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USE OF A DENSITY MODIFICATION TECHNIQUE FOR ISOLATION OF THE PLASMA MEMBRANE OF ROD OUTER SEGMENTS

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The external surface of rod outer segments contains receptors for the Jack Bean lectin, concanavalin A. Using isolated intact bovine rod outer segments, we have modified the density of the outer segment plasma membranes by means of polystyrene beads carrying covalently linked concanavalin A. After hypotonic lysis the bulk of the disk membranes can be removed and a plasma membrane fraction is isolated. The plasma membrane preparation contains 1.5% of total outer segment rhodopsin and 2.7% of total outer segment protein. It shows very little contamination with inner segment plasma membrane. Contamination with disk membranes appears to be low as well. Fatty acid analysis reveals that the plasma membranes are more saturated than the highly unsaturated disk membranes. Gel electrophoresis shows the presence of at least six additional polypeptides besides rhodopsin.

Rod outer segments of vertebrate photoreceptor cells are composed of a stack of hundreds of regularly piled disks surrounded by a plasma membrane. The disk membranes are the site of primary light reception while the plasma membrane contains a light modulated ionic conductance mechanism, that regulates the generation of the receptor potential. The coupling between photolysis of rhodopsin in the disk membrane and hyperpolarization of the plasma membrane is thought to be mediated by a cascade of only partially known reactions, mainly occurring in the rod outer segment cytosol [1,2].

In contrast to the disk membranes, the plasma membrane of rod outer segments has hardly been characterized by biochemical techniques and its isolation has never been reported. This is due to a combination of reasons: (1) The plasma membrane, even of small outer segments like those of cattle (1.1×15 μm), comprises only a small percentage ($\leqslant 6\%$) of the total rod outer segment membranes. (2) The rod outer segment plasma membrane also contains rhodopsin [3], which hampers its discrimination from disk membranes. (3) No specific marker enzymes or proteins are known for this plasma membrane. (4) Insufficient electronmicroscopic information is available for identification and purity assessment of this membrane. In spite of these difficulties the isolation of a well defined rod outer segment plasma membrane preparation is essential in order to render the functionally important structures in these membranes accessible to direct biochemical investigation.

To approach this problem we have devised a density modification technique by taking advantage of the presence of lectin binding sites at the extracellular surface of the rod outer segments [4]. Recent studies [5,6] indicate the presence of sites having affinity for wheat germ agglutinin, '*Ricinus communis* agglutinin' and concanavalin A, the latter sites, however, being much more abun-

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dant. Binding of intact isolated rod outer segments to concanavalin A, covalently coupled to polystyrene beads, increases the density of the bound plasma membrane as compared to the disk membranes and allows its subsequent separation. Binding of disk membranes to the immobilized concanavalin A is minimized by:

(1) lectin binding sites being only present at the inside of the disks [7],
(2) little tendency of disks to adapt the inside-out orientation [8], and
(3) covering of all binding sites on the beads by initially overloading them with outer segments.

Polystyrene beads containing 4% divinylbenzene as a cross-linker (200–400 mesh; PL Biochemicals) are diazotized according to Miller et al. [9]. Concanavalin A is covalently coupled to these beads in 50 mM sodium borate buffer, pH 8.8 at 2°C, containing 10 mM methyl-α-D-mannoside, 1 mM $CaCl_2$, 1 mM $MnCl_2$ and 1 mM dithioerythritol. The reaction, although almost complete within 3 h at 2°C, is allowed to proceed overnight at 4°C. The beads are washed with 500 mM NaCl containing 1 mM of each $CaCl_2$, $MnCl_2$ and dithioerythritol. Up to 0.7 mg concanavalin A is bound per ml bed volume.

