Proc. Natl. Acad. Sci. USA
Vol. 82, pp. 6716–6720, October 1985
Neurobiology

Calcium binding in pigmented and albino eyes

(melanin/calcium autoradiography/retinal pigment epithelium/tapetum lucidum)

URSULA C. DRÄGER

Harvard Medical School, 25 Shattuck Street, Boston, MA 02115

Communicated by David H. Hubel, June 14, 1985

ABSTRACT **The localization of calcium binding sites in eyes was determined autoradiographically after extracting endogenous Ca from tissue sections and replacing it with ^{45}Ca. The strongest labeling was associated with pigmented tissues due to the high concentration of melanin, which was shown to bind Ca effectively and in a pH-dependent fashion. The second strongest binding was over the tapetum lucidum of the cat eye, and moderate labeling was associated with eye muscles and epithelium and endothelium of the cornea. The neural retina was generally more lightly labeled than the surrounding tissue of the eye; here the plexiform layers stood out in comparison to the nuclear layers, as did a band located internal to the photoreceptor outer segments. The possibility that the Ca buffering capacity of melanin may represent the common denominator for the various neurological defects found in hypopigmentation mutants is discussed.**

Hypopigmentation mutants in mammals have two types of visual abnormalities that cannot be explained by insufficient light screening but point to another, unidentified function of pigment. The first abnormality is due to miswiring of optic connections; it includes the extensively studied defect in optic nerve crossing (1, 2) and probably also the eye movement defects (3–5). The second type is a dynamic defect that depends on direct contact of the neural retina with the retinal pigment epithelium (RPE): in optic nerve recordings from intact pearl mice, a hypopigmentation mutant not allelic with the albino, visual thresholds were elevated about 100 times at dim backgrounds, as compared to fully pigmented mice, but were about normal at bright ambient illumination (6); when recordings were done from ganglion cells in isolated retinas, however, visual thresholds of normal and pearl mice were indistinguishable (7). Defects in optic nerve crossing and eye movements are caused by mutations at any of several genes involved in pigmentation; severity of defects correlates roughly with the degree of impairment in pigmentation of the RPE and not with pigment in any other tissues (8–10). This is consistent with the notion that the defects are expressed in the embryo, because the RPE becomes pigmented at early stages of eye development, long before any other pigment appears in the organism; the choroid of the eye, for instance, assumes pigment only several days after birth in mice, at a time when optic connections are for the most part well established. It is not known with which pigment, if any, the light sensitivity defect may correlate, as it has only been described in pearl mice; a similar defect does seem to be present in albino mice (unpublished observations) and it may also be reflected in visual abnormalities found in albinos of several species, including humans, which cannot be explained by light scatter or aberrant crossing (11, 12).

METHODS AND RESULTS

Mice were perfused with paraformaldehyde and (in some cases) glutaraldehyde, and their eyes were dissected as eye cups, cut on a cryostat at 10 μm, extracted with 20 mM EGTA and 20 mM EDTA, and washed in pH 7.4 Tris buffer that had been stored over chelating resin. The sections were incubated for an hour with about 5 μm ^{45}Ca (New England Nuclear; specific activity 14 mCi/mg; 1 Ci = 37 GBq), washed thoroughly, and pressed for a few hours to a few days against pieces of 35-mm film (Kodak Tech Pan or Ilford HP5) together with controls to exclude chemography. Fig. 1*A* shows a direct print of the unstained, uncleared sections and Fig. 1*B* shows a print of the autoradiograph. There were no obvious indications of increased Ca binding to locations rich in phosphorylated neurofilaments (13) such as the optic nerve (arrow) under any of the conditions used here nor was there a difference with the state of light adaptation (upper versus lower eye cups in Fig. 1*A*). The one Ca binding site that overshadowed all more subtle inhomogeneities was associated with pigmented tissues: the left, heavily labeled pair of eyes was from a normally pigmented mouse (C57BL/6J) and the right pair was from a congenic albino mouse (C57BL/6J-c^{2J}). The back of the eye contains two layers of pigmented tissue: the RPE, which derives from the neural tube, and the choroid, in which the pigment is of neural crest origin. The contributions of these two layers to the Ca autoradiography were distinguished by comparing the eye of a steel-Dickie mouse (DBA/2J-Sl/Sl^d) to the other two; steel mutant mice are white with black eyes, because all neural-crest-derived pigment is missing but the neural-tube-derived pigment is normal (14). Fig. 1*C* shows autoradiographs of sections through whole eyes with appendages: in the steel mouse the Ca label in iris and ciliary body looks almost normal, but the band around the back of the eye is substantially thinner, as normally the choroid contributes the bulk of the pigment here.

