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A LIGHT SCATTERING STUDY ON THE ION PERMEABILITIES OF DARK-ADAPTED BOVINE ROD OUTER SEGMENT DISK MEMBRANES

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Summary

The ion permeability properties of dark adapted bovine rod outer segment disk membranes were studied using light scattering to monitor osmotic responses of disks to various salts and ionophores.

A preparation procedure is presented which provides very fresh rod outer segment material with mostly intact stacked disks, but with perforated plasma membrane. It is shown that in this preparation the disks (or rod sacs) are the only osmotically responding compartments and that these responses can be readily monitored by means of light-scattering techniques.

The disk membrane is found, under the conditions tested, to possess no measurable permeability to cations Na^+, Ca^{2+}, Mg^{2+} nor to the anions Cl^-, Br^-, NO_3^-, SO_4^{2-}, $H_2PO_4^-$ and HPO_4^{2-}. There is a considerable K^+ permeability, which can be completely abolished by millimolar amounts of divalent cations.

The proton permeability of the disk membrane is found to depend dramatically upon the preparation procedure and duration. The fresher the material used the lower is the proton permeability measured. In our freshest preparations, even after freeze-thawing in liquid nitrogen, the disks exhibit an H^+ permeability which is so low that it cannot be measured with the techniques used in this study.

Even in mitrochondrial or chloroplast membranes, in which proton gradients and therefore a low proton conductance play an essential role, such low proton permeabilities have not been found. This would suggest that proton gradients across the disk membrane could play an important role in the physiological function of the photoreceptor cell.

In summary it can be said that the disk membrane, apparently more than

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Abbreviation: Pipes, 1,4-piperazinediethanesulphonic acid.

any other natural membrane system studied so far, is capable of retaining ion gradients for extended periods of time.

Introduction

Visual transduction can be viewed as occurring in the outer segment of single retinal rod and cone cells. Both these types of outer segment consist of a lamellar membrane structure oriented perpendicular to the photoreceptor cell long axis [1—5]. Electron microscopic studies of rod outer segments have indicated that their lamellar structure consists of a stack of saccules (called disks), entirely separated from and completely enveloped by the plasma membrane. On the other hand, in the cones the lamellar structure appears to be an infolding of the plasma membrane along the entire length of the outer segment. Therefore, in the cone the interior of the disk-like flat membrane infoldings is continuous with the extracellular fluid [6—9], whereas the rod outer segment disk interior is a small compartment completely separated from both extradiskal (cytoplasmic) and extracellular space.

It appears likely that the characteristic compartmental structure of rod and cone outer segments is of direct importance to their function and functional differentiation. To substantiate this idea, one has to known the properties of the membrane which enclose these compartments, i.e., the permeability of the disk membrane to various solutes. We have therefore undertaken a systematic study of the disk membrane permeability to a number of cations and anions usually found in biological systems.

A convenient way to measure permeability properties of vesicular membrane systems is to study their osmotic behaviour in various solutes. In the case of the rod outer segment plasma membrane compartment this can be done quite easily by measuring the osmotically induced volume changes under the light microscope [10,11]. In the case of the much smaller disks, however, other techniques have to be applied, i.e. light scattering, packed pellet volume determination and radioactive tracer experiments. To date, this has been dealt with in two publications. The first of these is a short, preliminary research note by Brierly et al. [12], using cattle rod outer segment fragments, and the second is a paper by Heller et al. [13], using rod outer segments from frog retinae. Unfortunately the results of these studies are contradictory in some respects, principally because these investigators were not able to ascertain which membrane compartment, plasma, disk or both, participated in the osmotic volume changes taken as a measure for permeability properties. In this communication we describe rod outer segment volume changes under such conditions that they can be assigned unambiguously to changes in the internal volume of the disks.

To monitor the disk volume changes we use light scattering, a technique that has been shown to be a very sensitive probe for the volume of thylakoid [14], mitochondria [15], erythrocytes [16] and artificial phospholipid membranes [17] as well as rod outer segment fragments [18]. Furthermore, as Uhl et al. have shown [19], even very rapid light induced disk contractions can be visualised by this technique. We have chosen to use rod outer segment fragments with perforated plasma membrane, rather than disk preparations, since within

the time required to obtain isolated disks (at least 6 h) irreversible changes appear to occur in the disk membrane, which affect its properties dramatically. For example, as we shall demonstrate in this communication, the proton permeability of the disk membrane markedly increases with increased duration of the rod outer segment preparation.

