side, clockwise on the right). Shortening of the femoral axis in dorsoventral projection (not shown in Fig. 2) indicates that the proximal end elevates relative to the distal end in phases II and III; this movement may vary from as much as 90° to as little as 40°. The long axis of the femur also sweeps posteriorly during propulsion, starting at about 35° to 40° to the sagittal plane at phase I and ending at about 50° or more at phase III (Fig. 2A, C). At phase I the crural axis is nearly vertical although directed somewhat anterolaterally; the foot is directed laterally (Fig. 2A). In phases II and III the crural axis is approximately parallel to the sagittal plane, and the foot is rotated to point more posteriorly (Fig. 2, B and C). At the termination of phase III, the foot is directed posteriorly in a final thrusting movement (not illustrated). Except for the direction of movement and for the fact that the limb is lifted, phase IV is little different from phase II.

This analysis emphasizes some previously overlooked functional aspects of monotreme limbs and contradicts some common assumptions. Rotation is the principal locomotory movement of the humerus (*5*), not anteroposterior protraction as has been suggested (*3*). The antebrachium is directed ventromedially so that the manus, positioned approximately beneath the glenoid in phase II (Fig. 1B), supports the body in a more typically mammal-like stance than would be the case were the antebrachium perpendicular to the humerus as has been assumed (*6*). Femoral orientation, usually regarded as slightly anterior to the transverse plane (*6*), varies from approximately 35° to 50° from the sagittal plane and is thus similar to that in nonspecialized therians. Femoral elevation and depression in a 40° to 90° arc is likewise similar to a therian pattern. Statements that monotreme limbs are reptilian in posture (*4*) or that they sprawl in a manner comparable to that of lizards (*7*) are imprecise and with regard to the echidna, at least, are inaccurate. The limbs of the echidna support the body well off the ground, even when the animal is stationary or walking slowly. During propulsion, the femoral orientation and movement and the plantar contact of the manus beneath the glenoid are features found also in generalized therians that I have studied (for example, *Didelphis*). In contrast, the "sprawling" posture of most lizards (for example, *Iguana*) involves femoral angles that may vary from 50° (phase I) to 120° (phase III) and plantar contact of the manus lateral to the glenoid and elbow.

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Cones of Living Amphibian Eye: Selective Staining

Abstract. *The outer segments of cones are selectively stained by the chlortriazinyl dye Procion Yellow injected into the vitreous humor. Since the dye does not cross nerve cell membranes, the selective staining of cones is further evidence for structural differences between rod and cone outer segments. Specifically, it is believed that cone saccules are open to extracellular space whereas rod saccules are not.*

The terms rod and cone aptly describe the morphology of amphibian photoreceptor outer segments. In recent years evidence has gradually accumulated that these differences in form are accompanied by other important distinctions between the two. Specifically, the repeating lamellar membranes of cones appear to be contiguous with the cell membrane whereas those of rods do not (*1*); the rod basal lamellae regenerate continuously and thus migrate toward the pigment epithelium whereas those of the cones do not (*2*), and cones are the chief source of the early receptor potential (*3*) of the frog retina despite the preponderance of rod pigment (20:1) (*4*). To these distinctions we now add a striking disparity in staining reaction between rod and cone outer segments in the living animal, a disparity which we believe derives from the above-mentioned structural differences in outer segment membrane topography of the two types of photoreceptor.

We used the fluorescent dichlortriazinyl dye Procion Yellow (M-4RS, ICI America, Inc.) recently introduced by Stretton and Kravitz (*5*) as a single-cell marker in neurophysiology. Since it does not cross the cell membrane to leave a neuron once injected (*6*) and does not enter neural cells from extracellular space (*7*), it is virtually an ideal marker. Twenty-four hours after injection of 25 μl of a freshly made 0.5 to 4 percent aqueous solution of the dye into the vitreous humor, mudpuppies (*Necturus maculosus*) or frogs (*Rana pipiens*) were decapitated, and their eyes were enucleated and quenched in isopentane cooled to −130°C in a liquid nitrogen bath. Thereafter, the eyes were freeze-dried at −35°C for 3 days and then embedded in paraffin at 60°C. Sections were cut at either 5 or 12 μm, mounted in xylene and viewed in a Zeiss microscope equipped for fluorescence darkfield, fluorescence polarization, and birefringence observations.

