Studies on the retina and the pigment epithelium in hereditary canine ceroid lipofuscinosis

II. The subcellular distribution of lysosomal hydrolases and other enzymes

A. N. Siakotos,* D. Armstrong,* N. Koppang, and E. Connole

Observations on the progressive course of retinal degeneration in canine ceroid lipofuscinosis (CCL) showed dramatic changes in enzyme activity and subcellular compartmentation. Thus, in affected animals, a new particle containing high levels of hexosaminidase and galactosidase was found in fractions lighter than seen in controls. In the later stages of the disease and in normal aging, a progressive increase in dense fractions with high titer of acid lipase and acid phosphatase was observed. Peroxidase was found predominantly in the heavier fractions (1.24 to 1.28 gm/ml) and was lower than normal in affected retina and RPE. These fractions were located above the pigment granule fraction. Changes of peroxidase activity in the pigment granules were age dependent in controls, but a decrease of similar magnitude occurred much earlier in affected dogs. The accumulation of large numbers of dense bodies in the retina and RPE in CCL may indicate an impairment of intracellular digestive mechanisms. The early and marked reduction of peroxidase activity in affected dogs is an important indicator for major changes in the biochemistry of the entire eye in this disease. Therefore the initial pathogenic event seems to be the inability of affected cells to cope with peroxidative damage at an early stage, followed by an exaggerated attempt by the cells to digest accumulating lipopigments.

Key words: retinal degeneration, canine ceroid lipofuscinosis, enzyme, subcellular, peroxidase, peroxidative damage, lipopigments

In a number of inherited retinal degenerations, the pathogenic events leading to the loss of function in the retina, in particular the retinal pigment epithelium (RPE), are uncertain. In one retinal disorder in the rat (retinal dystrophy), the RPE appears to stop phagocytizing the rod outer segment (ROS) discs,1-3 but the same RPE does continue to phagocytize artificial substrates such as colloidal carbon.4,5 Custer and Bok6 have proposed that the loss of phagocytic capability in the RPE may be due to the loss of ROS membrane-recognition factor in the RCS rat. So, clearly there appears to be an unknown defect in the phagocytic mechanism of the RPE in this one form of retinal disease which
develops early in the life of the rat (30 to 60 days).

One of the consequences of impaired phagocytosis of ROS in the dystrophic rat is the impaired transfer of retinal to the RPE as ROS debris accumulates at the RPE-photoreceptor junction. Pathogenic mechanisms in the other inherited retinal degenerative disorders may be a result of malfunctions of the intracellular lysosomal digestive processes as a result of deficiencies in one or more lysosomal hydrolases. Another source of pathogenic changes may include peroxidative damage to subcellular membranes, specifically membrane-bound enzymes and membrane-specific ion barriers.

Koppang found marked accumulation of dense inclusion bodies in the central nervous system in canine ceroid lipofuscinosis (CCL). This accumulation of autofluorescent material, termed ceroid, is thought to represent the end stage of lipid peroxidation. Peroxidation of lipid-containing membranes does not seem to affect phagocytosis, since Neville et al. have observed a normal population of phagosomes in the RPE of dogs affected with CCL. However, the accumulation of large numbers of abnormal dense inclusion bodies may indicate an impairment of the intracellular digestive mechanisms.

Much attention has been focused on the functional role of lysosomes in the intracellular digestion processes of the normal and diseased RPE. The evidence for the active participation of this unique subcellular organelle, the lysosome, in retinal degenerations is confined to studies in the histochemistry, morphology, or biochemistry of whole tissues. In a previous report we have established that significant changes in lysosomal and other enzymes occur in whole retina and purified RPE cells in hereditary CCL, in which retinal degeneration is also observed.

In this report we have extended our previous study to localize the subcellular site of the observed changes in hydrolytic, peroxidative, and catalytic enzymes. The recent development of techniques for the fractionation of small samples of RPE provided an approach to the separation of individual subcellular fractions of the RPE in normal and affected animals with retinal degenerative diseases. In this study, the results of fractionating as little as one eye from both affected and unaffected animals in hereditary ceroid storage disease in the English setter dog are presented, and the observations on the progressive course of retinal degeneration in this disease are discussed.