In the embryonic RPE accumulation of Ca began with appearance of pigment at embryonic day E11. Fig. 2 shows coronal sections through heads of pigmented and albino embryos (about E14): here and in other sections it was evident that no tissue in the head or entire body labeled as intensely as the pigmented RPE; no comparable labeling was obvious in the albino. Affinity of Ca was not restricted to eyes of mice, but rather was apparent in all pigmented tissues of several vertebrates examined, and the intensity of the label always correlated with the degree of pigmentation.

To test whether the Ca binding is a property of melanin itself, synthetic melanin (Sigma) was put into dialysis tubing (3500 or 8000 molecular weight cutoff), extracted with 20 mM EGTA and 20 mM EDTA, washed, and dialyzed to equilibrium with different concentrations of labeled Ca and at different pH levels. Under all conditions the melanin fraction accumulated a substantial amount of the label, but the

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviation: RPE, retinal pigment epithelium.

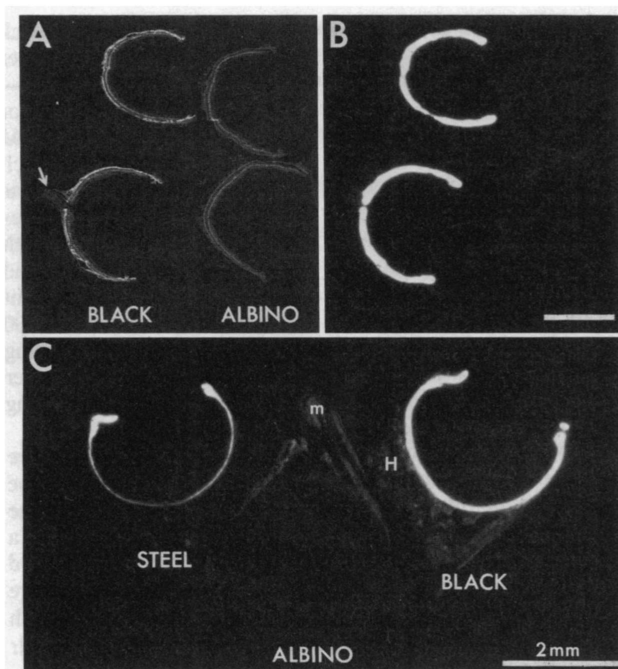

FIG. 1. Direct (negative) prints of unstained, noncleared sections through pigmented and albino eye cups (*A*) and of Ca autoradiograph (*B*). (*C*) Negative print of Ca autoradiograph from a section through eyes of steel, albino, and black mice. The steel mutant has only neural-tube-derived pigment. Note also labeling of the Harderian gland (H) and eye muscles (m).

binding (at 1 mM Ca) was about four times higher at neutral pH than at pH 4. This pH dependence resembled the pH dependence of Ca binding to eye cups, where it was more conveniently tested, as mouse eye cups are much smaller and contain their melanin better than the dialysis tubing. Eye cups, like the ones sectioned for Fig. 1*A*, were dissected from perfusion-fixed pigmented and albino mice, and endogenous

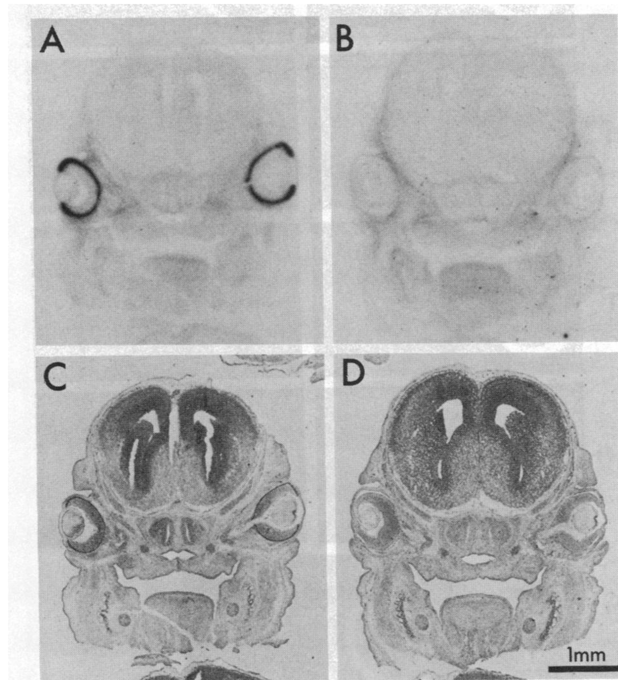

FIG. 2. Positive prints of unstained and noncleared coronal sections through heads of pigmented (*C*) and albino (*D*) embryos; autoradiographs are shown in *A* and *B*; sections were washed for 30 min following ^{45}Ca incubation and were exposed for 40 hr.