Methods

Rod outer segment preparation

Cattle eyes are obtained from a local abbatoir. The eyes are excised immediately after death of the animal and stored in a light-tight container at room temperature. Eyes of very young cows (heifers) are used preferentially since, in our hands, they yield about 60—80% more rhodopsin per retina than bullocks, old cows or old bulls. Usually 60 eyes are collected, which takes about 30 min. For regeneration purposes the eyes are kept at room temperature for an additional 30 min, are then cooled to 0—4°C and rapidly dissected under dim red light. The retinae are collected in ice-cold Ringer solution (0.5 ml per retina), containing 120 mM NaCl, 0.4 mM $CaCl_2$, 0.4 mM $MgCl_2$, 0.5 mM EDTA, 5 mM glucose and 15 mM phosphate, pH 7.2. EDTA is included in order to chelate Fe^{3+} which catalyses oxidative damage to the rod outer segments, and as a further precaution against such damage to the preparation all solutions were saturated with argon [20].

After completion of the dissection (20—30 min) the retinae are homogenized gently but thoroughly using mortar and pestle. To remove the gross debris the homogenate is filtered twice through a nylon mesh of pore size 30 μm. Without affecting the results we have recently replaced this procedure by vortexing the retinae vigorously for 30 s with subsequent filtering through the same nylon mesh. The crude rod outer segment suspension is layered on top of a sucrose cushion (density: 1.14, sucrose dissolved in Ringer solution) and centrifuged at 40 000 $\times g$ for 20 min in a Beckman SW 27 rotor.

Purified rod outer segments are harvested from the Ringer/sucrose interface with a syringe, the needle of which was bent at 90°. Two washing steps in ice-cold Ringer solution yield a pellet consisting mostly of intact rod outer segments with sealed plasma membrane (hereafter referred to as preparation I). Solubilizing preparation I in either sodium deoxycholate or Ammonyx-LO gives absorbance ratios A_{280}/A_{500} of between 2.3 and 2.5. Because we usually cannot increase the 500 nm absorption by addition of extraneous 11-*cis*-retinal, we conclude that significant amounts of bleached pigment are not present in our preparations.

Upon lysis of preparation I the absorbance ratio A_{280}/A_{500} falls to about 2.0 [21]. Subjection of preparation I to a Ficoll flotation procedure similar to that described by Smith et al. [22] yields a disk preparation with an absorbance ratio between 1.8 and 1.9 [21].

Preparation I is converted into preparation II by forcing the suspension twice very rapidly through a thin syringe needle packed with glass wool [19], and by subsequent freezing of 100 μl samples in liquid N_2. Preparation II was used throughout the reported experiments.

Light-scattering measurements

Thawed samples are suspended in the required salt solution (280 mosM), buffered with 5 mM Pipes, in a 10 mm square absorption cell, at an average rhodopsin concentration of 2 μM. All measurements were performed at pH 7 and 22°C, but essentially the same results were obtained at pH 6. Disk volume changes are monitored as changes in absorbance at 750 nm in a Cary 14 spectrophotometer. Neither during the preparation nor during the actual measurements had the rod outer segments been exposed to light of wavelength shorter than 650 nm.

Ionophores

The ionophores used are stored as concentrated ethanolic stock solutions in the dark at −40°C. They are introduced to the stirring test suspension with a microsyringe. The ethanol concentration in the reaction medium never exceeded 0.5%. The following ionophores were used:

2,4-Dinitrophenol (proton uncoupler);

Carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP, proton uncoupler);

Tributyl tin chloride (Cl^-/OH^- exchange carrier);

Valinomycin (ionophore specific for K^+);

Nigericin (K^+/H^+ exchange carrier);

X 537 A, very similar to 20 0006 (exchange carrier for H^+, monovalent, and divalent cations);

A 23187 (exchange carrier for H^+/divalent cations).

X 537 A, 20 0006 and Nigericin were a generous gift from W.E. Scott, Hoffmann LaRoche. A 23187 was a gift from Dr. R.L. Hamill, Eli Lilly and Co. Valiomycin, dinitrophenol and CCCP were from Sigma, and tributyl tin from Aldridge. Literature on the ionophores used can be found in the following review articles: Henderson et al. [23], Selwyn et al. [16], Reed and Lardy [24], Pressmann [25], Schadt and Häusler [26].

Results and Discussion

Study on the osmotic compartment responsible for the observed light-scattering increments

Interference from contaminating membrane material. Three strong pieces of evidence lead us to believe that the light scattering changes reported in this study originate exclusively in the rod outer segment:

(1) Our absorbance ratios A_{280}/A_{500} of 2.3—2.5 for unlysed and of 2.0 for lysed rods, as well as the value of 1.8—1.9 in the case of isolated disks, are as low as any ever reported in the literature. In particular the low values found for disk preparations leave little doubt that these preparations are pure, containing exclusively disk membrane intrinsic proteins and hence only disk membranes. These disks are isolated by floating all osmotically intact compartments present in preparation I on 5% Ficoll, and since only osmotically intact contaminating structures could interfere with our measurements, the absence of such structures in the disk preparation also implies their absence in preparation I and hence in preparation II.

(2) We could not detect any enzymatic activities in our rod outer segment

preparation which would be indicative of rod inner segment or mitochondrial contamination [27]. This is in agreement with Siebert et al. [28], who use a very similar preparation technique.

