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INTERFACIAL POTENTIALS AT THE DISK MEMBRANES OF ISOLATED INTACT CATTLE ROD OUTER SEGMENTS AS A FUNCTION OF THE OCCUPATION STATE OF THE INTRADISKAL CATION-EXCHANGE BINDING SITES

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Summary

Two different methods have been used to determine the interfacial potential at the disk membranes of intact isolated bovine rod outer segments:

(1) The photolysis products of rhodopsin are known to be dependent on pH. We have used this property in order to probe the interfacial potential at disk membranes which is considered to change the surface pH at the disk membrane seen by rhodopsin.

(2) The p*K* value of the amphiphilic pH-indicating dye neutral red (uncharged basic form) in water is 6.6, but adsorbed to disk membranes at least 7.8. This makes the distribution of neutral red between disk membranes and bulk water dependent on the interfacial potential at the disk membrane if the pH in the bulk solution is less than 7.8.

Both methods yielded comparable results on the influence of ions and ion carriers on the interfacial potential at disk membranes. In particular, we have studied the effect of different occupation states on the internal binding capacity (of rod outer segments) for divalent cations. In the presence of the ionophore A23187, addition of EDTA to a suspension of intact rod outer segments removed all endogenous divalent cations (Schnetkamp, P.P.M. (1979) Biochim. Biophys. Acta 554, 441—459) and resulted in an interfacial pH at the disk membrane surface of about 6.4, whereas the bulk pH was 7.4. Subsequent addition of 2 mM Mn^{2+} saturated the internal binding capacity and

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Abbreviations: SDS, sodium dodecyl sulphate; Mes, 4-morpholineethanesulphonic acid; Mops, 4-morpholinepropanesulphonic acid; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

resulted in an apparent shift towards alkaline pH of the surface pH at the disk membrane by 1.0—1.1 pH units. This could indicate a change of the interfacial potential by 60—65 mV. The same change of ionic conditions resulted in a change of the interfacial potential by 72 mV as determined from the partitioning behaviour of neutral red. These results were independent of the presence of H^+ ionophores such as carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone and gramicidin. We conclude that the above results can be explained by the presence of fixed net negative charges (charge density: 0.5—1.5 electronic charges/rhodopsin molecule) at the intradiskal membrane surface. That the above charge density can be attributed to the intradiskal membrane surface is inferred from the observation that the presence of A23187 was required for access of divalent cations to the membrane interface involved in both rod outer segments with an intact as well as with a leaky plasma membrane.

Introduction

Intact isolated cattle rod outer segments behave as a cation exchanger. They contain a large intracellular cation storage capacity (9 mol Ca^{2+}/mol rhodopsin) with a relatively broad selectivity, at which divalent cations and most likely also H^+ can be reversibly exchanged [1,2]. Access to these storage sites from the external medium through the plasma membrane can be mediated either by an endogenous exchange diffusion system or by external ionophores. The endogenous system operates with high ion selectivity and, under most conditions, does not allow net uptake of Ca^{2+} [2]. In the presence of the ionophore A23187, net transport of Ca^{2+} occurs and the intracellular storage capacity can be titrated with external Ca^{2+} [1]. In our preparations these storage sites are characterized by a single apparent binding constant. Light-stimulated release of Ca^{2+} from these sites is only apparent after addition of the ionophore A23187, and when the plasma membrane of the rod outer segment is made leaky [3]. Together with the predominant intradiskal location of endogenous Ca^{2+} in our rod outer segment preparations [1] this suggests that the Ca^{2+}-binding sites are predominantly located inside disks.

Phosphatidylserine is an important constituent of the bovine disk membrane (about 9 mol phosphatidylserine/mol rhodopsin, see Ref. 4). At physiological pH it most likely carries a net negative charge and may thus produce a negative interfacial potential at disk membranes. It is the objective of this communication to study the dependence of this interfacial potential on the ionic conditions and on the occupation state of the intracellular binding sites.

