Objective: To determine whether selective cone loss could explain the acquired tritan-like color confusion found in diabetic retinopathy.

Methods: Terminal deoxynucleotidyl transferase–mediated biotin-deoxyuridine triphosphate nick end labeling (TUNEL) was employed on paraffin sections of retinas from 5 donors with diabetic retinopathy. For quantitative analysis, postmortem retinas were obtained from 13 human donors; 7 from patients with various durations and stages of diabetic retinopathy (4 background, 3 proliferative) and 6 controls. Enzyme histochemical analysis for carbonic anhydrase (CA) was used to distinguish L/M-cones (positive for CA) from S-cones (negative for CA). Cone topography was determined by sampling 360° from 0.1 to 1.5 mm of foveal eccentricity and along the horizontal meridians from 1.5 to 15.0 mm.

Results: Rare cells in both the inner and outer nuclear layers of the diabetic eyes were positively labeled with the TUNEL method. The CA staining revealed incomplete and patchy losses of S-cones that were limited to the diabetic retinas. Statistically significant reduction in the density of S-cones was found at nearly all foveal eccentricities from 0.1 mm to 15.0 mm. This was not the case for the L/M-cones. On average, for all locations, the percentage of S-cones compared with L/M-cones was decreased by 21.0% ± 3.4% with respect to the controls.

Conclusion: The S-cones selectively die in diabetic retinopathy.

Clinical Relevance: Selective loss of S-cones may contribute to the tritan-like color vision deficit seen in patients with diabetic retinopathy.

Acquired color vision loss associated with retinal degenerative disorders usually results in color confusion along a blue-yellow axis, producing a tritan-like defect. At least 5 possible mechanisms have been proposed to explain this phenomenon.

- Filtering of the shorter wavelengths by the optical media. Brunescent cataracts and, perhaps, old vitreous or intraretinal blood would fall into this category, as would yellowish subretinal fluid and migration of retinal pigment epithelial cells into the retina.
- Paucity of S-cones (short wavelength–sensitive cones or blue cones). In humans, only about 9% of the cones are blue sensitive. If one assumes a linear system, loss of equal percentages of all 3 cone types would not necessarily produce a specific color confusion axis. However, because the S-cones are present in such small numbers, the loss of even a small percentage might cause considerable gaps in their matrix, which could be relatively more important than the loss of an equal percentage of L- or M-cones (long and medium wavelength–sensitive cones, or red and green cones, respectively).
- Limited response range of the S-cones. It has been observed that the luminance response range of the S-cones is narrower than for the L- and M-cones. If a disease were to result in the response ranges decreasing proportionally for all 3 types, the S-cones might saturate while the L- and M-cones would maintain some sensitivity.
- Heterogeneity in effect of retinal disease. The L/M-cones increase in density with decreasing foveal eccentricities; achieving a sharp maximum in the foveal center. The S-cones reach their greatest density within 1 or 2 arc degrees eccentricity from the foveal center. With smaller eccentricities, their numbers fall precipitously and they may even be absent within 8 arc minutes of the center. If retinal disease were to preferentially affect the parafoveal region (where S-cones are at their greatest concentration) and spare the fovea itself, a much greater percentage of S-cones would be injured. Tritan-like color confusion would then result.
- Selective cone fragility. The blue-sensitive cones (or their associated higher order neurons) might simply be more susceptible to stress than the L- or M-cones. Such a hypothesis is not unreasonable considering the fundamental biochemical and genetic differences that exist between them.
SUBJECTS, MATERIALS, AND METHODS

The experiments described in this section followed the tenets of the Declaration of Helsinki and were approved by the institutional review board of the University of Wisconsin Medical School.

TUNEL METHOD

Portions of 5 retinas from 5 subjects with varying degrees of diabetic retinopathy (subjects D1, D5, and D7 [Table] plus 2 additional eyes from 2 subjects that were not used for the quantitative study described here) and 1 postmortem control eye (without known retinal disease) were embedded in paraffin. Putatively apoptotic nuclei were labeled by the TUNEL method.22,53 Paraffin sections (4 μm thick) were mounted on slides treated with adhesive (Vectorbond; Vector Laboratories Inc, Burlingame, Calif), deparaffinized, and rehydrated with distilled water. All sections were digested with proteinase K and blocked for endogenous peroxidase. Terminal deoxynucleotidyl transferase was used to incorporate biotinylated dUTP at the sites of DNA breakdown. Biotinylated dUTP was visualized with streptavidin and diaminobenzidine. Rat small intestine with and without deoxyribonuclease added before incubation in TdT and biotin dUTP served as positive controls for the TUNEL method. Negative controls for the TUNEL method were incubated without biotinylated dUTP.