(3) In experiments indicating that the ion permeability of the membranes studied was very low, osmotically active vesicular contaminants, if present, would therefore also be impermeable under the same conditions and consequently would not perturb the measurements.

In experiments where a swelling was observed, the response was of an all-or-nothing nature and at no time did we observe a response which was a fraction of that maximally obtainable. This means that either there was no osmotically responding contaminating material present, or that it was present to some degree but absolutely impermeable to any of the ions tested. In neither case would interference occur.

Interference from osmotic compartments of the rod outer segments other than the disk. When isolated rod outer segment fragments (preparation II) are suspended in saline of varying osmolarity they respond osmotically and this response is accompanied by a distinct light scattering behaviour (Fig. 1). As the osmolarity increases the rod outer segment volume decreases, and this decrease manifests itself as an increase in turbidity. The linear relationship between $1/A_{750}$ and 1/osM shows that the osmotically responding compartments behave, for the most part, as ideal osmometers, obeying the Boyle-van't Hoff Law. At very low osmolarities there is a deviation from linearity, which was also reported by McConnell [18], who was the first to demonstrate the close relationship between rod outer segment volume (as determined by packed pellet volume studies) and light scattering. McConnell interpreted this deviation as an

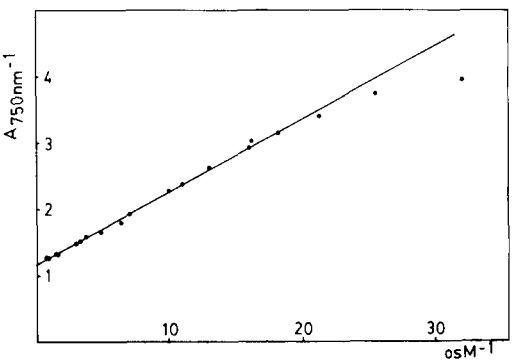


Fig. 1. Reciprocal of the turbidity (A_{750}) vs. reciprocal of the osmolarity for bovine rod outer segments (preparation II) suspended in KCl. Exactly the same slope is found for rod outer segments suspended in NaCl or sodium acetate. It is interesting to note that the intercept at the ordinate, i.e., the value for $1/A_{750}$, which is a measure of the rod outer segment volume at infinite osmotic pressure, is about 23% less than the volume found under isotonic conditions. If the osmotically responding compartments in this measurement were the disk lumen and the interdiskal matrix together, the totally collapsed volume at infinite osmotic pressure should amount to less than 50% of its volume under isotonic conditions, owing to the fact that rod outer segments do only swell or shrink in their longitudinal dimension [11,12] and that the disk lumen is about 20 Å, the interdiskal fluid 150 Å in thickness [40,41]. If, on the other hand, only the disks were to shrink, the centres of their two flat surfaces, which are approx. 75—80 Å apart, could approach each other by only 20 Å, thus reducing the total disk volume a maximum of 25%. This is very close to the observed value of 23% and would seem to indicate that it is actually the disk, and not the stack of disks or the interdiskal matrices, which respond osmotically under the conditions of the test.

indication of the possible involvement of more than one osmotic compartment in the observed volume changes. Therefore, if we are to use osmotic responses of rod outer segment fragments as a measure of permeability properties, the possible involvement of three distinct osmotic compartments must be considered. Two are enveloped by topologically closed membrane systems, namely plasma- and disk-membrane, and the third, the existence of which was postulated by Cohen [29], is contained in a gel-like matrix between the disks which is thought to exclude certain solutes, thus exhibiting osmotic responses.

In our preparation II the plasma membrane is no longer sealed (see also Ref. 19), since it allows rapid equilibration of Ca^{2+} [30], H^+ and ATP [30,31]:

(1) pH and [Ca^{2+}] have a pronounced effect on both kinetics and equilibrium constant of the metarhodopsin I-metarhodopsin II reaction. Low pH accelerates metarhodopsin I-metarhodopsin II and favours the formation of metarhodopsin II, whereas Ca^{2+} concentrations greater than 10 mM also accelerate metarhodopsin I-metarhodopsin II, but favour metarhodopsin I in the equilibrium. It therefore appears as though H^+ increases the rate of the forward reaction of metarhodopsin I-metarhodopsin II, whereas Ca^{2+} increases the rate of the reverse reaction, thus favouring metarhodopsin I in the equilibrium [30]. In order to exert this effect on the metarhodopsin I-metarhodopsin II reaction, the particular ion must have free access to the rhodopsin molecules in contact with the cytoplasm, i.e. H^+ or Ca^{2+} must be able to cross the plasma membrane if they are to affect metarhodopsin I-metarhodopsin II. In our preparation II both Ca^{2+} and H^+ exert their maximal effect on metarhodopsin I-metarhodopsin II in less than 15 s after they have been added to the reaction mixture. Their effect remains constant for over 20 min, thus indicating that complete equilibration has occurred *. On the other hand, as we shall demonstrate below, the compartments the volume changes of which we are monitoring in this examination do not allow any Ca^{2+} or H^+ movements across their boundaries unless suitable ion carriers are added.