Ca was extracted and replaced by labeled Ca at pH levels from 4 to 7.5, with a combination of acetate/Mes/Hepes buffers (Sigma). Fig. 3*A* shows the accumulations at 1 mM Ca concentration: labeling decreased with decrease in pH both in the pigmented and albino eye cups, indicating that protons compete with Ca at most of the Ca binding sites.

Under the conditions of Fig. 3*A* the pigmented eye cups accumulated about twice as much label as the albino ones: this difference is obvious but it does not approximate the one seen in the autoradiographs. The discrepancy appeared to be due to the choice of affinity conditions: the sections were incubated with 5 μM Ca and thoroughly washed, whereas the eye cups were brought to equilibrium with 1 mM Ca. To test this explanation, pigmented and albino eye cups were equilibrated with 1 mM labeled Ca in pH 7.4 Tris buffer and then vigorously washed; single eye cups were removed at defined times and assayed for radioactivity. Fig. 3*B* shows that the black eye cups again bound about twice as much Ca prior to washing as the albino ones, but the difference increased with washing time. After 5 hr the black eye cups contained about 15 times more label than the albino ones. This explains why rinse times, ranging from 5 min to 5 hr, seemed rather uncritical for the autoradiographical appearance of the pigment label: the overall labeling intensity decreased slowly, but the pigment signal-to-background noise increased; in the 10-μm sections the affinity difference, apparent in Fig. 3*B*, must be reached within minutes of rinse times. To illustrate some of the more subtle Ca binding sites, sections were rinsed for relatively short times or were overexposed with respect to the pigment label (or both). Fig. 1*C* shows the relatively high accumulation of label over eye muscles, and other examples are given in Fig. 4: the corneal epithelium and endothelium accumulated rather high amounts of Ca (Fig. 4*A*); in the neural retina the plexiform layers were relatively more heavily labeled than the nuclear layers, as was a line

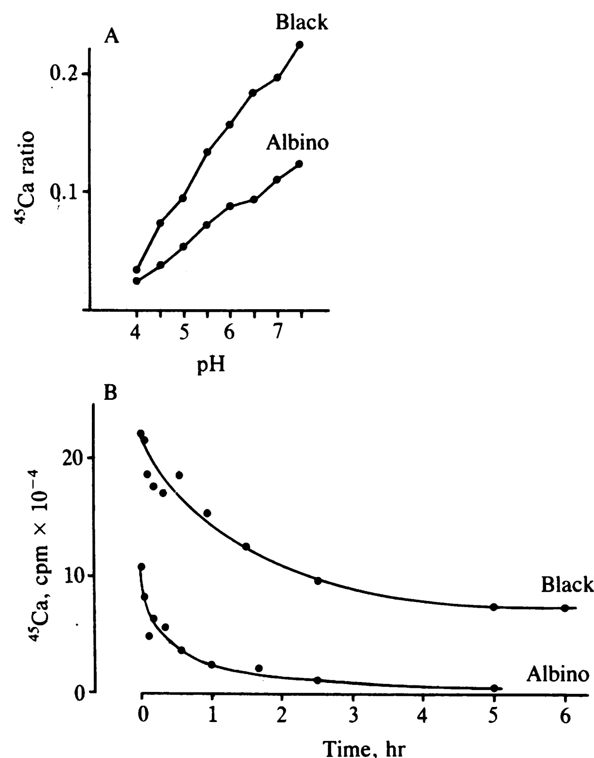

FIG. 3. Ca binding to pigmented and albino eye cups as a function of pH (*A*) and of rinse time (*B*). ^{45}Ca ratio indicates the total radioactivity of the eye cups divided by the total radioactivity of the buffers; buffer volumes were 1 ml; single eye cups weighed between 6 and 6.7 mg.