LABELING OF L/M-CONES FOR CARBONIC ANHYDRASE

Postmortem retinas were obtained from 13 human donors; 7 from patients with various durations and stages of diabetic retinopathy (cases D1-D7, Table) and 6 controls. The tissue was fixed in 4% phosphate-buffered paraformaldehyde (pH 7.2) for 24 to 48 hours at 4°C and then stored in 1% phosphate buffer (pH 7.4) at 4°C. Representative pieces of retina were removed and embedded in glycol methacrylate (GMA) (JB-4 Polysciences Inc, Warrington, Penn). To preserve enzyme activity, the tissue was embedded directly in GMA without the usual dehydration in graded alcohols. Instead, the specimens were gently agitated in graded concentrations of GMA monomer with benzoyl peroxide catalyst and distilled water, beginning with a 1:1 mixture. Polymerization was carried out at 0°C for 16 hours. Sections were cut 2 μm thick with the tissue oriented either radially or tangentially to the plane of the retina and reacted for CA according to a modified version15,54 of the Hansson method.15,56 This involved floating the tissue sections on the surface of a reacting medium consisting of 0.00715 mol/L of cobalt sulfate, 0.0526 mol/L of sulfuric acid, 0.0117 mol/L of potassium phosphate, and 0.157 mol/L of sodium bicarbonate for 4 to 8 minutes. The sections were rinsed by floating them on a 0.5% solution of ammonium sulfide for 1 minute and rinsing again on distilled water.

They were mounted on glass slides and counterstained with toluidine blue.

QUANTITATIVE ANALYSIS

The tissue sections were viewed with a microscope (Olympus BH-2; Olympus Corp, Lake Success, NY) and a digital camera (Sony 3-chip charge-coupled device [3CCD], Model DXC 960 MD; Sony Medical Systems, Montvale, NY). The digital image was then captured, and densities of the 2 cone types (positive and negative for CA) were measured using image analysis software (Optimas; Media Cybernetics LP, Bothell, Wash).

The location of any point on the retina can be specified by 2 coordinates; the distance from the fovea and the degree from the temporal meridian (ie, the 3-o’clock meridian for left eyes, the 9-o’clock meridian for right eyes). At 100 μm, 1 point was sampled every 45.0° (8 points); at 150 μm, every 30.0° (12 points); and at 200 μm, every 22.5° (16 points). Beyond 200 μm up to 1.5 mm, data were collected every 45.0° (8 points) at 200-μm intervals (Figure 1). A modification of the method of Curcio et al13 was used to create a smooth tessellation of the central retina, producing 2-dimensional topographical maps.

A method similar to that described by de Monasterio et al13 was employed for the peripheral retina. Cone topography was determined by sampling 360° (as described in the preceding paragraph) from 100 to 1500 μm of retinal eccentricity (distance from the foveal center) and then along the horizontal meridians from 3 to 15 mm. Four points were sampled near the horizontal meridian beyond 1.5 mm. All data for each eccentricity, regardless of direction, were then averaged (Figure 2). (The distances as given do not account for tissue shrinkage, which is about 15% for embedding in GMA. To convert millimeters to degrees of retinal eccentricity for the average adult eye, assuming 15% shrinkage, a factor of 3.96° per millimeter should be used.)