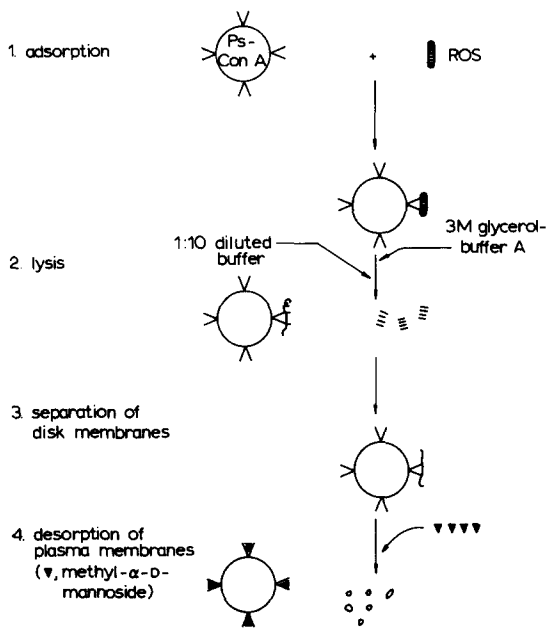
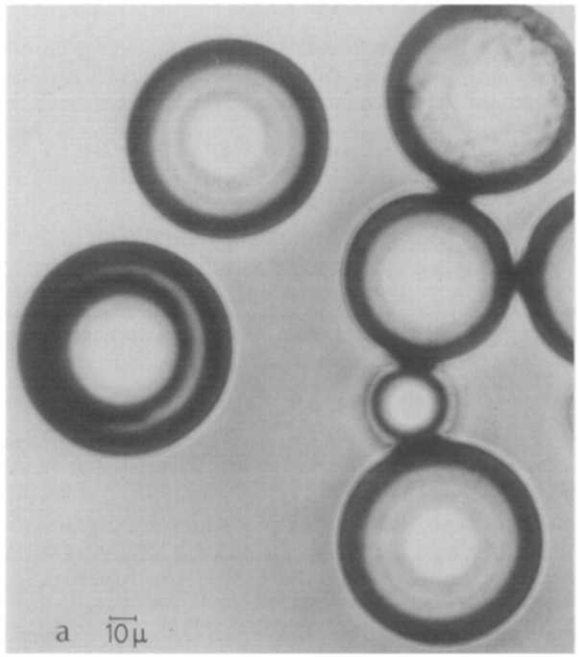
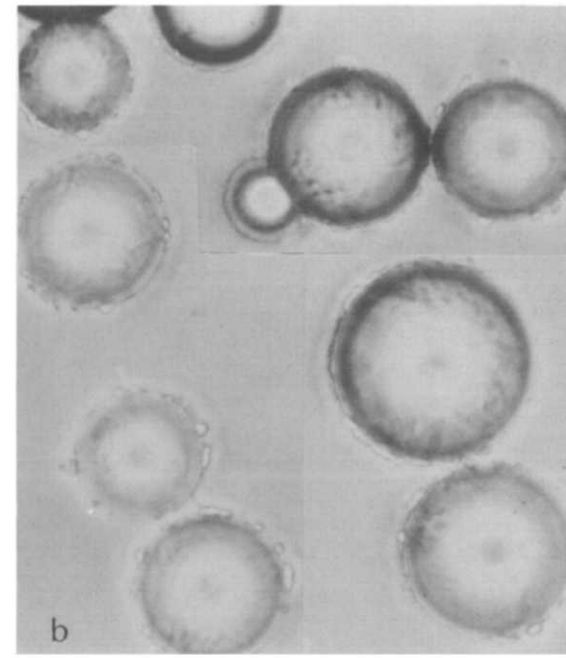


Fig. 1. Scheme for the isolation of outer segment plasma membranes on concanavalin A (Con A)-coated polystyrene (Ps) beads. After each wash the beads are allowed to settle for 10 min and the supernatant is removed by filtration through teflon screen. The recovery of the plasma membrane protein fraction is increased over 95% by raising the ionic strength of buffer B with NaCl to 0.5 M (Buffer C). After 2-fold dilution of the buffered media, membrane fractions are collected by centrifugation at 2°C, $10^5 \times g$, for 60 min.

Intact bovine rod outer segments are isolated and purified according to De Grip et al. [10]. All manipulations with rod outer segment preparations are performed in dim red light at 0–4°C and, where possible, under a nitrogen atmosphere. The rod outer segment-containing pellet is taken up in 20 mM Pipes (piperazine-*N*,*N'*-bis(2-ethanesulfonic acid)) buffer, pH 6.8, 130 mM NaCl, 10 mM KCl, 3 mM $MgCl_2$, 2 mM $CaCl_2$, 1 mM dithioerythritol, 0.1 mM EDTA (buffer A). The suspension is washed by centrifugation at $1000 \times g$ for 10 min at 4°C in order to remove sucrose. The pellet is carefully resuspended in the same buffer to a concentration of 0.3 mM rhodopsin. The isolation procedure finally adopted for the rod outer segment plasma membrane fraction consists of several steps (Fig. 1). Adsorption to the polystyrene-concanavalin A beads (Fig. 2) is carried out by adding 2 ml of the rod outer segment suspension to 6 ml of polystyrene-concanavalin A beads and the mixture is poured onto a small column (diameter 1 cm). The column bed is then washed with buffer A overnight to remove non-bound material. Subsequently the column is washed with one volume of buffer A, containing 3 M glycerol. Glycerol penetrates the rod outer segment quickly and renders the subsequent lysis step more efficient.