(2) The presence of ATP molecules in the cytoplasm is essential not only for the light-stimulated rhodopsin phosphorylation [31,32], but also for a rapid, light-triggered structural response of the disk membrane, which requires a specific Mg^{2+}-ATPase activity in the disk prior to illumination [27,33,34]. The immediate onset of both reactions in our preparation II clearly indicates that the plasma membrane no longer acts as an osmotic barrier *.

It is also possible to rule out the osmotic participation of the interdiskal gel in our permeability measurements. We take ionophore-induced rod outer segment volume changes as a measure for permeability properties. Ionophores can account only for ion transport across membranes, but not for ion adsorption or desorption at gel surfaces. Since the plasma membrane was shown to be leaky, the ionophore-induced osmotic responses in our test must originate in the disks, thus enabling us to correlate unequivocally light-scattering changes in our system with changes in the disk volume.

Ion gradients across the disk membrane and the action of ionophores

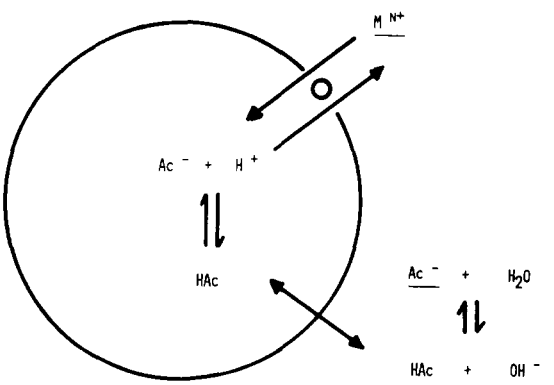
To use disk swelling as a measure of disk permeability to certain ions, an ion

* In contrast to this, we have found that in our preparation I an apparently intact plasma membrane excludes H^+, Ca^{2+} and ATP from the cytoplasm (unpublished results), as evident from the long incubation time required to obtain a maximal effect on rhodopsin and the disk membrane of the three ions.

gradient of the ion to be tested must first be established across the disk membrane. The rate of swelling, which accompanies the collapse of this gradient, is then a measure of the magnitude of the particular ion permeability. However, since we do not know the ion composition of the small aqueous disk lumen, the magnitude of this ion gradient is unknown. Secondly, even if there is an ion gradient across the disk membrane and the membrane is permeable to this particular ion, only very limited ion movements will occur unless there is an accompanying ion flux to restore electrical neutrality. For example, valinomycin, which specifically increases membrane permeability for K^+, does not cause any measurable disk volume changes if KCl is the suspending medium. Therefore, the disk membrane appears to be impermeable to Cl^- *. In our hands not even Nigericin, which allows a K^+/H^+ exchange across membranes, or valinomycin together with the uncoupler 2,4-dinitrophenol, cause disks in KCl to swell. This would indicate that the allowed K^+/H^+ exchange under these conditions is suppressed rapidly by the large proton depletion of the intra-diskal space which has a limited buffer capacity.

There is another possible interpretation of the above result: the disk lumen may already contain a high concentration of monovalent cations so that either there is no gradient across the disk membrane (in the case where the disks were already filled with K^+), or there is a 1 : 1 cation exchange, i.e., K^+ is moving into the disk and another monovalent cation, to which the disk membrane is permeable, is moving out simultaneously. This possibility, however, is precluded by the results given below.

A disk membrane anion permeability, which allows cations to equilibrate across the disk membrane at a rate proportional to the cation permeability of the ion tested, can be set up by using acetate as anion or by using Cl^- in the presence of trialkyl-tin compounds, which are known to permit rapid Cl^-/OH^- exchange in membranes. Scheme I illustrates how, in the presence of acetate, a



SCHEME I.

cation-acetate uptake into the disk occurs, provided the disk membrane is permeable to both H^+ and the particular cation. There will be a small amount of undissociated acetic acid inside and outside the disks which are, like other

* Brierley et al. [12] find a considerable swelling of rod outer segments in KCl in the presence of valinomycin. This seems to indicate that in their preparation there was a corresponding Cl^- permeability.

