Oval corneal opacities in beagles

III. Histochemical demonstration of stromal lipids without hyperlipidemia

Alan M. Roth, Marilyn B. Ekins, George O. Waring III, Lata M. Gupta, and Leon S. Rosenblatt

We found oval stromal acascular corneal opacities in 128 eyes of 75 beagles from 497 studied. There were three morphologic types that progressed in severity with time: nebular, racetrack, and white arc. Histochemical staining of the earliest morphologic type (nebular) revealed neutral fats, cholesterol, phospholipids, and sometimes fatty acids both intracellularly and extracellularly. We found no elevation of serum cholesterol or triglycerides except in dogs with the most advanced morphologic type (white arc) and no alteration in thyrometabolic function. We think that oval stromal opacities in beagle corneas are a primary disorder of corneal lipid metabolism closely resembling the central crystalline dystrophy of Schnyder and may be an animal model for this human disease.

Key words: corneal metabolism, primary lipid dystrophy, Schnyder's crystalline dystrophy, lipid histochemistry, animal model
Fig. 1. Nebular oval corneal opacity has homogeneous gray appearance with indistinct margins.

Fig. 2. Racetrack oval opacity has slightly depressed, brownish center.

Histochemistry is an imprecise art and that only a few techniques define lipids with certainty, we decided to perform a large battery of techniques to give the most comprehensive data. These may then be interpreted in light of the limitations of such testing and correlated with more precise biochemical analysis. We report here the histopathologic findings and histochemical demonstration of lipids in these opacities and show that serum lipid levels and thyroid hormone assays are similar in both affected beagles and age- and sex-matched controls.

**Materials and methods**

All beagles came from a colony involved in lifelong radionuclide toxicity studies at the Radiobiology Laboratory, University of California, Davis. As each dog was scheduled for sacrifice, we reviewed our previous records to determine
the type of corneal opacity present. After the dog was anesthetized with intravenous sodium pentobarbital, we verified the type of corneal opacity by inspection with a penlight and enucleated the eyes. We immediately removed a corneoscleral shell by cutting into the epichoroidal space 4 mm posterior to the limbus, excising the shell with scissors, and disinserting the ciliary body from the scleral spur. The cornea was placed epithelial side down on a silicone block and trisected through the oval opacity; one third was fixed in cold 2% glutaraldehyde in Millonig's buffer for electron microscopy, one third was frozen in liquid nitrogen for biochemical determinations, and one third was fixed in Baker's formol-calcium solution7 for light microscopy.

We recorded the nutritional and medical history of each dog. An experienced veterinary pathologist performed a complete autopsy.

**Histochetnistry.** We studied 29 corneas from 16 affected beagles that had the nebular type opacity and two corneas from two unaffected beagles. Three specimens from two dogs were blocked in paraffin only. Because routine histochemical stains

**Fig. 3.** White arc oval opacity is densely white, granular subepithelial deposit.

**Fig. 4.** Oval opacity in beagle from outside the colony studied shows double row of gray crystalloid deposits (left), which stand out prominently in retroillumination (right).
of sections cut from these blocks revealed no abnormal substances, because the ultrastructural studies on these specimens showed intracellular vacuoles and crystals suggestive of lipids (Spangler, Waring and Morrin, unpublished data), and because the clinical appearance was compatible with lipid deposits, we divided the formol-calcium-fixed tissue from each of the remaining 26 corneas into two pieces. We embedded one of these in paraffin and stained sections with hematoxylin and eosin, Masson’s trichrome method, the histochemical methods for demonstrating lipids (Table I). We stained sections of brain known to contain the different lipids with each corneal specimen as a control. Sections being analyzed for cholesterol were examined within 15 min of staining because of rapid oxidation of the reaction and because the sections were destroyed by the technique. Paraffin and frozen sections were also examined under plane-polarized light for birefringence.