It was not possible to include all points at every eccentricity. This was owing primarily to weak staining reaction or to tissue folding. The CA enzyme histochemical reaction is sensitive to autolysis and fixation. In some locations of some eyes, the reaction was too weak to distinguish CA-positive cones from CA-negative cones. Tissue folding during embedding presented occasional problems as well, making it difficult to obtain sections that were flat enough for adequate sampling. The central fovea (0 μm and 50 μm eccentricity) presented its own technical difficulties when this technique was employed. Carbonic anhydrase staining was particularly weak in this region, only yielding usable results in a few eyes. Also, the retina was thinner here so that during fixation and embedding, tissue shrinkage caused the photoreceptor layer to move vitreally, producing a concave outer surface opposite the fovea interna. Because of this, tangential sections through the fovea were generally not flat enough for analysis. Given these problems and the normal paucity of S-cones in the central fovea, statistical comparisons between diabetic retinopathy and control eyes are not meaningful for eccentricities of less than 100 μm with the use of this technique.
cesses is not an unreasonable assumption considering that 96% of the amino acid sequences of their opsins are homologous compared with only a 43% identity with S-cone opsin. Other biochemical similarities are suggested by their common histochemical staining patterns with opsin antibodies and with antibodies to the various S-antigen isoforms.

Previous work in our laboratory has shown that the latter mechanism, selective S-cone fragility, is strongly evident in the case of both human traumatic retinal detachment and experimental retinal detachment in the rhesus monkey. It is the L- and M-cones that are selectively (and probably equally) injured in glaucoma.

A range of disorders is ophthalmoscopically evident in diabetic retinopathy. Mild or background changes include capillary microaneurysms, hard exudates, small intraretinal hemorrhages, and cotton-wool spots (swellings of the axons of the nerve fiber layer). More advanced or preproliferative defects are composed of capillary dropout and intraretinal microangiopathy. Finally, severe abnormalities such as extrafoveal neovascularization, preretinal membrane formation with retinal traction detachment, and vitreous hemorrhages are seen in the proliferative form of the disease. Panretinal laser photocoagulation has been found to be of benefit in preventing visual loss in many patients with proliferative changes.

As with other retinal degenerative processes, the characteristic pattern of color vision loss in diabetic retinopathy is of the tritan-like or Verriest Type III. This selectivity of damage for the S-cone pathway was further demonstrated by Adams et al who measured the spectral sensitivities for each of 3 cone pathways in patients with diabetic macular edema. They found that the blue pathway was 40 times less sensitive than normal controls compared with the L- and M-pathways that were only 2.2 times less sensitive. More recently, Greenstein et al have carefully measured S-cone pathway sensitivity using an in-
cremement threshold technique and found the S-cone pathway to be specifically affected in diabetes. The L- and M-cone pathways showed less sensitivity loss. The rods seem to be less sensitive in diabetes as well.35,36

Tritan-like defects have been found in all stages of diabetic retinopathy. Kinne et al37 and Lakowski et al38 studied 549 patients with diabetes, most of whom had mild or no apparent retinopathy, and found that, despite normal visual acuity in most patients, color vision loss was common. More recent studies have confirmed the presence of type III loss even in patients with ophthalmoscopically absent or minimal retinopathy.37,39-41 Some controversy exists as to the correlation of the color defect with severity of the retinopathy. Kinne et al37 found that at all ages studied (group averages of 25 to 65 years old in 10-year increments), the color vision loss was worse in those subjects with at least some retinopathy. Moloney and Drury42 studied 66 young patients with diabetes (mean age, 27.7 years) and did not observe a relationship between stage of the disease and magnitude of the color vision loss. Bresnick et al43 on the other hand, examined 90 patients with diabetes (young and old) and concluded that the magnitude of the acquired tritan-like discrimination loss was correlated significantly with both the severity of the overall diabetic retinopathy and the severity of macular edema and hard exudate formation. An independent risk factor for tritan-like color vision loss seems to be the application of panretinal laser photoocoagulation,44-46 even though the macula is avoided in this treatment.

A question has arisen as to whether the tritan-like deficit in diabetes is prereceptoral, receptoral, or postreceptoral. In support of a prereceptoral mechanism, Lutze and Bresnick47 found that the lenses in patients with diabetes “yellow” at an accelerated rate. However, they later found that when correcting for these lens changes, areas of reduced S-cone system sensitivity were present. Using blue test spots on a bright yellow background, Terasaki et al48 concluded that the pattern of sensitivity loss cannot be caused by changes in preretinal screening. Even so, Tregear et al49 employed chromatic contrast threshold testing and concluded that the specific tritan deficits seen in patients with diabetes can be explained by lens yellowing rather than by selective damage to the S-cone system. Further complicating matters, Greenstein et al50 found psycho-physical evidence for postreceptoral sensitivity loss in patients with diabetes. Holopigian et al51 used 2-color dark-adapted thresholds as well as electrophysiologic testing and concluded that there were rod and cone receptoral as well as postreceptoral deficits.