**Methods and material**

Enzyme-grade sucrose was obtained from the Schwarz-Mann Co., Orangeburg, N. Y. The 4-methylumbelliferyl (4-MU) substrates were purchased from Research Products International Corp., Elk Grove Village, Ill. All other chemical reagents were obtained from the usual commercial sources.

**Source of English setters.** English setters affected and not affected with hereditary CCL were bred in Dr. Koppang's kennel in Norway. For the early stage of this disease (10 months), one eye was taken from a single affected dog and its unaffected littermate (Table I). In the older unaffected animals taken for a pooled sample, one eye was taken from the 14-month-old animal (A8) and pooled with two eyes of the affected dog (17A). In the experiment with older animals, three affected and three unaffected controls were sacrificed by electric shock. All animals were acclimated in the animal facility for at least 1 week prior to sacrifice. The room was maintained at 25°C, with a controlled 12 hr light cycle. In order to control the light-stimulated shedding of ROS's, all animals in this study were sacrificed 2 hr after the lights were turned on. This precaution was taken even though light-induced ROS shedding has been shown for the rat but not the dog.

**Preparation of retina and RPE cells.** The retina was dissected free of the underlying RPE as described earlier. Table I. Age and status of affected and control English setters

<table>
<thead>
<tr>
<th>Age at sacrifice (months)</th>
<th>Eyes in pooled sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>A3</td>
<td>10</td>
</tr>
<tr>
<td>A5</td>
<td>14</td>
</tr>
<tr>
<td>17A</td>
<td>22</td>
</tr>
<tr>
<td>A5</td>
<td>10</td>
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<td>A5</td>
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<td>A5</td>
<td>14</td>
</tr>
<tr>
<td>17A</td>
<td>22</td>
</tr>
</tbody>
</table>

**Table I. Age and status of affected and control English setters**

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The whole homogenate is prepared as described in Methods.

Fractionation of retina and RPE cells. The fractionation scheme for canine retina and RPE samples is given in Fig. 1. The purified retina and RPE cells were homogenized briefly with a Potter-Elvehjem homogenizer in 2 ml of 0.32M sucrose per eye and then transferred to a cell disruption bomb (No. 881455; Kontes Glass Co., Vineland, N. J.) The cell suspensions were treated with 250 psi nitrogen for 10 min and discharged into a centrifuge tube. The bomb was rinsed with additional sucrose, and the washes were combined with the disrupted cell suspensions. Next, the whole homogenate was centrifuged at 4,400 × g for 10 min in a Sorvall RC-3 centrifuge (HG-4 rotor) yielding a pellet (P1) and supernatant (S1). The P1 pellet was resuspended with a Potter-Elvehjem homogenizer in 1 to 2 ml of 0.32M sucrose and recentrifuged at 4,400 × g for 10 min in the Sorvall RC-3 centrifuge, yielding a supernatant (S2) and the 4,400 g pellet (P2). The supernatants S1 and S2 were pooled to make the 4,400 g supernatant and concentrated with dry Sephadex G-25 (coarse) to a volume of 1.0 ml. Then the concentrated supernatant was layered on a continuous linear gradient of 0.32M to 1.6M sucrose in the first experiment with the 10-month-old dogs, but a 1.0 ml cushion of 2M sucrose was added to the 14 ml polyallomer centrifuge tubes in the second experiment. This addition to the gradient provided better separation of the more dense particulates. The loaded gradient tubes were then centrifuged for 2 hr at 200,000 × g in an IEC SB-283 rotor (IEC B-60 ultracentrifuge) at 5° C. The purified RPE pigment granules were recovered as a black pellet at the bottom of the gradients fractionated from the 4,400 g pellet (Fig. 1). The separated gradients were fractionated on a 2490 Fractionator (Gilford Instrument Laboratories, Inc., Oberlin, Ohio), with 2.0M sucrose containing 10% sodium chloride used to displace the gradient. Each tube was fractionated into 300 fractions for a total of 40 fractions. Aliquots of alternate fractions were taken for protein determination. The refractive index at 20° was determined on 25 µl aliquots of every fifth fraction, with an Abbe 3L refractometer (Bausch & Lomb Co., Rochester, N. Y.). The remaining fractions were used to assay n-acetyl-β-D-glucosaminidase, acid phosphatase, β-galactosidase, acid lipase, peroxidase at pH 5.0 and 7.4, and catalase. The lysosomal enzymes were determined on odd fractions, and peroxidase
Figs. 2 to 17. Subcellular distribution of enzymes in retinas and RPE's of English setters. Enzyme data are given in specific activities on the ordinate and particulate densities (gm/ml) on the abscissa. The units of activity for catalase were Δ OD/mg protein/3 min; the peroxidases were expressed as Δ OD/mg protein/10 min; the lysosomal hydrolases were plotted as nmol/mg protein/hr.