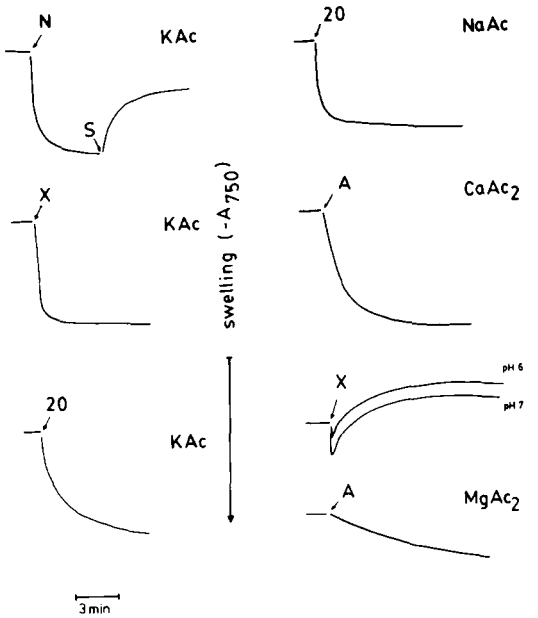


Fig. 2. Ionophore induced swelling of ROS in various salt solutions. Additions were: A, 5 μM (final concentration) A 23187; D, 100 μM (final concentration) DNP; N, 2 μM (final concentration) Nigericin; V, 1 μM (final concentration) Valinomycin; S, 200 mM (final concentration) sucrose; X, 25 μM (final concentration) X 537 A; 20, 25 μM (final concentration) 20 0006. Sucrose was added in one case to demonstrate the reversibility of the observed volume changes.

membrane systems [30,31], freely permeable to small uncharged molecules like acetic acid and NH_3. In the case where the disk membrane is permeable to both protons and the tested cation a rapid disk swelling should occur as a consequence of a fast proton/cation exchange across the membrane, accompanied by undissociated acetic acid and H_2O uptake by the disk. The process results in an overall cation acetate uptake.

Fig. 2 shows the rapid disk swelling (decrease in turbidity) observed in solutions of potassium, sodium, calcium and magnesium acetate. In these cases the required permeability for both protons and the given cations was provided by suitable ionophores. It was found that the extent and kinetics of the observed light-scattering increments were reproducible within less than 5%, when rod outer segments aliquots from the same preparation batch were used, and exhibited less than 20% variation from one preparation to the next. Their relative value in the various solutes, however, remained unchanged. The following information can be deduced from the results in Fig. 2:

(1) All the ionophores used possess the same ion-conducting properties in the disk membrane as they do in other membrane systems already studied.

(2) There are, in each suspending medium, considerable ion gradients across the disk membrane which, provided the membrane permeability allows it, cause a rapid ion influx accompanied by water uptake of the disks.

(3) The fact that the extent of disk swelling is very similar in potassium, sodium and calcium acetate indicates that the ion-gradients are similar as well. In each case the ionophore-induced decrease in absorbance amounted to

approx. 50%, corresponding to a decrease in osmolarity of about a factor of 4 (see Fig. 1).

(4) The extent of disk swelling in potassium acetate, induced by the highly specific K^+/H^+ exchange ionophore Nigericin, is not greater than the swelling induced by X 537 A or 20 0006. Since the latter two ionophores catalyse not only K^+/H^+ exchange, but also the exchange of monovalent metal ions with each other (in which case no swelling should be observed) and/or of two monovalent cations against one divalent cation, it appears that under our conditions a K^+/H^+ exchange is favoured over metal ion-metal ion exchange.

(5) The rate of K^+ uptake in the presence of X 537 A is clearly greater than in the presence of 20 0006, the bromine analogue of X 537 A. Therefore, in the disk membrane, X 537 A is a better K^+ ionophore than 20 0006. This is in contrast to the findings of Schadt and Häusler [26] that 20 0006 possesses a higher potassium specificity than X 537 A, when used in pure lipid systems.

(6) The ionophore A 23187, which catalyses the exchange of divalent cations against H^+, shows preference for Ca^{2+} over Mg^{2+} in this regard.

(7) The extent of disk swelling in sodium acetate in the presence of X 537 A is slightly smaller than the swelling observed in potassium acetate. This indicates that either internal cations compete with H^+ as exchange partners for the inflowing Na^+, or that the Na^+ gradient is smaller than the K^+ gradient due to some internally stored Na^+ in the disk.

(8) In the presence of calcium acetate and of an ionophore which allows metal ion-metal ion exchange, the initial rapid swelling is followed by an even greater shrinkage of the disks, probably due to the efflux of two monovalent cations for each Ca^{2+}. Naturally, this is pH dependent, i.e. is the more pronounced it is, the more protons are available in the extradiskal space.

(9) All the above results were obtained from rod outer segment preparations prepared in a saline medium containing Na^+ as the predominant cation. It is interesting to note that essentially the same results are obtained if the disks are prepared in media the predominant cation of which is K^+. In this case one would expect the disks to become loaded with K^+, with the result that little or no swelling be observed in the potassium acetate. In our hands there is no appreciable reduction in the swelling indicating that the disks possess a striking capability of retaining their original ionic milieu.

The metal ion permeabilities of the disk membrane

As outline in Scheme I and verified in the results of Fig. 2, disks undergo a rapid swelling when suspended in solutions of metal acetate, provided the disk membrane is permeable to both protons and the particular metal ion. The existence of highly specific proton ionophores (uncouplers) such as 2,4-dinitrophenol or carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) permits the use of the acetate system to test cation permeabilities of the disk membrane. In the presence of such proton ionophores, but without metal ion carriers, the rate of disk swelling should be essentially a direct measure of the natural metal ion permeability of the disk membrane.