Considering our previous results showing selective loss of S-cones in human retinal detachment,20 we wanted to determine if a similar pathogenic mechanism was involved in diabetic retinopathy, which might explain the observed color vision loss in this disease. Two methods were used to accomplish this goal. First, to allow a qualitative assessment of cell death, retinal tissue was labeled using the terminal deoxynucleotidyl transferase (TdT)–mediated biotin-deoxyuridine triphosphate (dUTP) nick end labeling (TUNEL) method. Second, a quantitative analysis comparing densities of S-cones as well as L/M-cones was conducted. Because of the great biochemical similarity of the L/M-cones, no convenient method has been developed to distinguish these 2 cone types in postmortem tissue. However, the technique of enzyme histochemical analysis for carbonic anhydrase (CA) described in the “Subjects, Materials, and Methods” section is a rapid method for distinguishing the S-cones (negative for CA) from the L/M-cones (positive for CA) in human eyes, so long as too much autolysis has not occurred.

Occasional nuclei were found to be positive by the TUNEL method in all 5 diabetic retinas tested but not in the controls. Cells positive for CA were found in both the inner and outer nuclear layers. In one case, a positively labeled nucleus (possibly from a cone)20 was seen that was external to the outer limiting layer (Figure 3).

Unlike the controls, the diabetic eyes showed patchy losses of CA-negative cones and rods (presumably S-cones and rods) (Figure 4). On average, there was reduction in the percentage of S-cones in all directions and at all eccentricities in the macula of the diabetic eyes.
compared with the control eyes (Figure 5). However, for individual eyes, these losses were sometimes irregular (Figure 6). A significantly lower density of S-cones was found at nearly all retinal eccentricities from 0.1 to 15.0 mm (Figure 7). By comparison, the mean density of L- and M-cones was significantly reduced at only 1.5 mm (Figure 8).

Since the S-cones normally have a regular distribution with respect to the L/M-cones,13,26 we reasoned that measuring the ratio of the S-cone to L/M-cone densities would be a sensitive measure since, being a ratio, it would compensate for artifacts such as irregular tissue shrinkage during embedding. Thus, as shown in Figure 9, the S-cones as a percentage of all cones were significantly reduced in the diabetic eyes at all but 3 eccentricities. On average, the percentage of S-cones compared with L/M-cones was decreased by 21.0%±3.4% (mean±SEM) in the eyes with diabetic retinopathy compared with controls.

COMMENT
Selective dropout of S-cones is a consistent finding in diabetic retinopathy. The loss is, on average, distributed rather evenly throughout the macula and along the horizontal meridian, extending at least to 15 mm beyond the fovea. Selective S-cone injury is similar to (though less extensive than) what is seen in retinal detachment26 but contrasts with the L/M-cone damage found in glaucoma.28

An effort was made to select eyes with a range of diabetic retinopathy. However, because complete ocular histories were not available for all patients and because of the small sample size, it was not possible to correlate S-cone loss and severity of disease. Simple inspection of the postmortem retinas with the dissecting microscope is not a substitute for detailed history because diabetic retinopathy can become inactive after several years, leaving little evidence of damage at this level.

Given the limited ocular histories for some of the subjects and especially for the controls, it is important to consider the other retinal diseases that might be common enough to act as confounding variables. The only entity that fits this category is glaucoma. Although macular degeneration is quite common among this age group, even mild forms of the disease are easily identified on inspection of the posterior poles with the dissecting microscope. No such eyes were included in this study. By contrast, glaucoma is often not evident in gross specimens. Moderate cupping of the optic nerve can be obscured by postmortem changes and fixation. Glaucoma is also a concern because it may58-60 (or may not61,62) be more prevalent in patients with diabetes mellitus. Fur-
Furthermore, glaucoma is now known to affect the photoreceptors in the outer retina. Even so, Klein, et al found that the difference in the prevalence of glaucoma between patients with older-onset diabetes and those without diabetes was only 2.2% (4.2% vs 2.0%, respectively), so it is unlikely to be a major factor in such a limited sample as ours. More importantly, most of the outer retinal damage in glaucoma seems to be concentrated in the L/M-cones, whereas the S-cones were selectively lost in our diabetic eyes.

