somal fractions and serum and evoked high incorporation of ^{59}Fe into RBC in the assay mice was actually Ep, a separate experiment was performed in which each pooled fraction was divided into three aliquots following membrane-disruptive procedures. One aliquot was incubated with saline, another was incubated with an identical volume of NRS, while the third was incubated with NRS followed by antiserum against Ep prepared from rabbits immunized against human urinary Ep as described by Schooley and Garcia (*14*). Excess antibody was removed by incubation of the mixtures with goat antiserum against rabbit gamma globulin before injection into the assay mice. The results indicate that treatment with antiserum against Ep abolishes the erythropoiesis-stimulating activity of the incubated mixtures in the assay animals. It is concluded, therefore, that the increased ^{59}Fe incorporation into RBC observed in these experiments is attributable to the formation and specific action of Ep. In this experiment with antibody against Ep, the Ep specific activity of the prezonal light mitochondrial fraction was 4 compared with values of 26 and 19 for the Triton-treated and -untreated lysosomes, respectively.

RICHARD M. LIBBIN
Department of Biology, Graduate School of Arts and Science, New York University, New York 10003
PHILIP PERSON
Veterans Administration Hospital, Brooklyn, New York 11209
ALBERT S. GORDON
Department of Biology, Graduate School of Arts and Science, New York University

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Topology of the Outer Segment Membranes of Retinal Rods and Cones Revealed by a Fluorescent Probe

Abstract. *When* N,N′*-didansyl cystine binds to the cell membranes of vertebrate rods and cones its fluorescence efficiency increases about 20-fold. The entire outer segments of living cones become brilliantly fluorescent. Stained live rods, as well as most freshly detached outer segments, are only weakly fluorescent, but they become brightly fluorescent within a few seconds if their plasma membranes are osmotically ruptured. The difference in staining of rod and cones suggests that disk membranes of rods are not continuous with the plasma membranes are osmotically ruptured. The difference in staining of rod and cones plasma membrane on outer segments of photoreceptors in electrophysiological and biochemical experiments, and to study the infolding pattern of rod and cone disks.*

The outer segments of vertebrate rods and cones consist largely of stacks of phospholipid pleats formed by regular infolding of the plasma membranes during cell growth (*1*). Electron microscopy suggests that the topological connectivity of rod membranes is different from that of cones. The infolded membranes of rods apparently detach from the plasma membrane to form stacks of closed, flattened sacs suspended in the cytoplasm. The infoldings of cones appear to remain continuous with the plasma membrane so that the space between alternate membranes is an extension of the extracellular fluid space (*2*). Experiments on the cable properties of live rod outer segments of rats indicate that the electrical capacitances of their plasma membranes are those of smooth cylindrical lipid bilayers. Thus, an electrical measurement seems to confirm that the internal sacs of rods are not continuous with the plasma membrane (*3*). This difference in topology of rods and cones is not a minor curiosity; it implies that excitation of rods by photons absorbed in the rhodopsin-bearing sacs cannot spread to the plasma membrane by electrical polarization of a membranous connection between the two structures. Instead, a chemical transmitter substance released by the rod disks might be needed to explain the ability of light to reduce the Na^+ permeability of the overlying plasma membrane (*4*). Since it is important to know if the structural and electrical evidence is correct, we now report a simple procedure for demonstrating the different topologies of the plasma membranes of retinal rods and cones on the basis of their staining behavior in solutions of the fluorochrome, *N,N′*-didansyl cystine, denoted here by DDC (*5*). Unlike both electron microscopy and measurement of electrical cable constants, the method is simple, fast, and potenti-