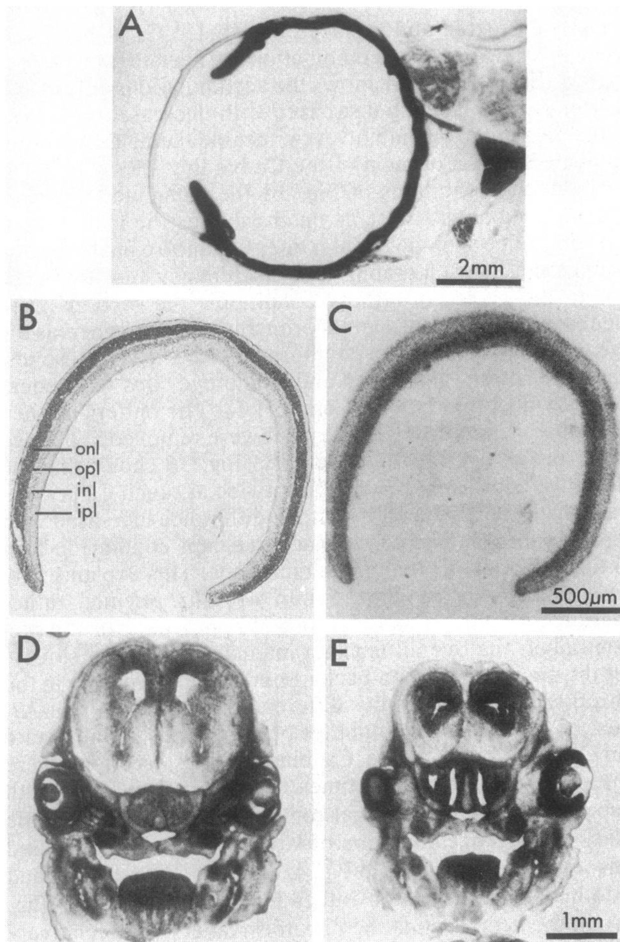

FIG. 4. Ca autoradiographs demonstrating nonpigment related binding. (*A*) Section through eye of ferret, washed for 5 min and overexposed (3 days): the heavy pigment labeling covers the entire neural retina; note the lighter labeling of corneal epithelia and eye muscles. (*B* and *C*) Print and autoradiograph of section through mouse retina (wash time, 15 sec; exposure, 1 week). The labeling seen here was the same in light- and dark-adapted retinas, and it was not altered when all incubations were done in phosphate buffer in order to inhibit phosphatases. Retinal layers: onl, outer nuclear layer; opl, outer plexiform layer; inl, inner nuclear layer; ipl, inner plexiform layer. (*D* and *E*) Autoradiographs of embryo sections from same block as in Fig. 2 (wash time, 15 sec; exposure, 4 days). The left section is from an albino and the right one is from a pigmented embryo at a somewhat more rostral level.

that localized to the outer edge of the retina but internal to the outer segments (Fig. 4 *B* and *C*); the embryo sections showed labeling patterns over different tissues that were as reproducible as a histological stain (Fig. 4 *D* and *E*).

An approximate value of the melanin content in the eye cups (about 50 μg) was obtained by comparing the optical density of Protosol-dissolved pigmented eye cups to albino eye cups with known amounts of synthetic melanin added. From the Ca binding to eye cups and from the dialysis experiments with synthetic melanin it was estimated that at 1 mM Ca concentration 1 g of melanin binds between 10 and 100 mg of Ca. The estimate for natural melanin was higher than for synthetic melanin, which might point to a Ca binding factor in pigmented melanosomes in addition to melanin. However, since the defect in the albino mutant is presumably restricted to the tyrosinase locus and since no elevated Ca binding was seen in albino RPE and choroid, it is more likely that the higher Ca/melanin estimate for eye cups reflects the tight and probably ordered packing of natural melanin in melanosomes.

The next question asked was to what extent other cations can interfere with the Ca binding. Ca-depleted sections of black and albino eyes were incubated in ^{45}Ca together with other divalent cations—magnesium, manganese, zinc, and barium—either at equimolar ratios (10 μM Ca^{2+} + 10 μM metal^{2+}) or with the other metals at higher concentration than Ca (10 μM Ca^{2+} + 5 mM metal^{2+}). Fig. 5 shows autoradiographs of such experiments: at low concentrations and equimolar conditions the Ca binding was not noticeably affected, but at 500 times higher concentrations the other metals partially interfered with the Ca binding; the effect of Mg was minimal, but Mn, Zn, and Ba substantially reduced the Ca binding. The monovalent cations sodium and potassium had no effect, even at 20,000 times the concentrations of Ca. The reduction of Ca binding probably reflects binding of the other cations to melanin, either at the same sites as Ca or at neighboring sites whose binding causes a reduction in Ca sites.