Findings from the TUNEL method for some of the nuclei in patients with diabetes were consistent with...
Figure 7. Density of S-cones as a function of retinal eccentricity ± SEM. An analysis of variance was used to examine whether S-cone density at various eccentricities differed between diabetic and control subjects. Eccentricity, a repeated-measures variable, was evaluated with Huynh-Feldt adjustments to the degrees of freedom to correct for correlated repeated measures. A type IV sum-of-squares error term was used owing to missing data at some eccentricities. S-cone densities differed significantly with eccentricity ($F_{1,8.92}=109.05, P<.001$). There was no overall difference in densities between the groups ($F_{1,2}=10.89, P=.08$). However, there was a significant difference between groups in S-cone density with eccentricity ($F_{1,8.92}=3.89, P<.005$). Asterisks indicate that the difference in means are significant ($P<.05$) by independent samples t tests assuming unequal variances and evaluated using a 1-tailed probability distribution due to the constraint that diabetic L/M-cone densities cannot exceed control values. The diabetic retinas show a significantly lower mean S-cone density at most eccentricities.

Figure 8. Density of L/M-cones as a function of retinal eccentricity ± SEM. These data were analyzed using the same analysis of variance design used for analyzing short (S) cones. Although densities change significantly with eccentricity ($F_{1,8.92}=32.05, P<.01$), there is no difference between groups ($F_{1,2}=1.95, P>.19$), nor do groups differ significantly by eccentricity ($F_{1,8.92}=0.41, P>.71$). Findings from the t tests performed for S-cones density data (Figure 7) were significant (asterisk) for only 1 of 13 tests.

Figure 9. Percentage of cones that are blue sensitive as a function of retinal eccentricity (means for all directions) extending to 15 mm ± SEM. A greater number of missing cells from the joint occurrence of the S and L/M-cone density data sets precluded performing an analysis of variance. Independent groups t tests were performed for the density data in Figure 7 and Figure 8; however, a 2-tailed test was used since the relative percentage of S-cones is not constrained and could increase if there were a decrease in L/M-cones. In patients with diabetes, most eccentricities have a significantly lower mean percentage of S-cones compared with the controls (asterisks). Note scale change at 2 mm eccentricity.

Cell death in the inner nuclear layer as well as the outer nuclear layers in diabetic retinopathy. In such a chronic disease, which can last for years or decades, it is expected that, even if cell death were common, only rare cells would be found positive by the TUNEL method. This is because in cells undergoing death by apoptosis, there is only a relatively brief time interval during which the nuclei may be found positive by the TUNEL method—perhaps as short 1 to 3 hours in normally cycling cells of the intestine, epidermis, lymphoid tissue, etc., and 8 to 11 hours for ganglion cells in axotomized rats.63 Finally, positive labeling with TUNEL method alone, though consistent with programmed cell death (apoptosis), does not rule out death by necrosis.64 Nevertheless, positive findings from the TUNEL method in our diabetic retinas and absence of such findings in the control retinas suggest that retinal cell death is occurring in diabetic retinopathy.

Although diabetic retinopathy is sometimes thought of as an inner retinal vasculopathy, loss of photoreceptors indicates damage to the outer retina as well. Outer retinal injury might be predicted, considering the studies showing that choroidal vasculopathy is an important element in this disease.65,66 Partial loss of S-cones could contribute to the color vision deficit in diabetic retinopathy. This does not rule out the possibility that factors in addition to photoreceptor death may be involved as well. Indeed, color vision deficits have been observed even in early diabetic retinopathy,67,68,69 including patients with diabetes with angiographically normal retinas.69 Unlike the findings of nearly total S-cone loss in human traumatic retinal detachment and experimental retinal detachment in monkeys, only a portion of the S-cones was lost. Therefore, the contribution of other prereceptoral or postreceptoral mechanisms cannot be ruled out.

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