**Serum lipid measurements.** We measured serum cholesterol and triglycerides in affected beagles and in age- and sex-matched control beagles on two occasions, 1 year apart. In December 1977, we paired each of 48 affected beagles (28 males, 20 females) with an unaffected beagle of the same age and sex (27 males, 21 females) in the same radiation group (65Sr, 226Ra, or not radiated). Some of the affected and control dogs were littermates. In January 1979, we re-examined the 29 surviving affected beagles (18 male, 11 female) and the 40 surviving controls (24 male, 16 female). The mean ages of the control and affected dogs were comparable for both sexes (male 12.5 years, female 12.6 years). After the dogs had fasted 6 hr, we collected 10 ml of clotted blood with a 20-gauge needle from the left jugular vein. The samples were immediately centrifuged at 3000 rpm at 4°C; the serum was removed and stored at 4°C. We measured total cholesterol by the Technicon AutoAnalyzer II (Clinical Method No. 24/preliminary March 1972) for the first determination and also sent random beagle serum samples to an independent laboratory that used the same methods. To confirm the accuracy of our serum lipid measurements, we employed standards of known lipid concentration and also sent random beagle serum samples to an independent laboratory that used the same methods.

We employed analysis of variance and regression analysis to compare serum lipid measurements from the total number of affected and control dogs, to compare each of the three affected subgroups (nebular, racetrack, and white arc) to each other and to the controls, and to compare sexes and ages. We used data only from beagles whose serum lipids were measured twice. Each of

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**Table I. Histochemical stains done for lipid**

<table>
<thead>
<tr>
<th>Reference</th>
<th>Type of reaction</th>
<th>Lipid demonstrated</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oil red O</td>
<td>7 Lipid-soluble dye</td>
<td>Neutral fat</td>
<td>High</td>
</tr>
<tr>
<td>Sudan black B</td>
<td>30 Lipid-soluble dye</td>
<td>Neutral fat especially phospholipid</td>
<td>Moderate</td>
</tr>
<tr>
<td>Baker</td>
<td>28 Acid hematin with pyridine extraction</td>
<td>Phospholipid</td>
<td>Low</td>
</tr>
<tr>
<td>Pearse</td>
<td>31 Copper phthalocyanin</td>
<td>Phospholipid</td>
<td>Low</td>
</tr>
<tr>
<td>Menschik</td>
<td>32 Nile blue sulfate</td>
<td>Phospholipid</td>
<td>Low</td>
</tr>
<tr>
<td>Landing et al.</td>
<td>33 Phosphomolybdic acid</td>
<td>Choline-containing phospholipid</td>
<td>Low</td>
</tr>
<tr>
<td>Fischler</td>
<td>27 Cupric soap—lithium hematoxylin</td>
<td>Fatty acid</td>
<td>Moderate</td>
</tr>
<tr>
<td>Holczinger</td>
<td>27 Cupric soap—rubeanic acid</td>
<td>Fatty acid</td>
<td>High</td>
</tr>
<tr>
<td>Schultz</td>
<td>27 Acetic—sulfuric acid</td>
<td>Cholesterol</td>
<td>Moderate</td>
</tr>
<tr>
<td>Adams</td>
<td>34 Perchloric acid—naphthoquinone</td>
<td>Cholesterol</td>
<td>High</td>
</tr>
<tr>
<td>Okamoto</td>
<td>27 Sulfuric—iodide</td>
<td>Cholesterol</td>
<td>Moderate</td>
</tr>
<tr>
<td>Bromine—Sudan black B</td>
<td>35 Bromination unmasking</td>
<td>Unmasked cholesterol</td>
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<tr>
<td>Mukherji</td>
<td>27 Bromine-silver</td>
<td>Unsaturated lipid</td>
<td>Moderate</td>
</tr>
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<td>Norton</td>
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<td>Osmium tetroxide</td>
<td>28 Lipid-soluble reagent</td>
<td>Unsaturated lipid</td>
<td>Low</td>
</tr>
<tr>
<td>Seligman—Ashbel</td>
<td>7 Naphthoic hydrazide</td>
<td>Active carbonyl lipids</td>
<td>Low</td>
</tr>
</tbody>
</table>

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the affected dogs was classed according to the lesion of its more severely affected eye. Between the first and second lipid determination, the opacities in some of the affected dogs progressed; thus the same dog may be in one affected subgroup in 1977 and in another in 1979.