If the Ca binding to pigmented layers of the eye is more than a coincidence and serves a function in vision, it could be argued (see below) that the light sensitivity defect in the hypopigmentation mutant pearl might reflect a defect in Ca regulation. There is, however, a problem: pearl's impaired light sensitivity is only obvious at dim backgrounds, but there is a large number of species adapted for night vision, which lack black pigment over the most important part of their retina, at the tapetum lucidum. This highly reflective layer behind the neural retina, which serves to bounce off nonabsorbed photons and give the photoreceptors a second chance, differs widely between species in composition and embryonic origin (15). In the cat, the species tested here, the tapetum is made up of modified choroidal cells. Sections through cat retina with tapetum and choroid were labeled with ^{45}Ca: as in mice there was massive accumulation in

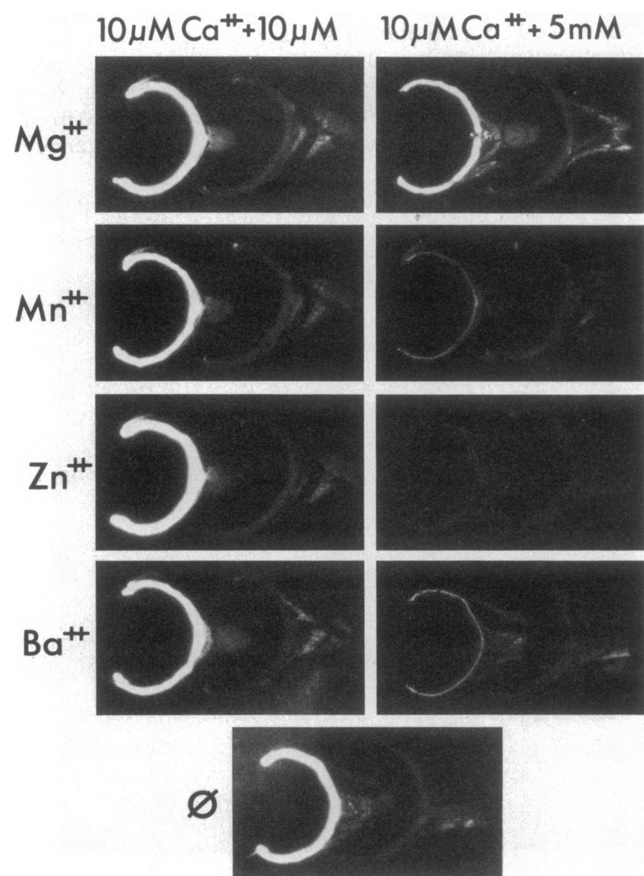

FIG. 5. Autoradiographs of sections through pigmented and albino eyes incubated with ^{45}Ca in the presence of other cations as indicated; 0 indicates no additional cation. [Direct (negative) prints.]

pigmented sites, but, in addition, there was a weaker accumulation over the tapetum, which, in Fig. 6, has been isolated by scraping away on the slide the melanin-containing choroid external to the tapetum; Fig. 6*A* shows a positive print of the unstained section, Fig. 6*B* identifies the tapetum through its autofluorescence (due to riboflavin; ref. 16), and Fig. 6*C* shows the overexposed autoradiograph; on shorter exposures the tapetum was labeled with about half the intensity as the choroid. Internal to the band of label corresponding to the tapetum a thin band can be seen in Fig. 6*C* over the neural retina, which appeared to localize internal to the outer segments, approximately to the level of the outer limiting membrane. This line of label may correspond to the outer band in the isolated mouse retina (Fig. 4*C*); what it represents is not clear.