Fig. 2. The 4,400 g supernatant fraction from the retina of an unaffected 10-month-old dog.

and catalase were assayed on the even samples. The data were expressed on graphs with the enzyme-specific activity on the ordinate and the density (gm/ml) on the abscissa. Specific activities were calculated as nanomoles per milligram of protein per hour for lysosomal hydrolases, change in optic density per milligram of protein per 3 min. for catalase; and change in optic density per milligram of protein per 10 min. for peroxidase. With this expression of data, age-related or disease-induced changes could be correlated and compared to specific changes in particles of the same density. Subcellular fractions with specific enzyme compositions, at a common-density region in the gradient, are referred to as bands or peaks. These terms are employed synonymously in this report.

Results

First experiment

Retina. The distribution of enzymes (specific activity) associated with subcellular particles in the retina of single dog eyes fractionated on sucrose density gradients for 10-month-old unaffected English setters are given in Figs. 2 to 5. Figs. 2 and 3 are subcellular enzyme distribution patterns for the 4,400 g supernatant fraction separated by density gradient centrifugation according to the procedure given in Fig. 1. The 4,400 g supernatant contains soluble cytoplasmic enzymes and lysosomal, mitochondrial, microsomal, and other particulate components. Figs. 4 and 5 were obtained from the 4,400 g pellet of the whole homogenate and fractionated on sucrose density gradients (Fig. 1). This pellet (4,400 g) contains cell membrane fragments and nuclei. The pigment fraction was assumed to be derived from fragments of RPE adhering to the retina. The retina 4,400 g pellet from the normal animal (A3), fractionated by the same scheme, is compared to the fractionated retinal pellet of an affected dog (A5) in Figs. 4 and 5.

The unaffected retinal 4,400 g supernatant fraction (Fig. 2) exhibited a peak containing acid phosphatase, lipase, and the pH 7.4 peroxidase at a density of 1.20 gm/ml. Other acid hydrolases, i.e., β-galactosidase and hexosaminidase, were found in very low concentration in the normal retinal supernatant. These two hydrolases were not definitely localized to a specific-density region in the un-
affected retina (Fig. 2). In marked contrast, the retinal pellet fraction (Fig. 4) revealed a discrete band of activity for $\beta$-galactosidase at 1.11 gm/ml and a diffuse band at 1.20 gm/ml for both lipase and hexosaminidase. In the 4,400 g retinal supernatant of the young affected dog eye (A5), the most striking change was the high concentration of acid hydrolases in multiple discrete fractions with different specific densities (Fig. 3). Acid phosphatase was found in a second major lighter fraction (density 1.14 gm/ml) and in the 1.20 gm/ml peak. This latter particulate fraction is also found in the unaffected canine retina (Fig. 2). Hexosaminidase was observed in three fractions (densities 1.16, 1.20, and 1.23 gm/ml), but these enzymes were not detectable as discrete fractions in the unaffected retina (Fig. 2). $\beta$-Galactosidase was observed not to be discretely localized in the unaffected retina within two diffuse bands with mean densities of 1.16 and 1.20 gm/ml (Fig. 3). Both acid phosphatase and acid lipase were also distributed over several densities. Two per-
oxidases, assayed at pH 5.0 and 7.4, were found in very low levels in the affected animal (Fig. 3), but catalase was observed in two peaks, unlike the unaffected retina (Fig. 2). The subcellular distribution of the acid hydrolases in the affected retinal pellet (Fig. 5) was different from both the unaffected retinal pellet (Fig. 4) and the affected retinal 4,400 g supernatant (Fig. 3), indicating no carry-over from the 4,400 g supernatant to the 4,400 g pellet fraction. However, both the affected 4,400 g retinal supernatant and the affected 4,400 g retinal pellet enzyme distribution had a fraction of β-galactosidase at 1.22 gm/ml (Figs. 3 and 5).