We have used two methods to assess the interfacial potential occurring at the disk membranes in rod outer segment preparations. The first method is based on the observation that, at room temperature, photolysis of rhodopsin at different pH values in the suspending medium yields different photoproducts on a time scale of some tens of seconds [5,6]. The previous interpretation that a pH-dependent equilibrium exists between metarhodopsin II (λ_{max} = 380 nm) and metarhodopsin I (λ_{max} = 45 nm) has been questioned recently be several authors (e.g., see Refs. 7 and 8). Disregarding the exact

mechanism, the observation remains that photolysis of rhodopsin at alkaline pH produces less absorption at 380 nm and more absorption remains at 485 nm as compared to photolysis of rhodopsin at acidic pH. Rhodopsin is an intrinsic membrane protein (predominantly within the disk membrane) and, thus, it can be expected that it senses the interfacial pH rather than the bulk pH. The interfacial pH in turn is dependent on the interfacial potential.

The second method uses the redistribution of the amphiphilic dye neutral red between the membrane phase and the bulk aqueous phase to probe changes of the interfacial potential upon changes of the ionic conditions. Neutral red is a pH indicator with a positively charge protonated form and an uncharged basic form. At least the uncharged form easily crosses membranes (similar to compounds like NH_3 and CH_3CO_2H) and may reach both interfaces of the disk membrane. We observed a large enrichment of neutral red in rod outer segment membranes concomitant with a shift of the apparent pK towards alkalinity. Therefore, under the conditions used in our study, the protonated positively charged form of neutral red predominates in the membranes, whereas the uncharged basic form predominates in the bulk water. We find that the combination of these properties makes the distribution of neutral red between the membrane phase and the bulk water sensitive to the interfacial potential.

Materials and Methods

Preparations and general procedures. All procedures with rod outer segments were carried out in darkness or in dim red light unless stated otherwise.

Intact and leaky rod outer segments were prepared from bovine retinas as described previously [9,10]. An an alternative to the cited method, intact rod outer segments were also made leaky by slow freeze-thawing (freezing by placing the suspension at −20°C). The 'frozen' leaky rod outer segments did not show retinol formation, whereas the chromophore could be reduced by externally added NADPH [9]. All rod outer segment preparations were stored as concentrated suspensions (100—200 μM rhodopsin) in the standard medium (600 mM sucrose, 5% (w/v) Ficoll 400, 20 mM Tris, adjusted to pH 7.4 with HCl) at 4°C. Directly before use, the stock suspension was diluted (generally 10-fold) with the standard medium omitting the Ficoll 400. The latter was omitted in the present experiments, since this improved the stability of the light-scattering properties. All experiments were carried out at room temperature (22°C). Ionophores (A23187, gramicidin, FCCP) were added as ethanolic solutions (final ethanol concentration 0.5%, v/v), which did not affect the integrity of the plasma membrane (judged by the rate of retinol formation, see Ref. 9).

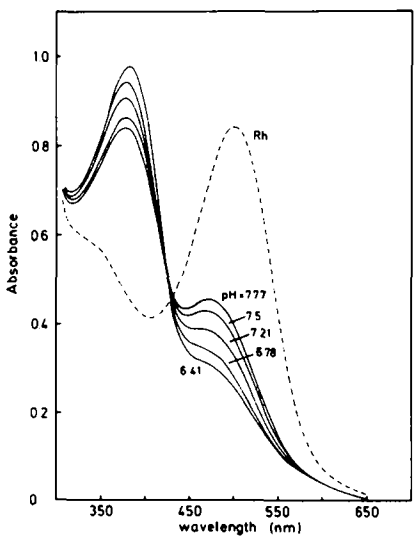
Spectrophotometric recordings. Spectrophotometric recordings were performed on a Beckman UV5260 spectrophotometer with the cuvette placed in front of the photomultiplier. Spectra were scanned with a scanning speed of 4 nm/s starting from the long-wavelength side. The absorption (apparent absorption due to light scattering) at 650 nm was arbitrarily set to zero.

Photolysis spectra. After recording the spectrum of the unbleached suspension of rod outer segments, it was exposed to white light for 10 s, which

bleached 80—90% of the rhodopsin present (depending on the ionic conditions). The bleaching percentage was determined by addition of Triton X-100 (1%) and NH_2OH (50 mM) following the usual procedure [11]. Spectral recordings were started 20 s, 3, 8, 15 and 25 min after the onset of illumination. For a given set of conditions in the suspending medium the bleaching procedure produced the photochemical equilibrium and proved to be strictly reproducible.