When viewed either by darkfield (exciting filter BG 12, barrier filter Zeiss 50) or between crossed polarizers (exciting filters BG 12 and BG 3 in series, no barrier filter) the cone outer segments appeared brilliant yellow-orange whereas the rod outer segments were a dim green outlined by a fine yellow margin (Fig. 1). The cones were several orders of magnitude brighter than the rods. In control preparations (no Procion Yellow) there was little difference in the intensity of the dim fluorescence emitted by rods and cones and no orange color whatever (Fig. 2).

In an attempt to clarify what appeared to be a selective binding of Procion Yellow to cone outer segments, polarization studies were carried out. Visual receptor outer segments are anisotropic, characteristically showing transversely oriented lamellar membranes, visual pigment dichroism, and positive uniaxial birefringence. Procion Yellow contains several planar aromatic rings and if binding to outer segment membranes involves adsorptive (Van

der Waals, and so forth) forces that make the dye molecule conform to the planar lamellar surface, the bound dye might be expected to be dichroic just as rhodopsin is. Such dichroism would be detected in linearly polarized exciting light if the bound Procion fluorescence emission intensity were found to be dependent on the angle between the long axis of the cone outer segment and the polarization direction of the exciting light. No such dependence was found. The trivial explanation that this negative result might be due to disruption of lamellar order caused by the preparation procedure (freeze-drying, paraffin embedding, microtome sectioning, and xylene mounting) is ruled out by ancillary experiments in which single mudpuppy cone outer segments, isolated in saline from fresh retinas of eyes injected with Procion Yellow, also showed localization of bright fluorescence but no dichroism. At the same time, the saline-mounted outer segments showed positive uniaxial birefringence. Both paraffin-embedded, freeze-dried outer segments and outer segments in slides prepared as described above showed positive uniaxial birefringence which implies the continued integrity of the repeating lamellar substructure of outer segments. Thus, we feel that Procion Yellow is bound isotropically to cone outer segment lamellae. Our results are consistent with the suggestion that binding is caused by chemical reaction of the triazinyl end of the molecule with proteins (*8*) or polysaccharides (*9*) of membranes, the chromophoric end of the molecule retaining a high degree of rotational mobility about an axis normal to the lamellar plane at each attachment site. If present, this type of binding should show no polarization of fluorescence emission as opposed to the 2:1 fluorescence polarization ratio to be expected if the dye is isotropically but rigidly bound (no rotational and torsional mobility). Although evaluation of the very dim emission of single receptors through crossed exciting and analyzing polarizers is difficult we found no evidence for fluorescence polarization.

Thus, we view the interaction of the dye Procion Yellow with neural cells in the following way (in collagenous tissue or those with substantial amounts of ground substance, obviously different chemical conditions obtain). On injection, either intra- or extracellularly, the dye molecule (molecular weight ~500) diffuses rapidly in the aqueous phase until it encounters a free hydroxyl,

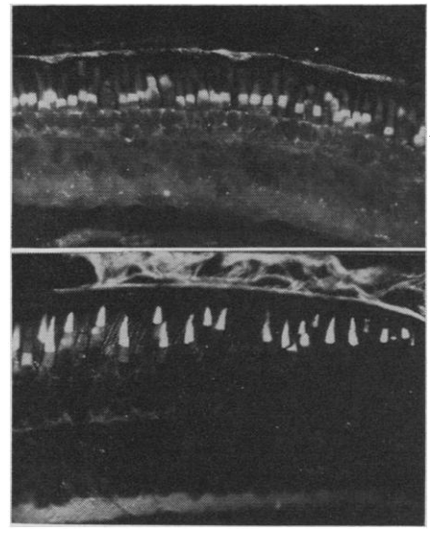

Fig. 1 (top). Brilliant cone outer segments stand out in stark relief from unstained rods in *Necturus* retina. Magnification × 500. Fig. 2 (bottom). Control retina. Neither rod nor cone outer segments are bright. Due to the prolonged exposure time necessary to photograph the dim fluorescence of rod and cone outer segments, the inner segments of these structures appear far brighter than in Fig. 1. Magnification × 320.