Separation of plasma membrane and disk membranes can be performed satisfactorily by means of hypotonic lysis and subsequent removal of the disk membranes by washing. Lysis is carried out by emptying the column into a tube containing five bed volumes of 10-fold diluted buffer A. After vortexing for 10 s, the beads are allowed to settle and the supernatant is decanted to remove the disk membrane vesicles. The beads are washed twice with buffer B (100 ml buffer A + 110 ml glycerol made up to 1000 ml with distilled water). The combined supernatants are filtered over nylon gauze (1200 mesh) to remove some residual beads, 2-fold diluted with water and the rod outer seg-





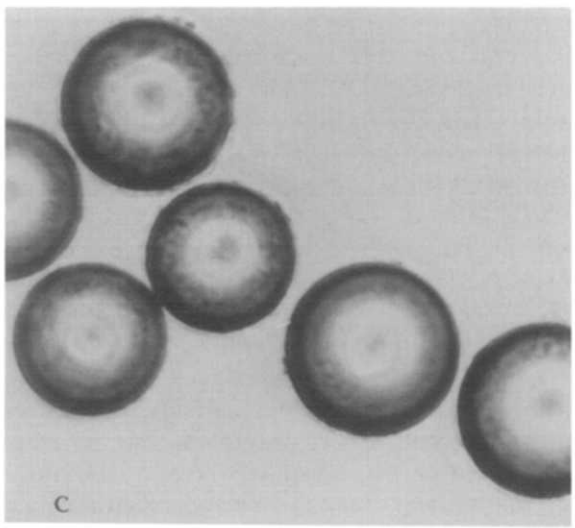


Fig. 2. Light micrographs of isolated rod outer segments and plasma membranes attached to concanavalin A (Con A)-coated polystyrene beads. (a) Con A-coated beads. (b) Isolated rod outer segments bound to the polystyrene beads. Unbound outer segments have been washed away. Rod outer segments are visible only in the focal plane. (c) Plasma membrane coated beads after hypotonic shock of outer segments and washing away the disk membrane fraction.

ment disk membranes are collected by centrifugation ($10^5 \times g$, 1 h). Desorption of the plasma membrane fraction is performed by washing the beads three times with buffer B containing 150 mM methyl-α-D-mannoside and 500 mM NaCl. The plasma membrane fraction is collected by centrifugation ($10^5 \times g$, 1 h) after 2-fold dilution with water. It contains up to 1.5% of the total rhodopsin and 2.7% of the total protein present in the outer segments originally applied to the column.

The identity of the disk membrane fraction is relatively easily established. Rhodopsin content, molar ratio phosphorus/rhodopsin (Table I), fatty acid composition (Table II) and gel pattern (Fig. 4, lane 3) match accepted values closely and demonstrate that we indeed are dealing with disk membranes.

Positive identification of the plasma membrane

TABLE I

PHOSPHOLIPID AND PROTEIN CONTENT OF BOVINE ROD OUTER SEGMENT MEMBRANE PREPARATIONS

Values ± S.E. for four preparations are given. Membrane preparations are washed twice in 10-fold diluted buffer A followed by centrifugation at 4°C, 100000 × *g*; 45 min. The protein ratio (rhodopsin protein/total protein) is calculated assuming 36000 as the molecular weight of rhodopsin [10]. Rhodopsin content is determined by measuring ΔA_{500} in 1% Ammonyx-LO, without correction for opsin content (4 ± 1%) in these preparations. Phosphate is determined using the method of Broekhuyse [26]. Protein is determined according to a modified Lowry procedure after Peterson [27] using bovine serum albumin as standard.

Rod outer segment fraction	mol phospholipid/ mol rhodopsin	Protein ratio (%)
Total rod outer segment membranes	63 ± 2	82 ± 5
Disk membranes	62 ± 2	88 ± 2
Plasma membranes	96 ± 6	45 ± 4

TABLE III

DISTRIBUTION OF $(Na^+ + K^+)$-ATPase AND 5′-NUCLEOTIDASE OVER ROD OUTER SEGMENT MEMBRANE PREPARATIONS

The values of four preparations are expressed as S.E. of the mean. All specific activities are expressed as μmol P_i/mg of protein per h; $(Na^+ + K^+)$-ATPase activity according to Bonting [8]; 5′-nucleotidase activity in a total volume of 0.75 ml containing 20 mM nonylglucose according to Heppel and Hilmoe [29]. The lower band is a small band just below the rod outer segment band which contains membrane material of uncertain (possibly Golgi, lysosomal, or synaptosomal, or inner segment plasma membrane) origin [10].

