-peripheral), and anterior to the equatorial area (peripheral) of the mature retinas.

Samples were homogenized in 600 μl of 50 mM sodium phosphate buffer (pH 7.0) using a motor-driven Teflon pestle with a 0.004–0.006 mm clearance at approximately 1600 rpm. The resulting tissue homogenate was centrifuged at 100,000g for 1 hr at 4°C. GSH-Px, GSH-S-tase, and cellular protein were measured in the supernatant. The activities of the enzymes were measured in the pellet in order to determine if the homogenization procedure was sufficient to release all of the cellular enzymes and soluble proteins. This was done by resuspending the pellet in 4% Triton X-100 and vortexing for 2 min. After an additional 10 min of incubation at room temperature in the presence of detergent, the suspension was centrifuged at 100,000g for 1 hr and the activity was measured in the supernatant. Greater than 95% of both enzymes was recovered in the supernatant, while less than 5% remained in the pellet, indicating that the homogenization and centrifugation procedures were sufficient to release most of the soluble enzymes.

**Determination of contamination by blood:** Blood contaminations in the supernatant fractions from premature and mature retinas were determined by measuring the hemoglobin content of these fractions (by the absorption at the Soret band). Relating this value to the hemoglobin content of a known volume of blood reflects the degree of contamination.

**Enzyme assays:** All reagents were dissolved in argon-purged, deionized water. GSH-Px activity was measured by a modification of the procedure described by Paglia and Valentine in which oxidation of glutathione was coupled to NADPH oxidation by glutathione reductase. The standard reaction mixture consisted of 5.0 mM glutathione, 0.6 mM cumene hydroperoxide, 50 mM sodium phosphate buffer (pH 7.0), 0.1 mM EDTA, an excess of glutathione reductase (1 unit/ml), 0.25 mM NADPH, and an appropriate amount of supernatant, which was approximately 50 μg of soluble retinal protein. The final volume of the reaction mixture was 1.0 ml. The reaction was initiated by the addition of cumene hydroperoxide and the oxidation of NADPH was followed spectrophotometrically at 340 nm.

GSH-S-tase activity was determined by measuring the conjugation of GSH with 1-chloro-2,4-dinitrobenzene (CDNB) as described by Habig et al. One milliliter of the assay mixture contains 1 mM CDNB, 1 mM GSH, 100 mM sodium phosphate buffer (pH 6.5), 1.0 mM EDTA, and an appropriate amount of retina supernatant. The reaction was initiated by the addition of CDNB and the formation of 5-2,4-dinitrophenylglutathione was followed spectrophotometrically at 340 nm. Whenever possible during pipetting, incubation, and storage, argon was blown gently over the reagent and the tubes were capped.

The non-enzymatic (spontaneous) oxidation of glutathione was determined by simultaneous assay of a system identical to the enzymatic one for both GSH-Px and GSH-S-tase assays, except for replacement of the homogenate by an equal volume of the buffer. The spontaneous reaction rate was subtracted from that determined for the sample. Boiled homogenate showed no difference from the spontaneous reaction. The protein concentration of the supernatant was determined in duplicate by the method of Lowry et al, using bovine serum albumin as a standard.

On the basis of the activities of these enzymes in the blood and the extent of contamination of the supernatant fractions, it was found that at most 2–5% of the enzyme activities of these fractions was due to blood contamination. Table 1 and Figure 1 were not corrected for this contamination.