RPE. In Fig. 6, the 4,400 g RPE supernatant fraction of the unaffected dog (A3) contained a definite single band (1.14 gm/ml) containing two hydrolases, acid phosphatase and β-galactosidase. Acid lipase and acid phosphatase were found in the 1.14 gm/ml band, but other peaks containing these latter two enzymes were also observed in 1.20 gm/ml regions. Both peroxidases were reduced in the 4,400 g supernatant fraction of the affected RPE. Peroxidase bands at 1.20 and 1.24 gm/ml were not detected (Fig. 7). Differences in the subcellular distribution of hexosaminidase and β-galactosidase in the affected RPE were very striking in the 1.06 to 1.12 gm/ml density region (Fig. 7), compared with the normal RPE supernatant fraction (Fig. 6). The RPE 4,400 g pellet fractions separated by density-gradient centrifugation contained two fractions of these two enzymes in common to both the unaffected and affected eye (Figs. 8 and 9). One band of hydrolases was observed at 1.17 to 1.22 gm/ml in the normal eye. In the affected eye, other major changes were seen in a very light fraction of particulates at a density of 1.06 to 1.10 gm/ml (Fig. 9). This band of particles was also found in the affected RPE supernatant and may represent a carry-over of the 4,400 g RPE supernatant to the RPE pellet sample or particles common to both samples. Otherwise the relative subcellular enzyme distribution in the RPE pellets had few features in common with the RPE Supernatant fractions (Figs. 6 and 7 vs. 8 and 9). As reported ear-
lier, catalase was not detected in the RPE of these dogs.

Second experiment

Retina. The subcellular distribution of enzymes from the pooled retinas of three dog eyes separated by sucrose density-gradient centrifugation on unaffected (14- to 22-month-old) and affected (23-month-old) English setters is given in Figs. 10 to 13. Figs. 10 and 11 are the subcellular enzyme distribution patterns for the 4,400 g retinal supernatant fraction (see Fig. 1). Figs. 12 and 13 were obtained from the 4,400 g retinal pellet centrifuged on the same density gradients.
Fig. 8. The 4,400 g pellet fraction from the RPE of an unaffected 10-month-old dog.

Fig. 9. The 4,400 g pellet fraction from the RPE of an affected 10-month-old dog.

Note that Figs. 10 to 13 were taken from gradients with a 1.0 ml. cushion of 2M sucrose, unlike Figs. 2 to 9. Hence, the separation of the more dense fractions was improved in the older specimens over the study with the younger animals (Figs. 2 to 9). The subcellular distribution of enzymes in the retina (Figs. 10 and 12) from two unaffected dogs (A8 and 17A) were compared with the retinal subcellular fractions of two affected dogs (3 and 5) in Figs. 11 and 13. In Fig. 10, the unaffected retinal 4,400 g supernatant exhibited a single catalase peak (1.22 gm/ml), and the affected dogs revealed a bimodal
peak at densities of 1.16 and 1.22 gm/ml. The subcellular distribution of acid phosphatase was also bimodal, in that both unaffected and affected retinas contained bands at 1.15 and 1.22 gm/ml. In contrast, the diseased animals had an increased level of acid phosphatase at 1.24 to 1.28 gm/ml. Similar increases in specific activity were observed for hexosaminidase and β-galactosidase at the same densities. Increases in specific activity were also observed for acid lipase in the same dense particle (1.24 to 1.28 gm/ml). When 4,400 g pellets of the retina were separated by density-gradient centrifugation, the diseased retinas contained subcellular fractions with very high titers of enzymes, probably bound to light pigment or heavy membrane particulates. Both peroxidases decreased in this
dense fraction (1.24 to 1.28 gm/ml). The disease in man and the dog appears to show a deficiency in the leukocyte pH 7.4 peroxidase. The most remarkable change in peroxidase distribution in the retina occurred in the affected 4,400 g retinal pellet fraction (Fig. 13). β-Galactosidase also increased in titer in the same heavy band (1.24 to 1.28 gm/ml). In addition, hexosaminidase, acid lipase, and both peroxidases also increased in the same dense particulate in the affected dogs, unlike the unaffected controls (Fig. 12) where the 4,400 g pellet fraction was practically without any marker enzymes. By comparing the subcellular enzyme distributions between affected animals at the 10-month and 23-month stage of the disease (Figs. 5 and 13), some similarities in enzyme composition of the separated particles were observed. However, in the older affected retinas, the 4,400 g retinal supernatant hexosaminidase had practically disappeared (Fig. 11).