Membrane fraction	$(Na^+ + K^+)$-ATPase	5′-Nucleotidase
Total rod outer segment membranes	1.17 ± 0.09	1.21 ± 0.06
Disk membranes	1.11 ± 0.12	1.31 ± 0.15
Plasma membranes	0.99 ± 0.08	1.10 ± 0.06
Lower band	20.60 ± 1.45	5.90 ± 0.76

fraction is much more difficult, since no component of this membrane is yet known which is absent from the disk membranes. Therefore, first we have looked for possible enzyme contamination

TABLE II

FATTY ACID COMPOSITION OF BOVINE ROD OUTER SEGMENT MEMBRANES

Values are given in mol% of total fatty acids ± S.E. ($n = 5$).

Fatty acid	Plasma membrane preparation	Disk membrane preparation	Total rod outer segment membranes
C 16:0	20.1 ± 0.8	17.6 ± 0.4	17.8 ± 0.3
C 16:1	4.0 ± 0.3	0.9 ± 0.2	1.0 ± 0.2
C 18:0	24.3 ± 1.0	21.6 ± 0.3	21.9 ± 0.4
C 18:1ω9	7.6 ± 0.3	4.1 ± 0.2	3.9 ± 0.2
C 18:2ω9	1.8 ± 0.2	0.8 ± 0.2	0.8 ± 0.1
C 20:4ω6	3.0 ± 0.5	4.0 ± 0.2	4.1 ± 0.3
C 22:4	1.6 ± 0.2	1.4 ± 0.1	1.6 ± 0.2
C 22:5ω6	2.2 ± 0.2	2.0 ± 0.1	2.1 ± 0.2
C 22:5ω3	1.5 ± 0.1	1.6 ± 0.1	1.6 ± 0.1
C 22:6	26.8 ± 1.9	42.6 ± 0.9	42.3 ± 0.4
C 24:4 C 24:5	1.8 ± 0.5	2.1 ± 0.4	2.3 ± 0.3
Unident. [a]	5.3	< 1	< 1

[a] Unidentified fatty acids contain mainly C 18:3 and C 20:4ω3.

within the plasma membrane fraction. The presence of plasma membranes from inner segments, which might have remained attached to the outer segments in our rod outer segment preparations [11], has to be considered. The specific activity of $(Na^+ + K^+)$-ATPase, demonstrated to be only present in inner segments [12,13], is equally low in rod outer segment disks and plasma membrane fractions, relative to the entire retina [14] or heavier fractions obtained after density centrifugation of retinal homogenates on sucrose gradients (Table III). The specific activity of 5′-nucleotidase, a common plasma membrane marker enzyme, does not significantly differ between the two rod outer segment membrane fractions. No cytochrome *c* oxidase activity could be measured in the membrane preparations. Hence, we can conclude that our plasma membrane preparation is not significantly contaminated with plasma membranes or mitochondria from the inner segment.

The question to what extent the plasma membrane fraction is contaminated with disk membranes is not easily answered. There are a number of differences between the two fractions. The plasma membrane fraction has a rhodopsin content of 45% which is much lower than the value of 88% of the disk membrane (Table I). Thus, the