Fig. 3 shows the swelling behaviour of rod outer segment disks in the presence of dinitrophenol in various salt suspensions. Using our technique it is apparent that the disks possess no measurable permeability for Ca^{2+}, Mg^{2+} and

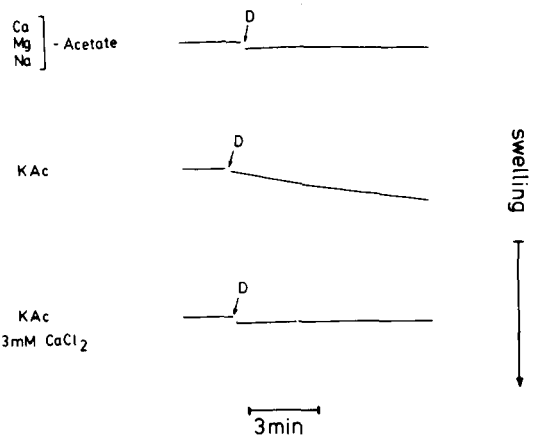


Fig. 3. Cation permeability test. Disk volume changes in calcium, magnesium, sodium and potassium acetate with and without 3 mM $CaCl_2$, upon addition of 100 μM dinitrophenol (D).

Na^+. A permeability of the disks to K^+ is detectable, but it can be completely abolished by small amounts of $CaCl_2$, an effect that is also observed in mitochondria [36].

It should be noted, however, that these experiments cannot give precise information on specific antiport systems probably present in the disk membrane. For identification of such systems, tracer experiments as carried out by Schnetkamp [37] are the method of choice. The low ion permeabilities found in this study by no means deny the existence of a $Ca^{2+}/Na^+/H^+$ antiport in the rod outer segment [37].

Proton permeability of the disk membrane

The method outlined above for measurements of membrane permeability to cations can also be adapted to measure the proton permeability of the disk membrane. In this case the membrane is made permeable to a specific cation, but not to protons. The observed swelling then becomes a measure of the hydrogen ion efflux from the disks. As valinomycin is the only known ionophore with absolute specificity for a single metal ion, i.e. K^+, the proton permeability

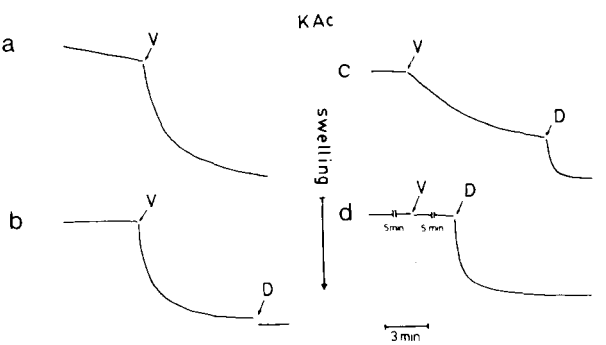


Fig. 4. Disk swelling in potassium acetate of disk preparations of different quality (see text). Ionophore additions were as described in the legend to Fig. 2.

tests were performed in potassium acetate solutions in the presence of valinomycin.

Fig. 4 shows the disk swelling in various preparations of rod outer segment fragments suspended in potassium acetate. Fig. 4a reports results from a rod outer segments preparation obtained from cattle eyes which had remained in the head of the dead animals for about 15—20 min before they were excised. In this case the dissection and density gradient procedure was performed by an inexperienced crew and about 3 h elapsed before the rod outer segments could be frozen. The disks swell even without the addition of valinomycin, indicating a considerable permeability of the disk membrane for both protons and potassium. After addition of valinomycin the swelling becomes very rapid, apparently not restricted by a limited proton permeability. Fig. 4b shows the results obtained from a rod outer segment preparation using similarly 'aged' cattle eyes, but then performed by a well-trained team, which had reduced the preparation time to about 2 h. There is no swelling in the absence of valinomycin, which indicates that the potassium permeability is much lower in this system. However, the proton permeability is still high as can be seen from the rapid disk swelling after addition of valinomycin. It should be borne in mind, however, that in the presence of such a high $[K^+]$ and of valinomycin in the extradiscal space a large diffusion potential should exist across the disk membrane, tending either to force protons or any other positive cations out of the disks, or acetate ions into them.