In addition, both peroxidases, pH 5.0 and 7.4, also showed similar decreases in specific activity in the same retina (Fig. 11).
when compared to the peroxidases in both age-matched unaffected controls (Figs. 2 and 10) as well as the earlier affected retina (Fig. 3).

RPE. In the older unaffected litters, acid lipase was apparently bound to dense particles (1.24 gm/ml) in the 4,400 g RPE pellet fraction (Fig. 16), but the same enzyme was soluble or bound to lighter fractions in the 4,400 g RPE supernatant (Fig. 14). It continued to show a large pool of particle-bound acid lipase in the dense fraction (1.22 and 1.24 gm/ml) (Figs. 15 and 17).

Both subcellular pools of the peroxidases were decreased in the affected RPE supernatant and pellet fractions (Figs. 15 and 17). The biochemical and morphological significance of the dense particulate fraction at 1.24 gm/ml in RPE pellet fractions is not obvious at this time, but the enzyme-specific
Fig. 16. The 4,400 g pellet fraction from the pooled RPE of unaffected 14- and 22-month-old dogs.

Table II. Specific activity of enzymes* in the RPE pigment fraction purified by density-gradient centrifugation

<table>
<thead>
<tr>
<th>Peroxidase</th>
<th>pH 5.0</th>
<th>pH 7.4</th>
<th>Catalase</th>
<th>β-Galactosidase</th>
<th>Hexosaminidase</th>
<th>Acid phosphatase</th>
<th>Acid lipase</th>
</tr>
</thead>
<tbody>
<tr>
<td>10-month-old dogs:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A3 (unaffected)</td>
<td>1205</td>
<td>65</td>
<td>n.d.†</td>
<td>—</td>
<td>23</td>
<td>39</td>
<td>39</td>
</tr>
<tr>
<td>A5 (affected)</td>
<td>105</td>
<td>20</td>
<td>n.d.</td>
<td>—</td>
<td>518</td>
<td>92</td>
<td>92</td>
</tr>
<tr>
<td>14- to 22-month-old dogs:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A8, 17A (unaffected)</td>
<td>65</td>
<td>88</td>
<td>n.d.</td>
<td>12</td>
<td>18</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>3,5 (affected)</td>
<td>88</td>
<td>110</td>
<td>n.d.</td>
<td>191</td>
<td>191</td>
<td>74</td>
<td>88</td>
</tr>
</tbody>
</table>

*The units of activity for catalase were ΔOD/mg protein/3 min; for the peroxidases, ΔOD/mg protein/10 min; for the lysosomal hydrolases, nmol/mg protein/hr.

†n.d. = none detected.

One eye from 14-month-old, 2 eyes from 23-month-old dogs.

activity of acid hydrolases in this fraction also appears to increase with age in the unaffected animal (Figs. 8 and 16). Other major differences between the unaffected and affected RPE 4,400 g pellet included the appearance of two new hexosaminidase bands in the affected pellet (Fig. 13): one at 1.20 gm/ml in the 4,400 g RPE supernatant sample (Fig. 16) and the second additional band at 1.14 gm/ml. These two new bands of hexosaminidase were not observed in the unaffected age-matched control (Fig. 16) where the single peak at 1.24 to 1.26 gm/ml was correlated with the acid lipase and β-galactosidase bands. In the affected animal (Fig. 17 the β-galactosidase and lipase bands remained at 1.24 to 1.26 gm/ml.