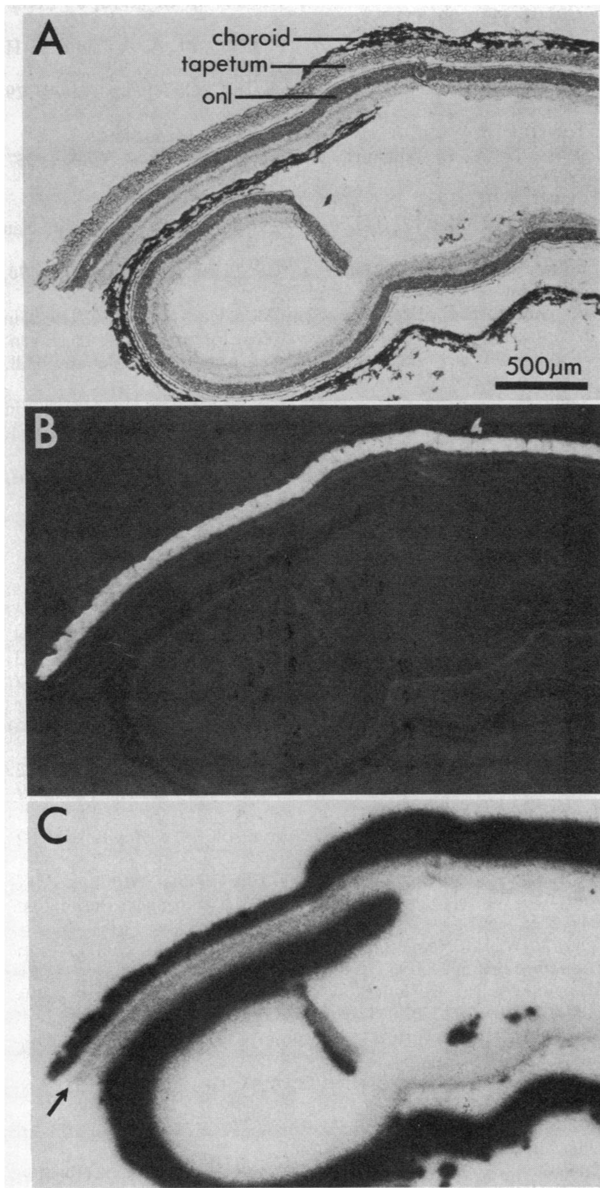

FIG. 6. Section through curled-up retina of cat. (*A*) Positive print of unstained tissue; onl, outer nuclear layer. (*B*) Same section viewed under fluorescence microscope (fluorescein isothiocyanate optics) showing autofluorescence of the tapetum lucidum. (*C*) Ca autoradiograph; note the labeling of the tapetum and of a thin layer internal to the outer segments (arrow). The relative ratios of labeling were not influenced by incubation of the sections in 10 mM $ZnCl_2$ prior to incubation with ^{45}Ca in order to replace zinc removed from the tapetum by the chelating agents.

DISCUSSION

Melanin consists of structurally ill-defined polymers containing indole-5,6-quinone units (17). Except for a role in photoprotection, its biological function is unknown, although its unusual chemical composition provides some clues: due to the high content of quinone and hydroquinone groups, it constitutes a biological redox polymer, and under all conditions it has free radicals, which are thought to make it a scavenger for photolytically or otherwise generated free radicals (18). In addition, melanin complexes metals with an affinity that increases with increasing valence and atomic weight of the metal (19, 20): this explains reports of highly variable and sometimes excessive amounts of heavy metals in pigmented tissues and has led to the proposal that one function of melanin is to scavenge potentially harmful metals (20, 21). When Ca was included in those studies, its binding to melanin was found to be rather weak and reversible as compared to heavier metals; the alkali metals did not bind at all (18, 20). Because under normal physiological conditions Ca and Mg are the only metals with melanin affinity that are present in more than trace amounts, the Ca binding to melanin must be strong enough to account for the very high concentration found in pigmented tissues: Hess (22) measured 15 mM Ca in the RPE of frogs. Similarly, direct binding of Ca to melanin would explain the present observation that there is no structure in the eye and probably the entire organism that binds Ca as effectively as pigmented tissue. Since binding of an ion of such physiological relevance as Ca is not likely to represent a scavenger function of melanin, the possibility arises that melanin may somehow contribute to Ca regulation; however, so far only circumstantial arguments favor this suggestion.

As most of the metabolic supply to the outer portions of photoreceptors comes from the choroidal blood circulation and via the RPE, unusual properties of the RPE are thought to reflect the need of photoreceptors, and as Ca levels in outer segments are tightly regulated, the high Ca concentration in pigmented tissues behind the retina could indicate an involvement of the RPE in this process. The limited observations on the tapetum of the cat might suggest that alternate mechanisms may exist in this species to partially compensate for the lack of Ca binding to melanin in amelanotic fundus parts. It is peculiar, however, that this binding is in the choroid, which is separated from the photoreceptors by the unpigmented RPE. In view of the diverse nature of tapeta in different species, no general conclusions are possible here. Nevertheless, an asssociation of Ca binding structures with eyes may be more than a coincidence, as it applies also to invertebrates, who use screening pigments that differ from melanin: Schröder *et al.* (23) measured >100 mM Ca in the pigment granules of crayfish eyes, which contain ommochromes, and, similarly, in Ca autoradiographs of eye pigment mutants in *Drosophila* the highest binding correlates with the presence of ommochromes (unpublished observations).

In vertebrate retinas the Ca concentration within rod outer segments is thought to be regulated to a large extent by exchange with the interphotoreceptor space: cytoplasmic free Ca is increased by the dark current and decreased by the Na^+/Ca^{2+} exchanger; free Ca is relatively high in the dark and low in the light (24–26). Recently it has been shown that Ca is probably not the internal messenger within rod outer segments, but it may influence phototransduction through regulation of cGMP metabolism (27, 28): through inhibition of guanylate cyclase and stimulation of phosphodiesterase, it may adjust cGMP activity to a middle working range (29, 30). As these actions should mimic the effects of light and as they should be relatively more operative in the dark, when free Ca levels are high, they could be described as possible mechanisms in dark adaptation. Lowering Ca around and within

photoreceptors increases the dark current but has different effects on the light response depending on the level of Ca depletion: relatively minor depletion results in sensitivity loss for dim flashes but a steeper light/response curve ("supralinearity"), and severe Ca depletion causes an overall potentiation in the light response (31–34). These effects evidently require actions of Ca beyond those identified so far. It is possible that the electrical events in the RPE triggered by the electrical activity in photoreceptors (35) could result in slight pH shifts in intracellular compartments, which may cause rapid and massive changes in free Ca due to the high capacity and affinity of the Ca buffer. Such a mechanism could influence the *rate* of Ca changes in photoreceptors. (There was no indication that the Ca binding tested here could be directly influenced by light.)