sulfhydryl, or amino group of any molecule (sugar, protein, nucleic acid) with which its chlortriazinyl group can react, forming a covalent bond between dye and biological molecule (*8*, *9*). If the biological molecule happens to be part of a larger composite, such as cell membrane or endoplasmic reticulum, the dye is no longer free to move translationally, although it may retain rotational mobility. If there is a high concentration of binding sites over a sufficiently large area and stoichiometrically sufficient dye, the high resulting mass of fluorescent material will permit easy visualization when properly excited. When the binding sites are on the outside of a cell envelope membrane, there is insufficient fluorescent mass (due to the small dimension of sections of one membrane surface) to permit ready visualization. However, highly convoluted membranes may provide a high mass of fluorescent material within a small viewing area allowing easy visualization of fluorescence. For this reason, we believe that amphibian cone outer segments are made visible by extracellular Procion Yellow which easily diffuses into the spaces between continuous, folded outer segment membranes to bind with the membrane. Rod outer segments do not stain similarly because their envelope membrane is not infolded. Rod outer segment lamellar membranes are instead pinched off from the envelope membrane and retain no contiguity with extracellular space [except for the few basal lamellar sacs that are newly formed and which in many instances are found to stain with Procion Yellow (*10*)]. Such a hypothesis leads to the suggestion that densely infolded membrane regions of other cells such as renal tubule, gut epithelium or electroplac brush border will be found to stain brightly with extracellular Procion Yellow.

Clearly, our results are compatible with those of Cohen (*10*, *11*), who, on the basis of experiments with lanthanum infiltration in the frog retina, concluded that rod outer segments are closed and cone outer segments are open to extracellular space. When taken together our experiments and Cohen's strongly support theories that link the large extracellular surface area of cone outer segments to the dominant role of the cone in the generation of early receptor potential (*3*).

To our knowledge, there are no previous reports of selective staining of cones in vivo. There has, however, been continued interest in selective staining of rods and cones in vitro (*12*). The pertinence of most in vitro methods to the present report is unclear. Since in all techniques in vitro aphysiological procedures–fixation, dehydration in fat solvents, microtome sectioning–are used which alter the structure and reactivity of rod and cone outer segments, it is not possible to judge a priori how much of the selectivity of staining is dependent on preparation.

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Defect in DNA Synthesis in Skin of Patients with Xeroderma Pigmentosum Demonstrated in vivo

Abstract. *Exposure of normal human skin in vivo to ultraviolet irradiation at wavelengths shorter than 320 nanometers stimulated an unscheduled DNA synthesis in all of the cell layers of the epidermis and in the upper dermal fibrocytes. The skin of patients with xeroderma pigmentosum did not show this response. Correlation of these findings with previous tissue culture studies suggests that the defect in repair of the damaged DNA in xeroderma pigmentosum cells occurs in vivo as well as in vitro.*

Stimulation of an unscheduled DNA synthesis by ultraviolet irradiation has been demonstrated by autoradiography in a number of mammalian cell types, including human skin cells in vivo and human fibroblasts in vitro with [^{3}H] thymidine (TdR) as the radioactive tracer (*1–3*). The unscheduled synthesis is represented by a uniform, sparse, nuclear labeling of cells not in the DNA replication phase of the cell cycle, whereas cells synthesizing DNA in preparation for division show a dense accumulation of silver grains. This unscheduled synthesis induced by ultraviolet irradiation is apparently missing or is greatly reduced in cultured fibroblasts from patients with the genetic disease xeroderma pigmentosum (XP) (*2*, *3*). This is a rare, inherited, autosomal, recessive disorder characterized by a peculiar sensitivity to ultraviolet radiation which results in multiple cutaneous malignancies within the first few years of life (*4*). We attempted to determine whether the defect in response to ultraviolet radiation noted in cultured XP fibroblasts could be demonstrated in the skin of these patients in vivo. Patients with the two clinical varieties of XP were studied; one individual was affected with skin changes only and two patients were affected with neurological and skin abnormalities (the de Santis-Cacchione syndrome). These were the same patients whose fibroblasts Cleaver studied initially (*2*). Eight normal subjects served as controls.