Photolysis of rhodopsin under variation of the medium pH at high ionic strength. The effect of medium pH on the photolysis products of rhodopsin was measured with lysed rod outer segments (lysis of intact rod outer segments by addition of 20 vol. of water, followed by centrifugation and resuspension in the desired medium). Suspending media contained 0.4 M NaCl and 60 mM of the following pH buffers. Below pH 6.5, buffers contained Mes, between pH 6.5 and 7.5 buffers contained Mops (both adjusted to the final pH with NaOH) and above pH 7.5 buffers contained Tris (adjusted to the final pH with HCl). The high electrolyte concentration in these media was assumed to reduce greatly the interfacial potential at the disk membranes so that the interfacial pH would not be expected to deviate significantly from the pH in the bulk solution. Fragmented rod outer segment preparations were not able to maintain pH gradients on the time scale of our experiments (see also Ref. 12).

Fig. 1 shows spectra recorded 20 s after the photolysis of rhodopsin as a



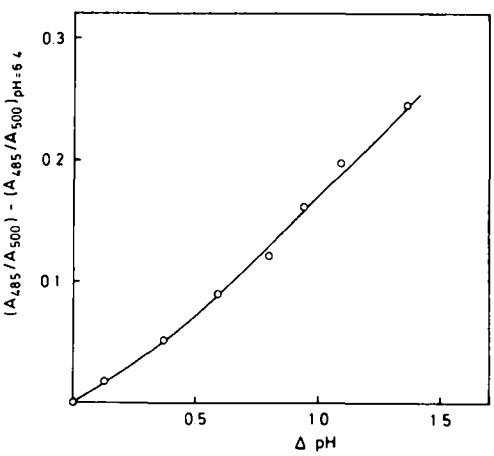


Fig. 1. Effect of the external pH on the photolysis products of rhodopsin in lysed rod outer segments membranes suspended in high salt solution. Lysed rod outer segment membranes, resuspended in 0.4 M NaCl and 60 mM pH buffer at the indicated pH, were bleached with white light for 10 s. After 20 s a spectrum was recorded. With increasing pH the absorption remaining at 485 nm increased and the absorption at 380 nm decreased. The medium pH increased as indicated from 6.41, 6.78, 7.21, 7.50 to 7.77. The spectrum marked Rh is the rhodopsin spectrum recorded before bleaching.

Fig. 2. pH calibration from the photolysis products of rhodopsin. The absorption remaining at 485 nm in spectra as shown in Fig. 1 was divided by the absorption at 500 nm before bleaching, and plotted against the increase in the medium pH. The value obtained at a medium of 6.4 was arbitrarily set to zero.

function of the pH. With increasing pH more absorption remained at 485 nm and less absorption arosed at 380 nm. Difference spectra reveal that the photoproduct, which increased at alkaline pH, has a maximal absorbance at 485 nm. The absorbance remaining at 485 nm (related to the amount of rhodopsin present before bleaching) was plotted against the increase in medium pH (Fig. 2). We observed that intact rod outer segments in the presence of EDTA and the ionophore A23187, and at a bulk pH of 7.4, showed photolysis spectra, which had an appearance similar to those of fragmented rod outer segments in a high electrolyte medium of pH 6.4. An increase in the electrolyte concentration in the suspending medium of intact rod outer segments in the presence of the appropriate ionophores resulted in photolysis spectra, which were more comparable to those observed in fragmented rod outer segments in high salt medium at a more alkaline pH. With the assumption that changes of the ionic conditions influence the interfacial pH only via the interfacial potential, we calibrated the latter by the pH shift towards alkalinity with the zero arbitrarily attributed to the most acidic apparent pH. When the same preparation was used and at rhodopsin concentrations greater than 15 μM, the different ionic conditions did not resulted in significant changes of the light-scattering properties of the intact rod outer segments. Following the above calibration procedure, the major source of errors, namely the differences in light-scattering properties between different rod outer segment preparations, could be eliminated.

In the above procedure, it was assumed that the various ionic conditions used in this study only affected the photolysis products of rhodopsin by their effect on the interfacial potential and, therewith, on the interfacial pH. The validity of this assumption will be discussed later.