Thyroid hormone assays. We tested thyrometabolic function in 24 affected beagles and in 39 age- and sex-matched control beagles. Blood was collected from the jugular vein and the serum was immediately separated as described above. The samples were stored at 4°C for no longer than 48 hr before analysis. Thyroxine (T4) levels were measured by the Quantimune T-4 Radioimmunoassay technique (Bio-Rad Laboratories Bulletin 4202, October 1978), and the binding capacity of thyroid hormone carrier proteins was assayed by the Quanta-Count T3 resin uptake method (Bio-Rad Bulletin 4212, March 1977). The free thyroid index (T3) was calculated by multiplying the T4 level and the T3 percent resin uptake (%RU).

We employed chi-square analysis to compare the results between control and affected dogs, between control dogs and those in each affected subgroup (nebular, racetrack and white arc), and between dogs in each subgroup. Statistical review was performed by Dr. Rosenblatt.

Results

Complete autopsies on all dogs revealed no changes suggestive of a systemic lipid metabolic disorder.

Histology and general histochemistry. Paraffin sections stained with hematoxylin and eosin and with Masson’s trichrome method demonstrated that the epithelium and its basal lamina generally were intact with occasional focal acanthosis (facet formation). Although Bowman’s layer is not normally present in beagle corneas (Morrin, Waring, Spangler, unpublished data), the normal anterior stroma is hypocellular, with interlacing collagen bundles. The major abnormality in the affected corneas was in this area, with disruption of collagen architecture, focal areas of collagen fibril fragmentation, stromal vacuole formation, and scattered enlarged vacuolated keratocytes. No blood vessels were seen.

Periodic acid–Schiff and Alcian blue reactions revealed no gross abnormalities in glycosaminoglycans. Plane-polarized light showed no birefringence in sections from paraffin blocks. Frozen sections, however, contained many doubly refractile crystals, primarily within keratocytes, but occasionally in the extracellular stroma. The posterior stroma, Descemet’s membrane, and endothelium were normal.

Lipid histochemistry. Table II shows the results of the histochemical reactions for lipids in 26 affected corneas. The right and left corneas from each dog usually stained similarly. All stains were positive on the known control tissue and showed no reaction in the two unaffected control corneas. The majority of positive stains were intracellular with many having extracellular staining as well. No case showed only extracellular stain, although a few had such dense overall reaction that we could not ascertain its location.

Stains for neutral fats (oil red O and Sudan black B) were positive in all corneas, primarily in the anterior half. In general, the intracellular stain was in large globules, whereas the stromal stain was in finer granules (Fig. 5). At least one of the methods for demonstrating cholesterol (Schultz, Okamoto, and Adams) was positive in each cornea (Fig. 6). The majority were positive by all three techniques. All corneas demonstrated phospholipids with at least one of four reactions for phospholipid (Baker, Pearse, Landing, and Menschik), and 12 of the 26 corneas demonstrated fatty acids by at least one of the tests (Fischler, Holczinger). The remainder of the histochemical reactions were negative, although sporadic intracellular staining was sometimes present.

Serum lipid measurements. There was no significant difference between affected and control dogs in either 1977 or 1979 for total cholesterol, C_HDL, C_LDL, and C_VLDL or triglycerides (Table III). The influence of sex on serum lipids was also negligible except that, in 1979, affected females showed higher triglycerides than affected males. The influence of age on serum lipids was less clear-cut (Table III). In 1977, there was a statistically significant trend for serum cholesterol to rise
Fig. 5. Neutral fat appears as large globules in keratocytes, whereas smaller extracellular granules accumulate in anterior stroma. (Frozen section, oil red O; ×200.)