The defect found in pearl mice, however, was related to the *steady state* of dark adaptation rather than its rate (6). If it were a reflection of a relative Ca deficit, one would have to postulate that the RPE somehow takes part in the regulation of Ca homeostasis in photoreceptors. In theory, it seems possible that the Ca concentration in the interphotoreceptor space may differ from the extracellular milieu, as the ionic composition here is to some extent controlled by the RPE (reviewed in ref. 36); in addition, in this context one wonders about the significance of the band of Ca binding internal to the outer segment layer (Figs. 4*C* and 6*C*). However, in recordings with Ca-sensitive electrodes from eye cups of light-adapted frogs, no obvious deviations from general extracellular levels were found (36). It may be of interest to measure Ca levels around photoreceptors at different illumination levels and to compare pigmented and albino eyes. Hypopigmentation is also associated with defects in the auditory system, which are believed to reflect a higher susceptibility to noise trauma (37). The only pigment in the auditory system is located in the inner ear, mainly in the stria vascularis, the tissue thought to generate the endolymph and, hence, to control the ionic milieu around the hair cells. Thus, in this respect a loose parallel exists between the strategic positions of the RPE and the stria vascularis.

In the developing mammalian visual system there is overwhelming evidence of an involvement of pigment in the correct formation of optic connections, although the mechanism is not clear. It has been proposed that pigment affects the routing of optic axons (38, 39). In addition, it can be argued that pigment is already involved earlier in the specification of the retina, since the RPE becomes pigmented at a time when the first ganglion cells leave the mitotic cycle and when the retina is presumably undergoing irreversible specification (40): lack of pigment does not infuence the retinal map, but it affects the determination of a property that makes optic axons either cross the midline of the brain or stay away from it, and the defect in optokinetic nystagmus can be most plausibly explained by a miswiring of inputs to one class of ganglion cells in temporal retina, resulting in an axial reversion in directional selectivity (41). When melanin is synthesized at a high rate in early eye development, the RPE may represent a temporary Ca sink, hence causing a lowering of Ca in cells of the adjoining neural retina with which it is connected via gap junctions at this stage (42, 43); this, in turn, might influence the timing sequence of other processes by slightly delaying the closure of gap junctions. In this case the albino abnormality may reflect a defect in temporal coordination (44). In addition, the emerging pigment may have an effect on Ca-dependent cell adhesion mechanisms, and a defect may relate to the abnormal course of optic axons found in hypopigmentation mutants (39).

I am grateful to G. Balkema, B. Baughman, L. Bodenstein, T. Fitzpatrick, S. Goldin, P. Hogan, M. Livingstone, and W. Schröder for valuable discussions and generous help; to S. Edelstein for technical assistance; and to J. Gagliardi for photography. Financial support was provided by National Institutes of Health Grant EY 01938 and a grant from the American Federation for Aging Research.