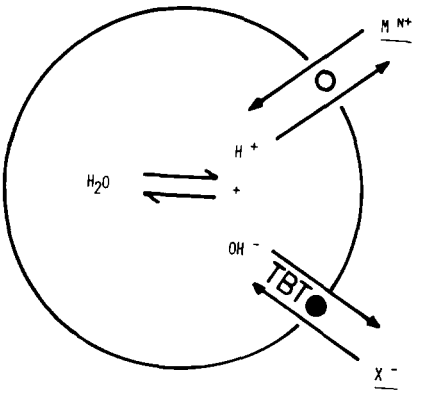
When we started to carry out our preparation procedure in the presence of 0.5 mM Ca · EDTA and in solutions saturated with argon to prevent oxidative damage of the rod outer segment [20] we found that the disk membrane permeabilities were markedly affected by these precautions, as can be seen from Fig. 4c. Swelling in potassium acetate and valinomycin is slowed down considerably, apparently slow H^+ efflux being the rate-limiting factor. It can be accelerated greatly by uncouplers such as dinitrophenol. In marked contrast, Fig. 4d shows the swelling behaviour of what we now consider our standard preparation II. In this case, where the eyes had remained in the head of the animal only 1 to 2 min after death, proton movements, even under high electric field conditions as established in the presence of a large K^+ gradient and valinomycin, apparently do not occur. Even after prolonged exposure of disks to potassium acetate and valinomycin the extent of disk swelling, initiated by addition of a protonophore, is only slightly reduced, indicating that the K^+ gradient remains relatively unchanged and is only very slowly dissipated, presumably by an exchange mechanism with another monovalent cation inside the disks.

Further evidence for the low proton permeability of the disk membrane

The ability of tributyl tin to induce selectively Cl^- permeability of the disk membrane gives rise to further possibilities to test for cation permeabilities of the disk membrane (Scheme II). As already mentioned above, disks suspended in NaCl, for instance, do not swell even in the presence of an ionophore which would allow Na^+/H^+ exchange. In addition, disks do not swell in NaCl upon addition or tributyl tin, as shown in Fig. 5. X 537A and tributyl tin together, however, cause a rapid swelling due to NaCl and H_2O uptake.

In KCl again disks so not swell upon addition of tributyl tin. Further addi-

SCHEME II. TBT, tributyl tin.



tion of valinomycin, however, causes a rapid swelling, the rate of which cannot be increased by uncouplers (Fig. 5). But in the presence of small amounts of $CaCl_2$, not only tributyl tin and valinomycin, but also a protonophore is required to produce a swelling of the disks.

To interpret these findings an exact knowledge of the action of tributyl tin is required. According to Mitchell and Moyle [38] tributyl tin allows Cl^- and OH^- to permeate freely the mitochondrial membrane, without a compulsory exchange. This would mean that both the neutral tributyl tin-Cl complex and the positive tributyl tin ion can cross the membrane. However, as Selwyn et al. [16] pointed out, there is good evidence to believe that only the uncharged tributyl tin-Cl or tributyl tin-OH complex can permeate, i.e. tributyl tin acts as an antiport. In the latter case, for a membrane suspended in a particular metal ion chloride salt to swell, the membrane must be permeable to Cl^-, OH^- (permeability provided by tributyl tin), to the particular metal ion (in our case K^+) and to protons, otherwise the disk interior would become too acidic. If tributyl tin functions only as an antiport, the data in Fig. 5 would indicate that the disk membrane is permeable to protons, unless there are small amounts of $CaCl_2$

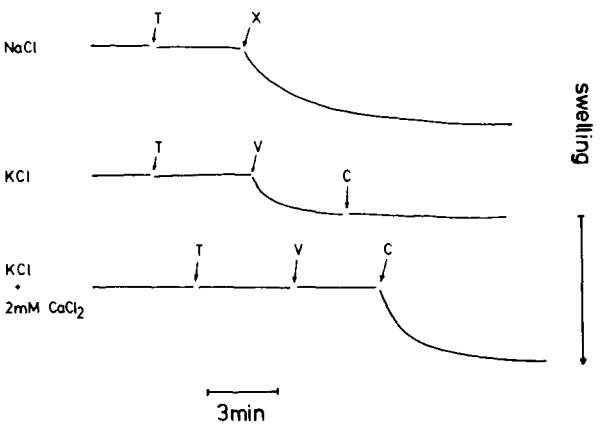


Fig. 5. Disk swelling in NaCl and KCl in the presence of 10 M tributyl tin and after addition of certain ionophores as described in Fig. 2. C, 10 μM CCCP.

present. This is in contrast to the results obtained in potassium acetate, where no $CaCl_2$ was required to make the disks proton-impermeable. Therefore we offer an alternative explanation of the findings in Fig. 5. In the presence of large concentrations of K^+ in the extradiskal space and after addition of valinomycin a large diffusion potential, positive inside the disks, would develop. Under these conditions the charged tributyl tin^+ cation, in which the positive charge is far more delocalised than in the case of H^+, could permeate the disk membrane down the electric potential gradient, i.e., from the inside of the disk to the outside. This would mean that disk swelling can occur even without the membrane being permeable to protons as suggested by the results obtained in potassium acetate. In terms of this model, the fact that in the presence of small amounts of $CaCl_2$ an uncoupler is required for the disks to swell would mean that, under these conditions, the tributyl tin cation could no longer cross the disk membrane, and would only function as an antiport as suggested by Selwyn et al. This interpretation is supported by the finding that disks swell in potassium acetate when DNP is present, even without addition of valinomycin, (Fig. 3, 2nd trace), but do not swell in KCl in the presence of tributyl tin, unless valinomycin is added (Fig. 5, 2nd trace). Apparently the low K^+ permeability of the disk precluded the build-up of a K^+ diffusion potential large enough to overcome the lack of a proton translocating system in the experiment of Fig. 5.2, i.e. under these conditions tributyl tin acts exclusively as an antiport. As to the effect of Ca^{2+} (Fig. 5.3), it appears possible that the existing diffusion potential facilitates Ca^{2+} binding to the extradiskal surface, thereby changing the surface charge dramatically and preventing the release of the tributyl tin cation into the extradiskal space.