Neutral red spectra. Neutral red (3-amino-7-dimethylamino-2-methylphenazine) was purchased from Sigma (Munich, F.R.G.). Neutral red spectra at different pH values were recorded in 100 mM of the pH buffers mentioned before except for the spectra recorded in the standard medium (600 mM sucrose, 20 mM Tris, adjusted to pH 7.4 with HCl) omitting Ficoll 400. Spectra of neutral red in rod outer segment membranes were recorded against a sample of the same rod outer segment suspension in the reference cuvette. The uncharged basic form of neutral red has a maximal absorbance at 440—450 nm, while the protonated positively charged forms absorbs maximally at 525—545 nm (the molar absorbance as well as λ_{max} varies with the environment, see Results).

Determination of the neutral red partition coefficient. Aliquots of a suspension of intact rod outer segments in the standard medium (final rhodopsin concentration 25—35 μM) were incubated for 10 min with neutral red (final concentration ranged between 10 and 200 μM) and with A23187, EDTA and $MnCl_2$ (as indicated). Longer incubation times did not alter the results. Subsequently, the samples were centrifuged (3500 $\times g$, 20 min, 15°C) and aliquots of the supernatant were appropriately diluted with the standard medium containing 1% SDS. The latter was added to shift all neutral red molecules into the protonated form, which has the highest molar absorbance. The amount of neutral red present in the supernatant was determined spectrophotometrically and the amount of neutral red present in the rod outer segment membranes

was calculated. The amount of neutral red present in the aqueous compartments of rod outer segments was neglected (total intracellular aqueous volume was always less than 1% of the volume of the external medium).

Results

Photolysis products of rhodopsin under different ionic conditions

Fig. 3 shows photolysis spectra of rhodopsin in suspensions of intact rod outer segments in the standard medium with variation in the divalent cation concentration. Spectrum 2 was recorded in the presence of EDTA, which chelated at least the external divalent cations present in the preparation. When, in addition to EDTA, the ionophore A23187 was added to the suspension, the internal divalent cations were nearly completely removed from the rod outer segments [1]. Under this condition, the first spectrum recorded after the photolysis of rhodopsin showed less absorption at 485 nm as compared to that recorded without A23187 (cf. spectra 1 and 2). A similar change could be