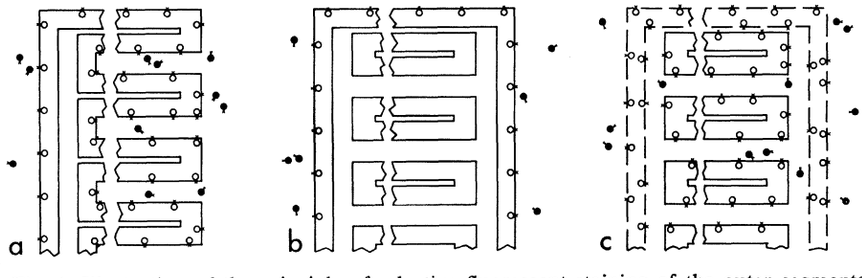

Fig. 1. Illustration of the principle of selective fluorescent staining of the outer segments of (a) cones, (b) intact rods, and (c) stripped rods with broken plasma membranes. Closed symbols indicate unbound, weakly fluorescent probe molecules. Open symbols denote bound, highly fluorescent probes.

ally useful in distinguishing topological differences among populations of intact photoreceptors and populations of isolated rod outer segments used in biochemical experiments.

The method depends on several properties of the stain. It must bind strongly to membranes with a marked increase in its fluorescence efficiency, it must diffuse freely in aqueous media but not through membranes, and it must be nontoxic at useful staining concentrations. When a rod is exposed to such a stain its plasma membrane will become fluorescent, but the internal disk membranes of rods will remain unstained, and thus nonfluorescent (Fig. 1b). A cone should become fluorescent throughout its entire outer segment (Fig. 1a). If the plasma membrane of the rod is ruptured, the rod outer segment should also fluoresce brightly, since the sides of the sacs originally in contact with the cytoplasm will come into contact with the unbound stain (Fig. 1c).

We have examined several water-soluble fluorescent substances for these properties (*6*). Fluorescein, rhodamine G, and their NH_2-binding isothiocyanate derivatives are too fluorescent in aqueous solution or do not show sufficient binding to membranes. Procion yellow, which couples covalently to primary amines and which has been shown to stain cone outer segments selectively (*7*), is only weakly fluorescent and its specificity for membranes is questionable (*8*). Solutions (0.1 to 1 m*M*) of *N*-dansyl isoleucine and 8-anilino-1-naphthalenesulfonic acid in physiological Ringer solution quickly stain rod and cone outer segments so that they become brilliantly fluorescent. However, the staining occurs in all outer segments, whether or not they possess an intact plasma membrane. *N*-Dansyl glucosamine and *N*-dansyl cysteic acid do not seem to stain photoreceptor membranes at all. *N*-Dansyl cysteine possesses all the sought-for properties, as does its more stable dimer DDC.

Figure 2 shows evidence of this selective staining of rods. A retina from a dark-adapted frog (*Rana pipiens*) was removed in darkness and quickly dipped into a drop of 2 percent agarose in frog Ringer solution on a sapphire plate held at 38°C. The agar, containing detached outer segments of rods and cones, was spread out into a layer about 10 μm thick by compression against a polyethylene film. The entire assembly was quickly cooled to 5°C for 10 seconds to gel the agarose, and the polyethylene film was peeled off. The sapphire plate with its adherent layer of immobilized outer segments was installed in a flow chamber through which various solutions could be pumped while the outer segments were observed by phase and fluorescence microscopy. The washout time of the chamber was less than 2 seconds and the penetration of DDC and other small molecules into the agar was complete in about the same time.

All experiments were conducted at 25°C. A group of fresh rods in phase contrast 2 minutes after detachment is