The extraordinarily low proton conductance of the disk membrane, found in our freshest preparations, is surprising in the light of work of Dratz and coworkers (personal communication). They report the presence of considerable amounts of free fatty acids in the disk membrane, which are known to act as uncouplers by transporting H^+ across lipid bilayers and membranes [39]. Their presence in disks should preclude the low proton permeability that we observe in our preparations. It appears possible, however, that there are phospholipase present in the retina which create free fatty acids during the course of the preparation procedure.

Anion permeabilities of the disk membrane

The principle of the test for anion permeability of the disk membrane is shown in Scheme III. This is analogous to Scheme I, except that here the ammonium salt of the anion whose permeability properties are under test is used. NH_3, in its uncharged form, readily equilibrates across membranes. In this system, however, the anion influx is accompanied by a proton influx rather than an efflux as in Scheme I.

Fig. 6 shows that disks suspended in ammonium chloride do not swell in the presence of an uncoupler, thus indicating the very low Cl^- permeability of the disk membrane. Addition of tributyl-tin, a compound that allows Cl^-/OH^- exchange in membranes, causes a rapid swelling due to the now allowed NH_4Cl uptake. Furthermore, disks fail to swell in the presence of a protonophore, when they are suspended in the ammonium salts of sulphate, phosphate, nitrate

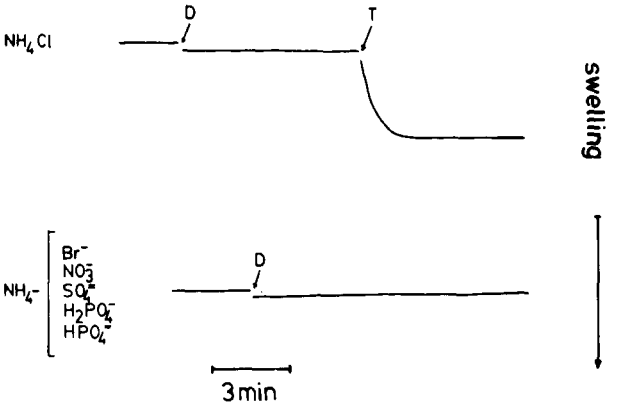
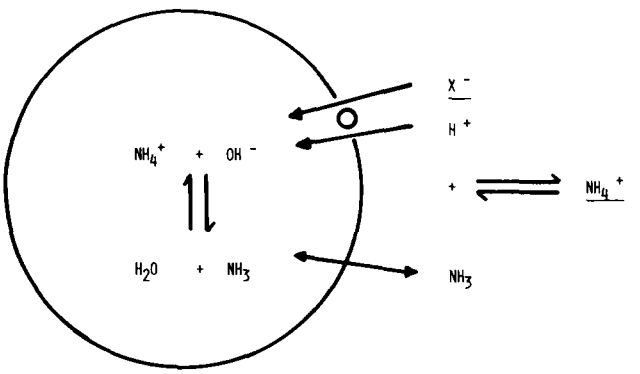


Fig. 6. Anion permeability tests using the ammonium salts of the anions to be tested. The fact that 100 μM DNP alone cannot induce a disk swelling in any of the ammonium salts tested demonstrates the very low disk membrane permeability to these anions. T, 10 μM TBT.

and bromide. This suggests that the disk membrane has no measurable permeability for these anions.



SCHEME III.

Comparison of the permeability properties of the disk membrane and other membrane systems

Permeabilities of the membranes of mitochondria, thylakoids and erythrocytes have been studied extensively [15,16,23,35,36]. All membrane systems were found to be permeable in some degree to a number of ionic species. Rod outer segment disk membranes, on the other hand, exhibit a striking impermeability to most ions. The only exception is K^+, and its permeability, as in the case of mitochondria, can be suppressed by the addition of small amounts of divalent cations.

Particularly surprising, however, is the low H^+ permeability of the disk membrane. It appears to be even lower than the H^+ conductance of the membranes of mitochondria and thylakoids. Both these systems are presumably compartmented so as to favour the development of H^+ gradients, and to permit passage

of the H^+ only through the transmembrane ATPase when ATP is manufactured. The extremely low H^+ permeability of the disk membrane suggests that H^+ gradients across the disk membrane may also play a significant physiological role in the case of the photoreceptor cell.

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