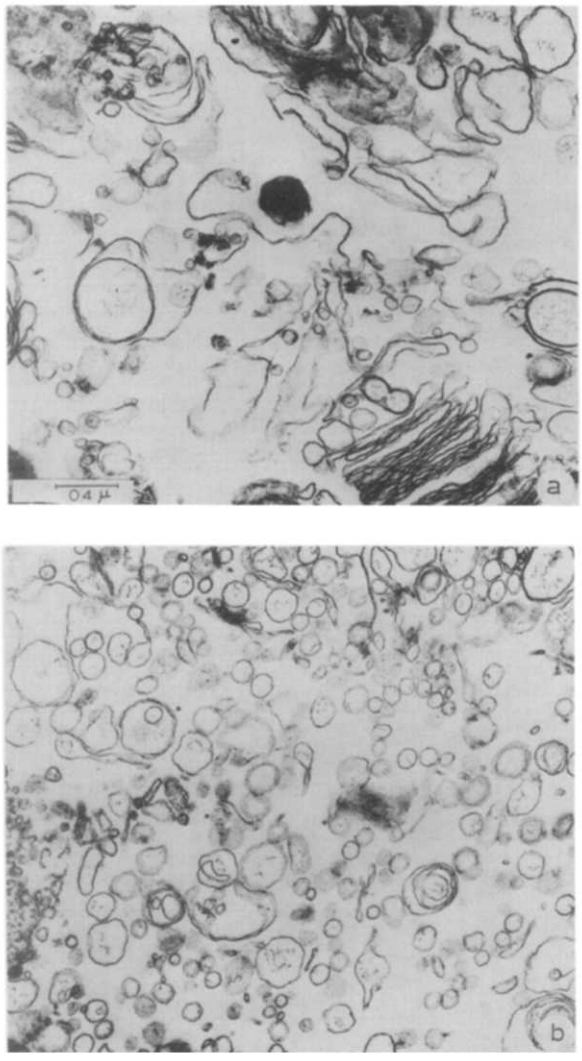


Fig. 3. Electron micrographs of outer segment disk membranes (a) and plasma membranes (b). After their isolation the membranes are suspended in isotonic Pipes buffer (pH 6.8). Samples are fixed at 4°C by adding one volume of 6% glutaraldehyde in Pipes buffer. Micrographs are obtained in collaboration with Dr. J. Olive and Prof. Dr. E.L. Benedetti, Institute de Biologie Moleculaire, Université de Paris VII, France.

rhodopsin/protein ratio of the plasma membrane is about half that in disk membranes. Table II shows that the fatty acid composition of both membrane preparations, as determined in lipid extracts [15] by gas chromatography [16], differs significantly. Disk membranes contain high amounts of docosa-hexaenoic (C 22:6) acyl chains: 43 mol% of total fatty acids, in good agreement with earlier reports [17,18]. The plasma membrane fraction contains only 27 mol% C 22:6, a decrease which parallels the ratio rhodopsin/total membrane protein. Overall, the fatty acids in the plasma membrane fraction are more saturated than in the disk membranes.

It is clear that, if the plasma membrane would not contain rhodopsin, contamination with disk membranes might be as high as 50%, considering its C 22:6 and rhodopsin content. However the presence of rhodopsin in the plasma membrane is indisputable [3,19,20], although its density has not been determined. Therefore, contamination with disk membranes is certainly less than 50%. In view of the following data, we estimate this contamination to be less than 20%. (1) In the large number of plasma membrane preparations ($n = 15$) isolated so far, the rhodopsin content was never lower than 40% (w/w) of the total protein content and, in addition, independent of the rhodopsin recovery. If the actual rhodopsin content would be significantly lower than 40%, a larger variance would be expected. (2) Disk membranes obtained upon lysis do not bind to polystyrene-concanavalin A beads. (3) Micrographs of the plasma membrane fraction (Fig. 3) show little contamination with vesicles of the size typical for disk membranes. In fact, one has to reckon with the presence of disk membranes 'in statu nascendi'. These disks at the base of the outer segment, have not yet been pinched off from the plasma membrane. It can easily be calculated that they could constitute up to 20% of the total 'surrounding' membrane of an outer segment.

Analysis of the polypeptide composition of disk membranes and the plasma membrane fraction by means of SDS-polyacrylamide gel electrophoresis (Fig. 4, lanes 3 and 4, respectively) leads to the conclusion that the latter contains several proteins not present in the disk membranes. The plasma membrane fraction extensively washed in the presence of 1 mM EDTA, contains proteins of 230, 226, 160, 125, 110, 66, 57, 55, 54 and 33 kDa, besides rhodopsin. Disk membranes similarly treated, contain rhodopsin as the predominant membrane protein (partially as a dimer) with small amounts of 230 kDa [21], 160, 125, 66, 57 and 55 kDa proteins. The proteins of 226, 110 and 33 kDa seem specific for the plasma membrane frac-