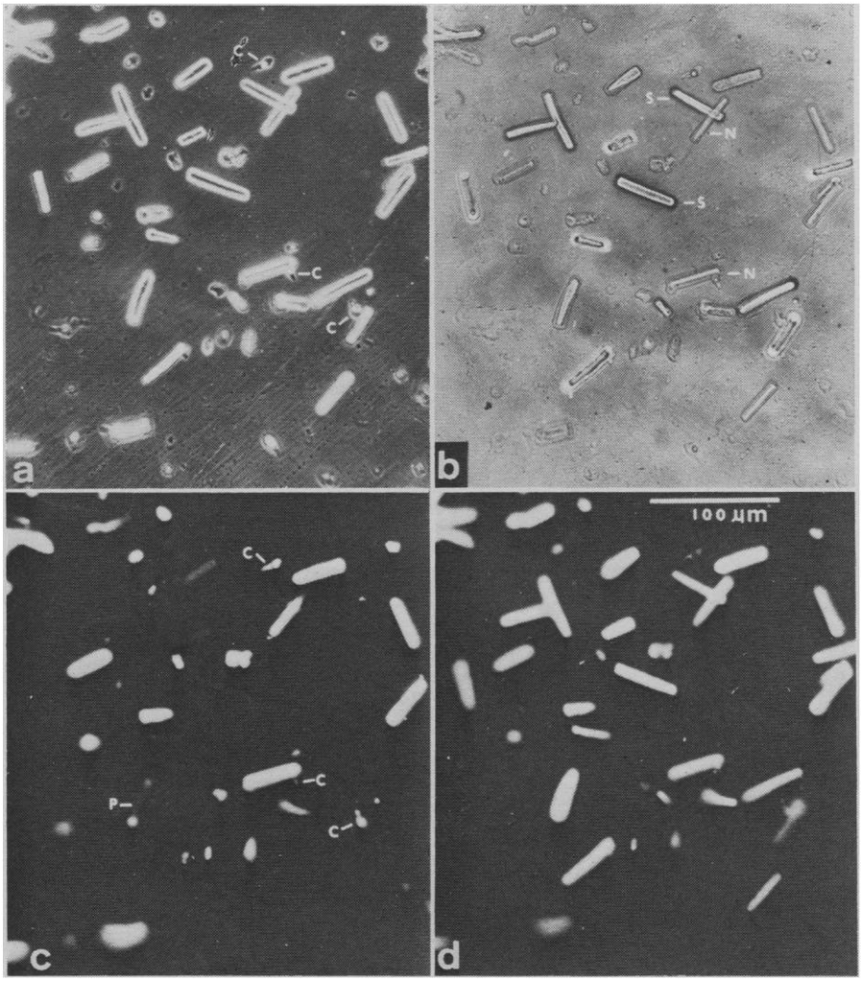

Fig. 2. Comparison of selective DDC staining and osmotic shrinking behavior of intact and stripped frog rod outer segments. (a) Phase contrast micrograph of isolated rod and cone outer segments immobilized in a 10-μm-thick layer of 2 percent agarose Ringer gel in a flow chamber. Three small cones, *C*, are visible. (b) Print made by photographic subtraction of (a) from a phase micrograph of the same field taken 10 seconds later with hypertonic DDC frog Ringer solution (frog Ringer + 230 m*M* added NaCl + 10 μ*M* DDC) flowing past the rods. Images of rods such as *N* which do not shrink osmotically are almost invisible after subtraction. Rods such as *S* which do shrink show enhanced contrast of their contours. Note the four rods in the upper half of the photographs forming two "tees." Both rods in the left-hand tee shrink, but only the rod pointing toward 10 o'clock in the right-hand tee shrinks. Measurements on large numbers of isolated rods in free suspension indicate that the hypertonic Ringer solution causes class *S* rods to shorten by 20 ± 2 percent and class *N* rods to shrink less than 2 percent. Clearly two discrete classes of shrinking behavior exist. (c) Fluorescence micrograph of field 20 seconds after exposure to hypertonic DDC Ringer solution. All rods that shrink fluoresce very faintly due to DDC bound to the plasma membrane. Those that do not shrink stain intensely. Compare the two tees described in (b). One rod, *P*, stains only at its ends. The three cone outer segments, *C*, in the field all stain brightly. Excitation, 365 nm; fluorescence photographed through a filter transmitting wavelengths > 450 nm. (d) Fluorescence micrograph 10 minutes later after rods had been exposed to hypotonic DDC Ringer (50 m*M* NaCl, 10 μ*M* DDC) for 1 minute. Now all rods immediately stain with DDC and fail to shrink when reexposed to hypertonic Ringer. Their plasma membranes are now permeable to DDC.