Purified RPE pigment granule fractions from both experiments. The purified pigment fraction separated from the 4,400 g RPE pellet on a sucrose density gradient was assayed for various enzyme activities (Table II). At the 10-month stage of the disease, an 11-fold decrease was noted in the pH 5.0 peroxidase activity (A5). The affected 23-month-old dogs (3 and 5) showed only a slight additional decrease in this enzyme with age. The changes seen in the 14- to 22-month-old unaffected dogs also included a dramatic reduction in the specific activity of the pH 5.0 peroxidase...
Fig. 17. The 4,400 g pellet fraction from the pooled RPE of affected 23-month-old dogs.

level over the specific activity of the control dog (A3) seen 12 months earlier. The changes observed with the pH 7.4 peroxidase were reduced in the affected 10-month-old animal (A5), but the reduction was only threefold. Aging changes observed in the pH 7.4 peroxidase fraction also showed slight increase in the peroxidase specific activity in the unaffected animals as compared with the fivefold elevation in the older affected dogs.

The lysosomal hydrolases exhibited marked increases in enzyme activities in the 10-month-old affected dog (A5), with the hexosaminidase activity increasing 22-fold. This increased level of acid hydrolases was also observed in the older affected animals. The affected dogs (3 and 5) showed a 10-fold increase in hexosaminidase over unaffected controls. β-Galactosidase also showed similar changes in specific activity (16-fold increase), whereas the other lysosomal hydrolases did not show marked changes over the previous 10-month-old values for affected and unaffected controls.

Discussion

One goal of this study was to establish whether the approach to the fractionation of the RPE developed earlier was useful for the isolation and study of subcellular fractions of the RPE and retina from as little as one or two eyes in animals with various forms of inherited retinal degenerations. We also wished to know whether the quantities of subcellular fractions obtained from these small samples were adequate for biochemical studies on the sequence of the pathogenic events in the progressive course of an inherited retinal degeneration.

In the biochemical studies of the subcellular particulates separated from the retina and purified RPE, it was obvious that dramatic changes were apparent in the subcellular compartments of several acid hydrolases, as well as peroxidase and catalase. That these enzyme distributions continued to change over the progressive course of this inherited retinal degeneration (CCL) was apparent in both the retina and RPE. Major changes were observed both in the retina and in the RPE fractions. The pathogenic changes in the younger retina were associated with the appearance of additional subcellular particles containing lysosomal hydrolases and at least two bands of catalase. In the RPE, the subcellular compartmentation of the acid hydrolases was quite different in the affected animals when compared with unaffected dogs. Two enzymes, β-galactosidase and hexosaminidase, revealed a great increase of
lighter particulate-bound enzymes. Of special note was the loss of the pH 7.4 peroxidase in the diseased RPE localized at the 1.20 gm/ml density, probably lysosomal.

In the later stages of the disease (23-month-old dogs), a progressive increase in dense fractions with very high titers of acid lipase was observed, in both the affected retina and RPE fractions. This altered distribution indicated a change in the subcellular compartment of this enzyme which was probably essential for hydrolyzing lipids of ingested ROS’s.27, 28 The subcellular distribution of acid phosphatase was also shifted to more dense particulates.

Of special note was the discovery of a different subcellular fraction at a density of 1.24 gm/ml in the RPE. This subcellular fraction was found to be of low specific activity in the unaffected 10-month-old animal, but it increased with both age (Fig. 16) and the progressive course of this disease (Fig. 17). The level of this particulate fraction, as monitored by lipase, was twice the specific activity seen during normal aging. Likewise, the affected 10-month-old dog (Fig. 9) had acid lipase levels higher than those of normal 23-month-old dog (Fig. 16). In contrast, age-related increases of enzyme titers in more dense particulates were not observed in the retina, but disease-induced increases were also noted in the 1.26 to 1.28 gm/ml density and fraction. The markedly deranged subcellular distribution of enzymes seen at the 10-month stage of the disease indicates that the major pathogenic events in the subcellular pathology of this form of retinal degeneration occurs much earlier than electroencephalogram changes which are not observed until 21-22 months of age.13

The dense fractions (1.26 to 1.28 gm/ml) appearing with age are probably lipopigment in nature, similar or identical to lipofuscin or ceroid.12 Siakotos and Koppang29 have reported that these pigments accumulate or bind lysosomal hydrolases in very high concentration in brain, liver, and heart. Since Neville et al.13 have demonstrated that ceroid accumulates in the ganglion cells, other cells of the retina, and RPE of dogs affected with CCL, the increases seen in particle-bound enzymes may be related to the accumulation of ceroid in this disorder. The similar changes seen with the normal aging process may be due to the increase of another autofluorescent lipopigment (lipofuscin) in the RPE.