Table II. Results of histochemical stains for lipid

<table>
<thead>
<tr>
<th>Dog and cornea No.</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
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<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
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<td>+++</td>
<td>+++</td>
<td>+++</td>
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<td>++</td>
</tr>
<tr>
<td>Sudan black B</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
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<td>++</td>
</tr>
<tr>
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<td>+++</td>
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<td>++</td>
</tr>
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<td>+++</td>
<td>+++</td>
<td>+++</td>
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<tr>
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<td>+++</td>
<td>+++</td>
<td>+++</td>
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</tr>
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<td>+++</td>
<td>+</td>
<td>++</td>
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</tr>
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</tr>
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<td>-</td>
</tr>
<tr>
<td>Seligman-Ashbel</td>
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<td>+</td>
<td>++</td>
<td>+</td>
<td>++</td>
<td>++</td>
</tr>
</tbody>
</table>

- = Negative; + = mildly positive; ++ = moderately positive; +++ = strongly positive.

Dogs D and M supplied only one cornea each.

*With pyridine extraction control.
Fig. 6. Cholesterol accumulates predominantly in keratocytes of anterior stroma. (Frozen section, Okamoto’s method; ×200.)
Table III. Serum lipid measurements (mg/dl) in beagles with oval opacities and unaffected controls

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<th></th>
<th>Total cholesterol</th>
<th>C&lt;sub&gt;HDL&lt;/sub&gt;</th>
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<td></td>
<td></td>
</tr>
<tr>
<td>Male:</td>
<td></td>
<td></td>
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<tr>
<td>Mean</td>
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<td>222.2</td>
</tr>
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<td>S.D.</td>
<td>43.6</td>
<td>76.2</td>
</tr>
<tr>
<td>N</td>
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<tr>
<td>Female:</td>
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<td></td>
</tr>
<tr>
<td>Mean</td>
<td>201.0</td>
<td>220.0</td>
</tr>
<tr>
<td>S.D.</td>
<td>56.9</td>
<td>61.1</td>
</tr>
<tr>
<td>N</td>
<td>21</td>
<td>20</td>
</tr>
</tbody>
</table>

Analysis of variance*:
- By sex: NS
- By group: NS
- By sex and group: NS

Linear regression against age:
- Male: r = 0.40, p(r) = 0.04
- Female: r = 0.57, p(r) = 0.01

First sample = 1977; second sample = 1979; NS = not significant = p >0.05.

* Normal vs. abnormal; male vs. female.

with age, but this was not confirmed in 1979. When we analyzed total serum cholesterol, C<sub>HDL</sub>, C<sub>LDL</sub>, and C<sub>VLDL</sub> measurements in the three different clinical patterns in 1977, dogs with white arc opacities consistently had higher absolute values, but we could not confirm these differences in 1979 (Table IV).

Thyroid hormone assays. The 39 control dogs had T<sub>3</sub> %RU of 35.2% ± 4.6, T<sub>4</sub> level of 1.37 μg/100 ml ± 0.68, and T<sub>7</sub> of 0.48. Eight beagles with the nebular opacity had T<sub>3</sub> %RU of 34.6% ± 5.4, T<sub>4</sub> level of 1.45 μg/100 ml ± 5.4, and T<sub>7</sub> of 0.51. Eight dogs with the race track lesion showed T<sub>3</sub> %RU of 36.6% ± 5.1, T<sub>4</sub> levels of 1.46 μg/100 ml ± 0.32 and T<sub>7</sub> of 0.55. Six animals with the white arc corneal opacity had T<sub>3</sub> %RU of 35.3% ± 4.2, T<sub>4</sub> level of 1.70 μg/100 ml ± 0.70 and T<sub>7</sub> of 0.57. Chi-square analysis showed no significant difference in T<sub>3</sub> %RU, T<sub>4</sub>, or T<sub>7</sub> between control and affected dogs, between control dogs and those in each subgroup, or between beagles in each subgroup.