shown in Fig. 2a. All look refractile and unbroken, but only some of them shrink when exposed to a Ringer solution containing added NaCl (230 m*M*) and DDC (10 μM). This treatment is known to cause freshly isolated light-adapted outer segments to shrink as if they were osmometers whose envelope membranes were impermeable to NaCl (*9*). The population of outer segments that shrink in hypertonic Ringer solution can be seen in Fig. 2b. This print is made by photographic subtraction (*10*) of Fig. 2a from an image of the same field in hypertonic Ringer solution. The rods that shrink (some of which are labeled *S*) show marked contrast because their images are not identical in the two pictures. The rods that do not shrink are much less prominent in the difference print. Figure 2c shows the dansyl fluorescence of the same group 20 seconds later. The rods can be divided into three types: those fluorescing brightly throughout their bulk (*N*), those fluorescing so dimly as to be almost invisible (*S*), and a few rods, such as one marked *P*, staining partially at one or both ends. The faint staining of the *S* class is presumably due to DDC bound to the external surface of their plasma membranes, because the faint autofluorescence of rods fades too rapidly to be seen by the photographic technique used here. Since the plasma membrane of a frog rod outer segment comprises less than 1 percent of its total membrane area, its staining by DDC will contribute little fluorescence in comparison with that of stained disks.

Without exception in many experiments, the rods or parts of rods that stain do not shrink, and vice versa. Nevertheless, all the agar-embedded outer segments can be made to stain. If the preparation is exposed for 1 minute to a hypotonic solution containing 50 m*M* NaCl instead of the usual 115 m*M* all the outer segments immediately become fluorescent in DDC (Fig. 2d). Simultaneously, they lose their ability to shrink in hypertonic NaCl Ringer solution as if their plasma membranes were now freely permeable to NaCl. The onset of staining in such outer segments is sudden and occurs after a short but unpredictable delay, as if the failure of the plasma membrane were catastrophic. Unlike agar-bound rods, outer segments suspended in hypotonic Ringer solution do not all stain with DDC even though they are greatly deformed.

An unknown mechanism binds DDC to outer segments. There is no covalent link to rhodopsin (*11*). Sulfhydryl groups do not seem to be involved, since rods treated for 10 minutes with α-iodoacetamide stain as quickly and intensely as do untreated ones. Nor does 1 m*M* dithiothreitol accelerate the normal 3-minute half-time for washout of the stained rods. Preliminary studies suggest that less than 1 mole of DDC is bound per mole of rhodopsin in 100 μM DDC solutions.

Staining with DDC is useful in assaying the average intactness of the plasma membranes of isolated outer segments in aqueous suspensions. A suspension of freshly harvested frog rods in a large volume of 10 μM DDC Ringer solution is weakly fluorescent at 470 nm, because a few stripped rods are present. Some fluorescence of unbound DDC is also seen, of course, but it is weak and displaced to longer wavelengths than that of the bound species (*12*). When exposed to freezing and thawing or sonication (100 watts, 20 khz, 1 minute in 1 ml of 10 μM DDC Ringer solution) the rods become brightly fluorescent. The fluorescence obtained is proportional to the original number of outer segments in the suspension. A suspension containing a high proportion of stripped rod outer segments is proportionately more fluorescent in 10 μM DDC Ringer solution than one whose rods are mostly intact.

DDC staining can be used to detect presumably nonfunctional rods in electrophysiological preparations of isolated live retinas (Fig. 3A). Yet at 10 μM, DDC does not obviously affect dark current, photocurrent, or fast photovoltage of rat rods. DDC is also useful in locating cone outer segments (Fig. 3B) in a retinal mosaic. A 1-minute exposure to 10 μM DDC is sufficient to make the outer segment of a single goldfish cone (Fig. 3C) brightly fluorescent (Fig. 3D) even though its plasma membrane behaves as if impermeable to the stain. This can be seen by noting that the concentrated mitochondria in the cone ellipsoid in Fig. 3D are only weakly fluorescent (due mainly to fluo-