**Results.** The specific activities of GSH-Px and GSH-S-tase for all retinas examined are shown in Table 1. The specific activity of GSH-Px in the premature retina was twice the activity of the mature retina from both the central and peripheral regions. A significant elevation in the levels of GSH-Px and
Table 1. Distribution of GSH-Px and GSH-S-tase in premature and mature human retina

<table>
<thead>
<tr>
<th>Donor tissue</th>
<th>Retinal region</th>
<th>GSH-Px*</th>
<th>GSH-S-tase*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>&lt;95.8±21.2f n=13</td>
<td>626.0±182.0f n=13</td>
</tr>
<tr>
<td>NPremature retina</td>
<td>Vascular</td>
<td>127.6±19.6f n=13</td>
<td>616.0±186.0f n=13</td>
</tr>
<tr>
<td></td>
<td>Avascular</td>
<td>45.6±16.4 n=16</td>
<td>448.1±185.3 n=16</td>
</tr>
<tr>
<td>Mature retina</td>
<td>Central</td>
<td>51.6±15.4 n=14</td>
<td>521.6±170.3 n=14</td>
</tr>
<tr>
<td></td>
<td>Mid-peripheral</td>
<td>56.6±20.8 n=16</td>
<td>531.8±162.8 n=16</td>
</tr>
</tbody>
</table>

* Specific activities of GSH-Px and GSH-S-tase are expressed in nmole GSH oxidized/min/mg cytoplasmic protein.
† Significantly higher than mature central retina at P < 0.0005.
‡ Significantly higher than the vascular region of premature retina at P < 0.0005.
£ Significantly higher than mature peripheral retina at P < 0.0005.
† Significant higher than mature central retina at P < 0.005.

GSH-S-tase (52.4% and 28.4%, respectively) was found in the central region of premature retinas compared to the same region of mature retinas. Moreover, both enzymes showed greater activities in the far peripheral region of premature retinas compared to mature retinas; however, only the GSH-Px increase was significant (55.6%). Comparing the two regions from premature retinas alone, we have found a significantly greater GSH-Px activity in the avascular region (24.9%), while GSH-S-tase showed no regional difference.

The specific activities of GSH-Px and GSH-S-tase in two regions of mature and premature retinas are presented in Figure 1 as a function of postnatal and gestational age. There is no correlation between the activity of either enzyme and gestational age in premature retinas. However, there was a significant decrease in GSH-Px as a function of postnatal age in both central and peripheral retina (P < 0.02, P < 0.001, respectively). There were no age-related changes in GSH-S-tase activities. There is a significant increase (P < 0.01) in the activities of GSH-S-tase in the avascular region of premature retinas from infants who survived longer than 24 hr and received vitamin E supplements (dotted lines in Fig. 1), while no differences were observed in the vascular region.

Discussion. It has been known for a long time that vitamin E functions by terminating lipid-free radicals.
at the site of their formation in the membrane, while GSH-Px and GSH-S-tase are involved in the decomposition of hydroperoxides and other xenobiotic factors. Since premature retinas are deficient in vitamin E in both the vascular and the avascular regions, the early antioxidant protection is provided in part by GSH-Px and GSH-S-tase, whose activities are substantially greater than those present in mature retinas. The levels of vitamin C are also greater in premature retinas than in adults, suggesting a role for this water soluble antioxidant in protecting the premature retina. However, with an oxidant challenge, GSH-Px, GSH-tase and ascorbate are not able to fully protect the premature retina from oxidative damage.

Supplementation with vitamin E leads to an increase in vitamin E in both vascular and avascular regions in infants greater than 27 weeks gestational age. This is the same minimum age where supplementation decreases the severity of ROP. Thus, it appears that the retinas of premature infants less than 27 weeks gestational age have less antioxidant protection than adults and, when challenged with oxygen, are vulnerable to development of ROP. Since vitamin E is the only antioxidant measured thus far that is lower in premature retinas as compared to adults, it seems likely that this compound plays a major role in providing antioxidant protection in these developing retinas.

**Key words:** glutathione peroxidase, glutathione-S-transferase, retinopathy of prematurity, premature infants, retina

**Acknowledgments.** We thank The Lions Eyes of Texas Eye Bank for supplying mature eyes and Dr. Frank Kretzer for obtaining premature infant eyes. We are grateful to Dr. Laurence Rapp and Dr. John Penn for their critical review of this manuscript. Our thanks go to Janice Jackson for typing the manuscript.

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