Comment

Relatively little is known about the composition, source, and metabolism of normal corneal lipids. There is no evidence for lipid storage in the normal cornea in vivo and lipids are probably limited to corneal cell membranes in the form of neutral fats (predominantly cholesterol and its esters) and phospholipids (primarily sphingomyelin). These are relatively stable and there appears to be a low lipid turnover rate. Normal corneal stromal cells can synthesize lipids and can incorporate labeled phosphorus into phospholipids.

Lipids appear abnormally in the human cornea in a variety of patterns (Table V). Three factors may contribute to these lipid deposits: (1) stromal blood vessels such as those in old scars or in limbal masses, (2) abnormal systemic lipid metabolism such as type II hyperlipoproteinemia or Tangier disease, and (3) primary corneal abnormalities such as central crystalline corneal dystrophy.
We searched for these three contributing factors in beagles with oval lipid corneal opacities. The corneas were avascular both clinically and histopathologically. In a few cases, corneal ulceration had occurred in conjunction with the oval opacities, and the corneas vascularized secondarily.

Serum lipid measurements in affected dogs were normal. The environment, nutrition, and medical care of this colony were carefully monitored. A complete autopsy on each dog revealed neither atherosclerosis nor excess lipid deposits in parenchymal organs. Our initial analysis of serum cholesterol suggested elevated C<sub>HDL</sub> in dogs with the race-track and white arc patterns. However, further statistical analysis and a second sampling of affected and control dogs failed to reveal statistically significant differences (Table IV). Moreover corneal opacities have not been described in reports of hyperlipemic beagles. Therefore we concluded that a systemic disorder of lipid metabolism did not contribute to the oval lipid corneal opacities.

However, we did observe consistently higher levels of serum cholesterol in dogs with the white arc type opacity, although the level was not always statistically significant and the number of these dogs was small (Table IV). It is possible that these subepithelial white deposits, which we presume are lipids but have not studied histochemically, are related to the elevated serum lipids. On the other hand, although we found clinical progression of these opacities from nebular to race-track to white arc types, we have not been able to document either a concurrent rise in serum lipids or increased serum lipids with age (Tables III and IV).

In hypothyroidism, serum cholesterol levels may be elevated because of altered metabolism; this metabolic effect has been reported in beagles. Because our pilot study suggested that affected beagles had elevated serum cholesterol levels, we measured thyroxin and thyroglobin binding capacity, cal-

<table>
<thead>
<tr>
<th>First sample</th>
<th>Second sample</th>
<th>First sample</th>
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<tbody>
<tr>
<td>Control</td>
<td>Opacity</td>
<td>Control</td>
<td>Opacity</td>
</tr>
<tr>
<td>39.5</td>
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<tr>
<td>0.05</td>
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NS = not significant; p = 0.02

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Table IV. Total serum cholesterol measurements in beagles with nebular, race track, and white arc oval opacities

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<td>Racetrack</td>
<td>White arc</td>
<td>Control</td>
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<td></td>
<td>NS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>By type</td>
<td>p = 0.001</td>
<td></td>
<td></td>
<td></td>
<td>NS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>By sex and type</td>
<td>NS</td>
<td></td>
<td></td>
<td></td>
<td>NS</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

First sample = 1977; second sample = 1979; NS = not significant = p > 0.05.

*Normal vs. abnormal; male vs. female.