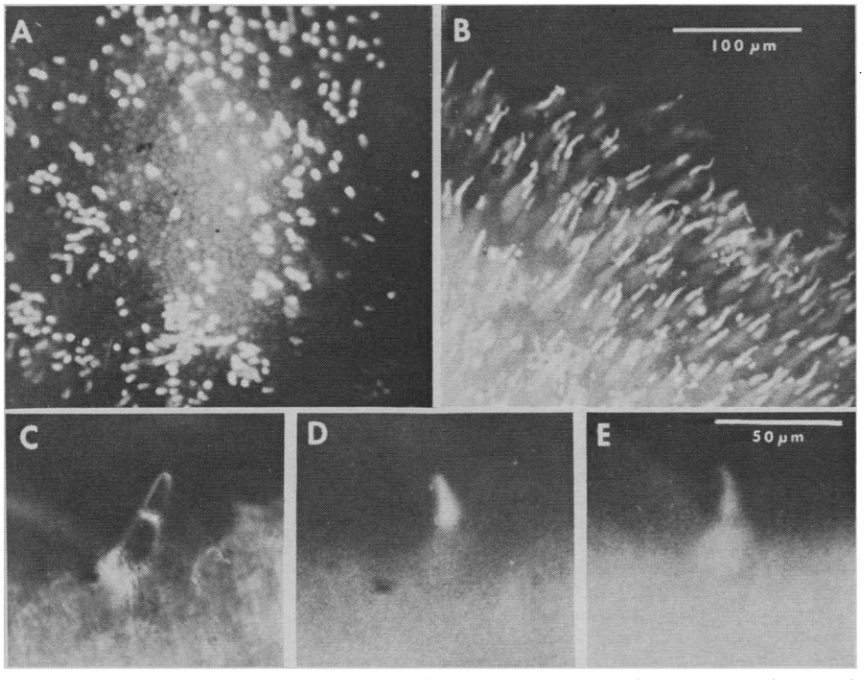

Fig. 3. Effects of membrane topology on DDC staining. (A) Fluorescence micrograph of fresh frog retina stained 1 minute with 10 μM DDC. The receptor layer is seen from the distal ends of the rods. The receptor mosaic is complete, but many of the rods stain as if their plasm membranes were stripped. (B) Live goldfish retina stained for 2 minutes in 10 μM DDC Ringer solution. The rods in this area of retina have mainly been pulled off with the pigment epithelium. Those remaining are not fluorescent. The cone outer segments are brightly fluorescent. (C) Phase micrograph of a single goldfish cone at the edge of a piece of retina in a flow chamber. (D) Fluorescence of same cone after 4 minutes of exposure to 10 μM DDC Ringer solution. The entire outer segment is brightly fluorescent. (E) Same cone after 2 minutes in hypotonic DDC Ringer solution. The cell becomes leaky to DDC and now both the outer segment and the mitochondria of the inner segment are brightly fluorescent with bound DDC. Excitation, 365 nm with vertical dark-field illumination; fluorescence photographed through a cutoff filter ($>$ 450 nm).

rescence scattered from the deeper retinal layers). If the cone is treated for 2 minutes with a hypotonic solution containing 50 m*M* NaCl, however, reapplying 10 μM DDC now stains the mitochondrial membranes as well as the outer segments. Thus, the presumed topological difference between rod and cone outer segment membranes suggested by electron microscopic and electrical evidence is confirmed by the DDC staining test.

S. YOSHIKAMI
W. E. ROBINSON
W. A. HAGINS
National Institutes of Health, Bethesda, Maryland 20014

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5. Dansyl is an abbreviation for 5-dimethylamino-1-naphthalenesulfonyl.
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8. Our attempts to demonstrate selective staining of cone outer segments confirmed the previous report (*7*), but such preparations also showed many rod and cone nuclei stained with the fluorochrome in cells whose outer segments were unstained. We do not know if this result is due to variation in the staining properties of different batches of Procion yellow dyes. Until their structures are published and pure samples are available, the Procion dyes will be difficult to use in reproducible experiments.
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10. Photographs of the microscopic fields were made on Polaroid type 55 P/N sheet film before (B) and during (D) exposure of the layer of rods in agarose to hypertonic Ringer solution. A contact print of the negative of B was made on a Kodak Lantern slide plate developed to a contrast of $\gamma = 1$. The exposure of the plate was adjusted so that when it was superimposed on the negative of B, a uniform gray appearance almost devoid of contrast was seen. The plate was then superimposed on the negative of D and printed on grade 4 (high contrast) photographic paper.
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12. The fluorescence emission maximum of DDC dissolved in Ringer solution is at 550 ± 5 nm while that of DDC bound to rod outer segments is at 505 ± 5 nm.