1. Lund, R. D. (1965) *Science* **149**, 1506–1507.
2. Guillery, R. W. (1982) *Contrib. Sens. Physiol.* **7**, 39–73.
3. Hahnenberger, R. W. (1977) *Exp. Eye Res.* **25**, 9–17.
4. Collewijn, H., Winterson, B. J. & Dubois, M. F. W. (1978) *Sciences* **199**, 1351–1353.
5. Balkema, G. W., Mangini, N. J., Pinto, L. H. & Vanable, J. W. (1984) *Invest. Ophthalmol. Vis. Sci.* **25**, 795–800.
6. Balkema, G. W., Pinto, L. H., Dräger, U. C. & Vanable, J. W. (1981) *J. Neurosci.* **1**, 1320–1329.
7. Balkema, G. W., Mangini, N. J. & Pinto, L. H. (1983) *Science* **219**, 1085–1087.
8. Sanderson, K. J., Guillery, R. W. & Shakelford, R. M. (1974) *J. Comp. Neurol.* **154**, 225–248.
9. LaVail, J. H., Nixon, R. A. & Sidman, R. L. (1978) *J. Comp. Neurol.* **182**, 399–422.
10. Mangini, N. J., Vanable, J. W., Williams, M. A. & Pinto, L. H. (1985) *J. Comp. Neurol.*, in press.
11. Creel, D. J., Dustman, R. E. & Beck, E. C. (1970) *Exp. Neurol.* **29**, 298–309.
12. Tomei, F. & Wirth, A. (1978) *Vis. Res.* **18**, 1465–1466.
13. Jones, S. M. & Williams, R. C.. (1982) *J. Biol. Chem.* **257**, 9902–9905.
14. Mayer, T. C. (1972) *Dev. Biol.* **23**, 297–309.
15. Rodieck, R. W. (1973) *The Vertebrate Retina* (Freeman, San Francisco).
16. Elliott, J. H. & Futterman, S. (1963) *Arch. Ophthalmol.* **70**, 531–534.
17. Fitzpatrick, T. B., Szabo, G., Seiji, M. & Quevedo, W. C. (1979) in *Dermatology in General Medicine*, eds. Fitzpatrick, T. B., Eisen, A. Z., Wolff, K., Freedberg, I. M. & Austen, K. F. (McGraw-Hill, New York), pp. 131–163.
18. Sealy, R. C., Felix, C. C., Hyde, J. S. & Swartz, H. M. (1980) in *Free Radicals in Biology*, ed. Pryor, W. A. (Academic, New York), Vol. 4, pp. 209–259.
19. Bruenger, F. W., Stover, B. J. & Atherton, D. R. (1967) *Radiat. Res.* **32**, 1–12.
20. Potts, A. M. & Au, P. C. (1976) *Exp. Eye Res.* **22**, 487–491.
21. Sowden, E. & Pirie, A. (1958) *Biochem. J.* **70**, 716–717.
22. Hess, H. H. (1975) *Exp. Eye Res.* **21**, 471–479.
23. Schröder, W., Frings, D. & Stieve, H. (1980) *Scanning Electron Microsc.* **2**, 647–654.
24. Yau, K.-W. & Nakatani, K. (1984) *Nature (London)* **309**, 352–354.
25. Yau, K.-W. & Nakatani, K. (1984) *Nature (London)* **311**, 661–663.
26. MacLeish, P. R., Schwartz, E. A. & Tachibana, M. (1984) *J. Physiol.* **348**, 645–664.
27. Fesenko, E. E., Kolesnikov, S. S. & Lyubarsky, A. L. (1985) *Nature (London)* **313**, 310–313.
28. Yau, K.-W. & Nakatani, K. (1985) *Nature (London)*, **313**, 579–582.
29. Troyer, E. W., Hall, I. A. & Ferrendelli, J. A. (1978) *J. Neurochem.* **31**, 825–833.
30. Robinson, P. R., Kawamura, S., Abramson, B. & Bownds, M. D. (1980) *J. Gen. Physiol.* **76**, 631–645.
31. Yoshikami, S. & Hagins, W. A. (1973) in *Biochemistry and Physiology of Visual Pigments*. ed. Langer, H. (Springer, Berlin), pp. 245–255.
32. Yau, K.-W., McNaughton, P. A. & Hodgkin, A. L. (1981) *Nature (London)* **292**, 502–505.
33. Hodgkin, A. L., McNaughton, P. A., Nunn, B. J. & Yau, K.-W. (1984) *J. Physiol.* **350**, 649–680.
34. Matthews, H. R., Torre, V. & Lamb, T. D. (1985) *Nature (London)* **313**, 582–585.
35. Steinberg, R. H., Linsenmeier, R. A. & Griff, E. R. (1983) *Vis. Res.* **23**, 1315–1323.
36. Kaila, K., Voipio, J. & Akerman, K. E. O. (1984) *Invest. Ophthalmol. Vis. Sci.* **25**, 1395–1401.
37. Conlee, J. W., Parks, T. N., Romero, C. & Creel, D. J. (1984) *J. Comp. Neurol.* **225**, 141–148.
38. Strongin, A. C. & Guillery, R. W. (1981) *J. Neurosci.* **1**, 1193–1204.
39. Silver, J. & Sapiro, J. (1981) *J. Comp. Neurol.* **202**, 521–538.
40. Dräger, U. (1985) *Proc. R. Soc. London Ser. B* **224**, 57–77.
41. Winterson, B. J. & Collewijn, H. (1981) *Brain Res.* **220**, 31–49.
42. Hayes, B. P. (1976) *Anat. Embryol.* **150**, 99–111.
43. Fujisawa, H., Morioka, H. & Watanabe, K. (1976) *J. Cell Sci.* **22**, 285–296.
44. Murakami, D., Sesma, M. A. & Rowe, M. H. (1982) *Brain Behav. Evol.* **21**, 67–113.