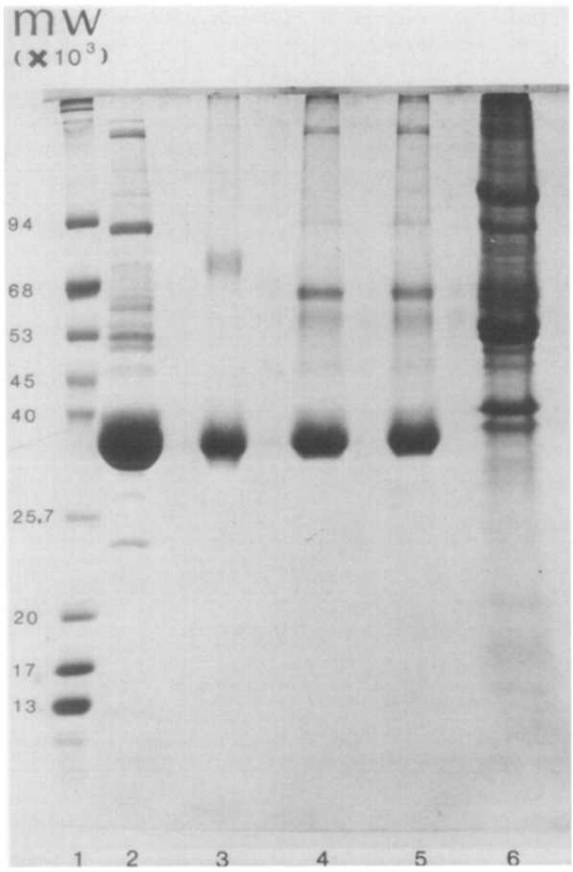


Fig. 4. SDS-polyacrylamide slab gel of rod outer segment membrane fractions. The standard discontinuous gel system is adapted from Laemmli (Nature (1970) 227, 680–685). A linear polyacrylamide gradient (10–15% acrylamide) is prepared from a stock solution of 40% (w/v) acrylamide/1.07% (w/v) bisacrylamide. Membrane samples were solubilized at 0°C in order to avoid di- and trimerization of rhodopsin as much as possible. Lane 1, molecular weight calibration: phosphorylase A (94 kDa); bovine serum albumin (68 kDa); glutamate dehydrogenase (53 kDa); ovalbumin (45 kDa); aldolase (40 kDa); chymotrypsinogen (25.7 kDa); αA_2-chain of lens crystallin (20 kDa); myoglobin (17 kDa); and cytochrome *c* (13 kDa). Lane 2, rod outer segments (72 μg protein); lane 3, disk membranes (35 μg); lane 4, plasma membrane fraction, EDTA-washed in 50 mM Tris-HCl buffer (pH 7.4), 2 mM EDTA (39 μg); lane 5, plasma membrane fraction washed in Tris buffer, containing 5 mM $MgCl_2$ and 25 mM KCl (TMK buffer) (48 μg); lane 6, pellet ($10^5 \times g$, 1 h) after extraction of total rod outer segment membranes with 1% Triton X-100 in TMK buffer (80 μg).

tion. The 33 kDa protein is a galactose containing protein, which has affinity for the *Ricinus communis* lectin-120 (data not shown). This lectin is known to bind to the rod outer segment plasma membrane. An almost 2-times higher galactose content of the plasma membrane fraction (per mg protein) also suggests the presence of a different glycoprotein population.

Electron microscopy has only yielded indirect information on the plasma membrane fraction (Fig. 3). Parts of ciliary structures are present in the plasma membrane fraction. This could explain the presence of an actin-like protein (43 kDa; p*I* 6.1), together with other cytoskeletal proteins of 55–57 kDa. These proteins are also present in a Triton X-100 extract of rod outer segment membranes containing axonemes [22]. In our hands the Triton X-100 pellet ($10^5 \times g$, 1 h) of rod outer segment membrane proteins contains about 1% of total rod outer segment membrane protein (Fig. 4, lane 6).

The lectin-based density modification technique described here, has some analogy to the polylysine coated bead technique [23], but is more specific as well as more easily reversible. For isolation of rod outer segment plasma membrane we exploit the observation that the external surface of the rod outer segments contains receptors for the Jack bean lectin, concanavalin A. When using intact sealed bovine outer segments, the intradiscal concanavalin A-binding sites remain masked and only plasma membrane will be bound to the concanavalin A, covalently linked to the polystyrene beads. Thus we have been able to isolate a plasma membrane fraction containing 1.5% of total rod outer segment rhodopsin with very low contamination by inner segment plasma membranes and, presumably, low contamination by disk membranes.

To our knowledge, this is the first published procedure yielding a relatively pure rod outer segment plasma membrane preparation. This should permit biochemical study of the highly specific function of this membrane in visual excitation [1,24,25]. So far, we already observed distinct differences of disk and plasma membranes in rhodopsin content, fatty acid distribution and protein composition. Further analysis of this outer membrane preparation with respect to phospholipid and protein composition (glycoproteins, carbohydrate composition) and additional purity control with immuno-labeling techniques are in progress.

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