13. We thank W. H. Cobbs for discussion.

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Centric Fusion, Satellite DNA, and DNA Polarity in Mouse Chromosomes

Abstract. *A fluorescent staining technique has demonstrated a contralateral arrangement of fluorescent spots in the centromeric region of mouse metacentric chromosomes which have resulted from centric fusion. The results suggest that centric fusion involves the maintenance of DNA polarity through the centromere and that the thymidine-rich chain of satellite DNA in the centromeric region is associated with the same DNA chain in every mouse autosome.*

There is a fluorometrically detectable lateral asymmetry in the centromeric region of mouse (*Mus musculus*) chromosomes (*1*). This asymmetry was detected through the use of a newly developed chromosome staining technique based on the quenching by 5-bromodeoxyuridine (BrdU) of the fluorescence of the dye 33258 Hoechst (*2*). In the absence of BrdU treatment, the centromeric region of mouse chromosomes (except the Y chromosome) fluoresces brightly and uniformly when stained with 33258 Hoechst (*3*). In contrast, when the chromosomes of mouse cells grown for one generation in the presence of BrdU are stained with 33258 Hoechst, there is a marked difference in the fluorescence between the halves of the centromeres, such that half of the centromeric region of each chromosome (except the Y chromosome) shows a brightly fluorescent spot (*1*). The staining properties and pattern of segregation during cell division of this fluorescent spot suggest that it is associated with the thymidine-rich chain of satellite DNA in the centromeric region. [One chain of mouse satellite DNA contains 45 percent thymidine, and the other chain contains 22 percent (*4*).]

The mouse cells used in the previous experiments involving the BrdU-Hoechst treatment were cells of permanent lines (RAG and NCTC 2472), and they contained a few metacentric chromosomes not present in diploid mouse cells. After one cycle of replication in BrdU, two brightly fluorescent spots were observed in each metacentric chromosome. The spots were arranged contralaterally, one on either side of the centromere and located on the opposite arms of the chromosome (*1*). Although the results led to speculations concerning DNA polarity and the arrangement of satellite DNA, no firm conclusions could be drawn because it was not known whether the metacentric chromosomes in these cells had arisen by misdivision of the centromere or by Robertsonian centric fusion of telocentric chromosomes.

We now describe the centromeric staining properties of metacentric chromosomes known to be the result of Robertsonian centric fusion of telocentric chromosomes. The cells for this study were derived from two different strains of mice. One of these strains is the tobacco mouse (*Mus poschiavinus*), which contains seven pairs of metacentric chromosomes resulting from centric fusion (*5*). The other strain is the 36(TW/TW TA/TA) strain which contains two pairs of metacentric chromosomes resulting from centric fusion (*6*) (these mice are referred to below as 36T mice). The tobacco mice were provided by Dr. Alfred Gropp, and the 36T mice were provided by Dr. Beverly White.

Male tobacco mice were bred with female mice of the CD-1 strain (Charles River), which contain no metacentric chromosomes. (The mice resulting from this cross will be referred to as TM/C mice.) Male and female 36T mice were bred with each other. At 15 to 18 days after fertilization, the embryos were removed and minced into small fragments, which were further dissociated by trypsinization. The cells were then cultured in F10 medium supplemented with 15 percent fetal calf serum. For the chromosome analyses, the cells were grown in medium containing $10^{-5}M$ BrdU for either 17 or 31 hours—that is, one or two cycles of DNA replication, respectively. The cells were harvested, fixed, stained with 33258 Hoechst, and photographed (*2*). The 33258 Hoechst was generously provided by Dr. H. Loewe, Hoechst AG, Frankfurt.

The fluorescent staining patterns of the chromosomes of TM/C cells are shown in Fig. 1. These cells are heterozygous for each of the seven metacentric chromosomes of the tobacco mouse. After one cycle of replication in BrdU, the centromeric region of each metacentric chromosome exhibits two brightly fluorescent spots, arranged one on either side of the centromere and on opposite arms of the chromo-