The clinically undamaged skin of each subject was irradiated with 13.6 $\times$ 10^6 erg/cm^2 of ultraviolet energy at wavelengths shorter than 320 nm (Hanovia hot quartz contact lamp). Fifteen minutes later, [^{3}H]TdR (10 μc; 11 c/mmole) was injected intradermally into the irradiated and adjacent nonirradiated skin of each subject. Biopsies of the injected sites were obtained 1 hour later, and the tissue was fixed in a mixture of formalin, acetic acid, and alcohol. After the specimens were dehydrated, they were embedded in paraplast and sectioned at 4 μm. The sections were then coated with Kodak nuclear track emulsion (type NTB-2). The tissue was exposed for

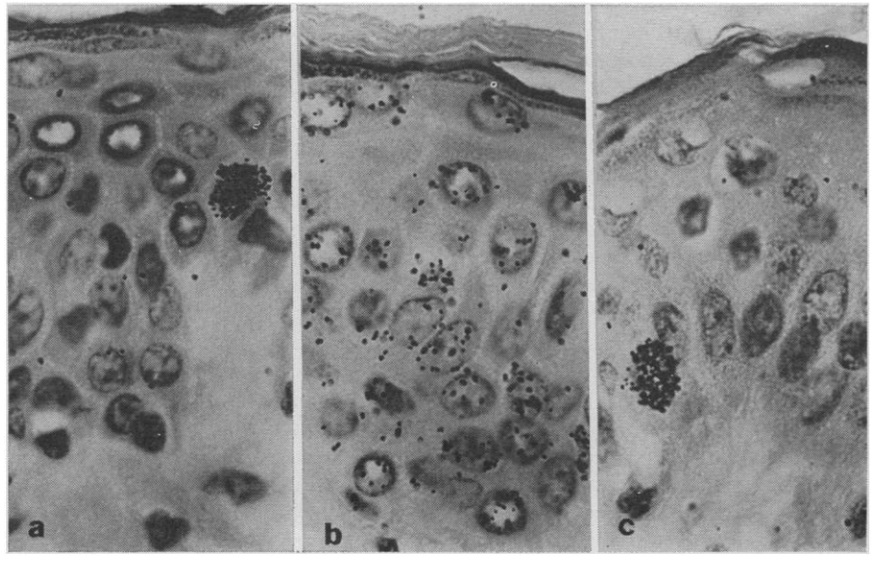

Fig. 1. (a) Normal human epidermis not subjected to ultraviolet irradiation. The autoradiograph shows the dense labeling of one basal cell in the premitotic DNA synthesis phase of the cell cycle. (b) Normal human epidermis subjected to ultraviolet irradiation. The autoradiograph shows sparse labeling of cells throughout the epidermis. (c) Epidermis of a patient with XP subjected to ultraviolet irradiation. Autoradiograph shows one densely labeled basal cell, but no sparse labeling.

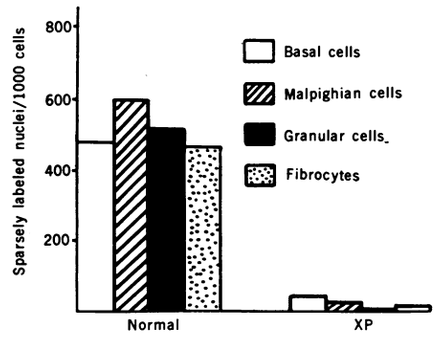

Fig. 2. The mean values for sparsely labeled nuclei (3 to 15 silver grains) in normal skin subjected to ultraviolet irradiation (eight subjects) are compared with mean values for sparsely labeled nuclei in skin of XP patients subjected to ultraviolet irradiation (three patients). The difference between the counts in each cell type of the normal skin and skin of patients with XP revealed a $P < .01$ with the t-test for comparison of means and standard deviations. The subjects were injected with [^{3}H] TdR 15 minutes after ultraviolet irradiation.