The age-related changes appear to be confined to the RPE. Streeter30 and Hogan31 have shown that autofluorescent lipopigments accumulate with age in the RPE, and Kolb and Gouras32 have observed the accumulation of autofluorescent lipopigments in a case of human retinitis pigmentosa. It is tempting to assign names to the peaks of the subcellular marker enzymes and the new fraction seen in the affected canine retina and RPE. Even though the quantity of subcellular particles isolated in the fractionation of one to three eyes from both unaffected and affected canine eyes was suitable for biochemical studies, the quantities of such sub-

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cellular fractions was not adequate for electron microscopic identification of the morphologic species in the specific enzyme fractions. New techniques have since been developed which will permit the preparation of such small samples for electron microscopy. Further studies should not suffer from the absence of a morphologic correlation with the biochemical studies of retinal degeneration in the dog.

The significance of the shifts of enzyme compartments in the usual aging canine eye and in the eyes of dogs with inherited retinal degeneration is not clear at this time. The classic review of Hogan on the role of the RPE in the phagocytosis and recycling of metabolic components of the ROS’s provides a base for examining the consequences of impairment in the intracellular digestive pathways. In this form of retinal degeneration (CCL), the accumulation of intracellular autofluorescent lipopigments marks the inability of the lysosomal digestive system to degrade these products of lipid peroxidation. In addition, marked derangements were observed in the distribution of intracellular lysosomal hydrolases. The probable consequences of these changes would be less efficient recycling of lipids and protein as a result of the impaired digestive pathways.

The intracellular pathogenic events leading to altered enzyme compartmentalization is unknown but is probably related to interruption in the normal packaging of lysosomal enzymes, e.g., endoplasmic reticulum to Golgi to primary lysosomes to secondary lysosomes. If the newly synthesized acid hydrolases remained in the endoplasmic reticulum, increases in acid hydrolase activity would be observed in the less dense microsomal fractions. This phenomenon was in fact observed in our present study. With the recent discovery by LaVail and confirmation by Bassinger et al. of the cyclic shedding of ROS’s induced by light, any interference in the intracellular RPE digestive mechanism or the nature of the phagocytized ROS’s would lead to the accumulation of undegraded masses of tertiary lysosomes or residual bodies in the cytoplasm of the RPE. In the study reported here, the accumulation of such products appeared to be correlated with the appearance of a more dense fraction with aging and disease.

The recent studies of Feeney and Mixon on the intracellular fusion of lysosomes and phagosomes in the RPE have suggested that RPE lysosomes are assembled in response to an acute phagocytic challenge, such as during the light-induced cyclic shedding of ROS’s and the subsequent phagocytosis of the ROS’s by the RPE cell. Also the same authors suggested that degradation of the ingested phagolysosomes occurs less rapidly during the ingestion of inert particles. The consequences of this rationale in the pathogenesis of canine RPE may be the marked stimulation of the fusion of lysosomes early in the disease and the gradual decrease of most enzymes within normal subcellular compartments with the continual increase in abnormal particulates. The continuing attempt at autophagy and fusion with lysosomes would result in large amounts of lysosomal hydrolases being trapped within the lipopigment matrix.

In addition, the marked decrease in the purified pigment granule peroxidases observed early in the course of the disease provides a clue to another and perhaps more important pathogenic mechanism in this disorder, that is, the diminution of the normal protective role of peroxidase. In the absence of a normal complement of soluble and membrane-bound peroxidases, abnormal material accumulates within inclusion bodies by the normal autophagic response of the RPE cell. Therefore the initial pathogenic event seems to be the inability of affected cells to cope with peroxidative damage because of the reduced level of peroxidase, and this early lesion is followed by the exaggerated attempt by the RPE cell to digest the accumulating masses of lipopigment.

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