Table V. Corneal stromal lipid deposits in man

<table>
<thead>
<tr>
<th>Type</th>
<th>Reference</th>
<th>Clinical appearance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corneal arcus</td>
<td></td>
<td>Gray paralimbal arc with lucid interval</td>
</tr>
<tr>
<td>Normal aging &gt;40 years</td>
<td>37,38</td>
<td></td>
</tr>
<tr>
<td>Hyperlipoproteinemia II and III, &lt;40 years</td>
<td>39</td>
<td></td>
</tr>
<tr>
<td>Secondary (pre-existing stromal vessels)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vascularized scar</td>
<td>40</td>
<td>Gray-white, random pattern, spiculated margin</td>
</tr>
<tr>
<td>Limbal mass (e.g., dermoid, nevus)</td>
<td></td>
<td>Gray arc, lucid interval</td>
</tr>
<tr>
<td>Primary (no known pre-existing corneal pathology)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Limbal</td>
<td>41</td>
<td>D-shaped, yellow, solid, deep stromal, initially vascular</td>
</tr>
<tr>
<td>Central</td>
<td>42</td>
<td>Round, yellow, initially avascular</td>
</tr>
<tr>
<td>White ring (of Coats)</td>
<td>40</td>
<td>Small, white, round with gray dots</td>
</tr>
<tr>
<td>Central crystalline dystrophy (of Schnyder)</td>
<td>21-26</td>
<td>Central anterior ring of fine, white crystals, hazy gray background, corneal arcus</td>
</tr>
<tr>
<td>Systemic disorders</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tangier disease (decreased C&lt;sub&gt;HDL&lt;/sub&gt; in serum)</td>
<td>43,44</td>
<td>Diffuse fine gray spots</td>
</tr>
<tr>
<td>Leechithin cholesterol acyltranferase deficiency</td>
<td>45-47</td>
<td>Diffuse fine gray spots</td>
</tr>
<tr>
<td>Juvenile xanthogranuloma</td>
<td>48</td>
<td>Flat, yellow, vascularized limbal mass</td>
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Table VI. Corneal stromal lipid deposits in man

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culated T<sub>3</sub>, and found no significant differences between affected and control dogs or dogs with different subgroups of opacities. Thus thyroid dysfunction does not play a part in the pathogenesis of the oval opacities.

We think that oval stromal corneal opacities in beagles represent a primary disorder of corneal lipid metabolism. Of the human disorders in which lipid is deposited in the cornea, these opacities most closely resemble the central crystalline corneal dystrophy of Schnyder. Both occur in avascular, non-inflamed corneas, are bilateral, and appear relatively symmetrical. The clinical appearance of both includes a ring-shaped configuration in which a central gray stromal opacity that is most dense superficially is studded with subepithelial white crystals. In ad-
advanced cases of both disorders, the opacity may occupy full-thickness stroma. Histochecmically, both contain cholesterol and neutral fats, whereas the beagle corneas also have histochemical evidence of phospholipids and sometimes fatty acids. Both have similar ultrastructure with elongated crystals, extracellular vacuoles and debris, disruption of collagen lamellae, hyperplasia of keratocyte organelles, and stromal cellular degeneration. Hyperlipoproteinemia has been found in some patients with central crystalline corneal dystrophy and in family members of affected individuals. Although we did not find hyperlipidemia in affected beagles, the dogs with white arc opacities did have serum cholesterol levels higher than the control population, which may contribute to their clinically more advanced opacity. Bron and colleagues speculated that the stromal keratocytes in central crystalline corneal dystrophy are unable to properly metabolize lipids, so that local lipid deposits occur. Elevated serum lipids then further overload the cells and increase the lipid deposition. In man, the disorder is autosomal dominant; it is unfortunate that we cannot determine the hereditary influences in our beagle colony because of the carefully planned outbreeding. Oval corneal lipid opacities in beagles may be an animal model of the human corneal dystrophy of Schnyder.

The demonstration of lipids by histochemical techniques is a tedious process. The methods are not well documented, many reagents are difficult to obtain, and only after diligent search by laboratory personnel can a meaningful battery of lipid stains be performed (Table I). Even when histochemical methods demonstrate the presence and location of lipids, distinguishing among the classes of lipids is difficult. Many of the reactions are nonspecific for the different types of lipid and can give false-positive and/or false-negative results. The presence of other types of compounds in the tissue may cause capricious staining. Diffusion from the sections can occur and subcellular fractionation has been found. Biochemical analysis of lipids is necessary to accurately identify and quantitate the varieties in the oval opacities; we are presently performing these studies on affected beagle corneas.

REFERENCES

15. Wolf HG, Della Rosa RJ, and Andersen AC: Nutri-