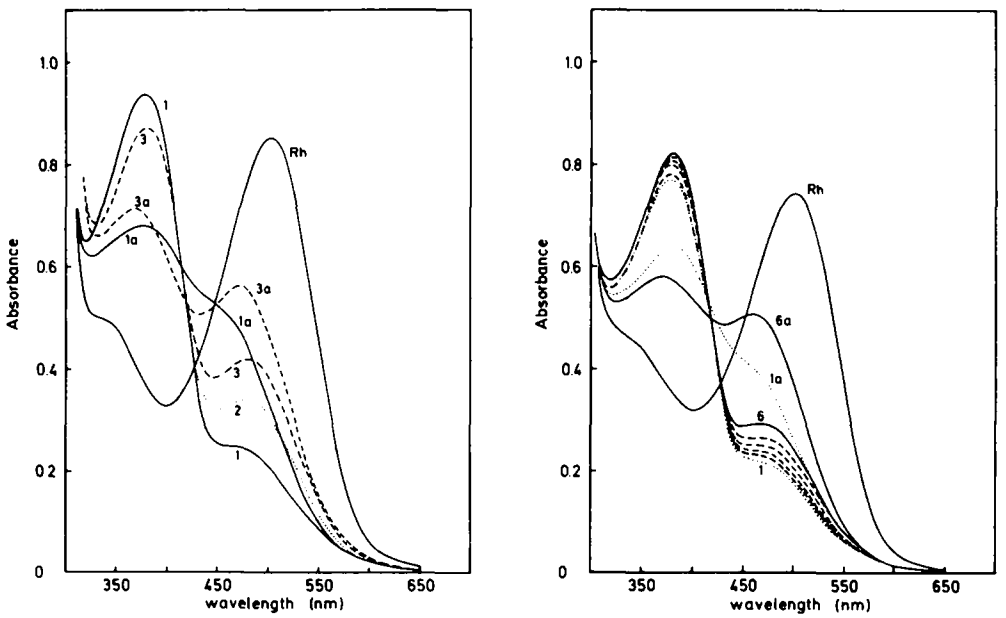


Fig. 3. Effect of EDTA and Mn^{2+} on the photolysis products in intact rod outer segments. Photolysis spectra of suspensions of intact rod outer segments in the standard medium are shown (spectrum before bleaching is marked Rh) following an 85% bleaching. Spectra 1—3 were recorded 20 s after the onset of illumination, spectra 1a and 3a were recorded after 25 min. Additions to the standard medium (600 mM sucrose, 0.5% Ficoll 400, 20 mM Tris, adjusted to pH 7.4 with HCl): (1) 0.5 mM EDTA + 5 μM A23187; (2) 0.5 mM EDTA; (3) 2 mM $MnCl_2$ + 5 μM A23187.

Fig. 4. The effect of $CH_3CO_2NH_4$ on the photolysis products in intact rod outer segments. Photolysis spectra of suspensions of intact rod outer segments in the standard medium were obtained following the same procedure as in Fig. 3. Additions to the standard medium: 0.5 mM EDTA, 5 μM A23187 and from spectra 1 to 6 at increasing concentrations 0, 2, 5, 10, 20 and 50 mM $CH_3CO_2NH_4$, concomitant with an increase of the absorption remaining at 485 nm. Using the calibration curve shown in Fig. 2, the apparent pH shifts which accompanied the increase in the concentration of $CH_3CO_2NH_4$ amounted to 0.17, 0.30, 0.41, 0.51 and 0.61 pH units, respectively, Spectra 1—6 were recorded 20 s after bleaching, spectra 1a and 61 15 min after bleaching.

obtained with fragmented rod outer segments suspended in a high-electrolyte medium by incrasing the medium pH (Fig. 1). Comparing these two results we infer that the interfacial pH in intact rod outer segments is 6.4 at the pH-sensitive site of rhodopsin under these conditions (bulk pH 7.4, removal of divalent cations by EDTA via the ionophore). For this estimate, the different light-scattering properties of intact rod outer segments as compared to lysed rod outer segments were considered. When the external concentration of divalent cations was increased by adding 2 mM $MnCl_2$ without A23187, the photolysis spectrum observed was nearly the same as that obtained after addition of EDTA in the absence of A23187 (not shown). In contrast, addition of Mn^{2+} in the presence of A23187 resulted in a photolysis spectrum which showed substantially more absorption at 485 nm as compared to that observed when EDTA with or without A23187 was added (Fig. 3, cf. spectrum 3 with spectrum 1 and 2). The difference between spectrum 1 and 3 was similar to that observed for fragmented rod outer segments in high salt solutions when the external pH was increased from 6.4 to about 7.4—7.5 (cf. Figs. 1 and 3).

We have followed the development of the slow photoproducts and found a clean metarhodopsin II to metarhodopsin III transition in the presence of A23187 and either EDTA or Mn^{2+} *. The spectral transitions observed suggest that the distinct photolytic behaviour with different concentrations of divalent cations is not the result of a transient H^+ gradient caused by the exchange of Mn^{2+} and H^+ mediated by A23187 (this ionophore exchanges divalent cations and H^+, see Refs. 13 and 14). A similar conclusion can be drawn from the attendant observation that inclusion of H^+ ionophores (5 μM FCCP or 3 μM gramicidin) did not noticeably change the above results for the cases in which A23187 and either EDTA or Mn^{2+} were present.

In the experiments shown in Fig. 3, we investigated the effect of divalent cations on the photolysis products of rhodopsin in intact rod outer segments suspended in a medium or relatively low salt concentration (about 15 mM Tris-HCl). The results were compared with photolysis spectra of fragmented rod outer segments in high salt solutions at various external pH values. In the experiment shown in Fig. 4, the effect of a 1 : 1 electrolyte on the photolysis products of rhodopsin in intact rod outer segments was investigated. In the case of divalent cations, the accessibility of all intracellular compartments was provided for by the addition of the ionophore A23187. In the case of a 1 : 1 electrolyte, accessibility was ensured by application of $CH_3CO_2NH_4$, an intrinsically permeant electrolyte. This electrolyte rapidly equilibrates across biological membranes due to the permeability of the neutral species NH_3 and CH_3CO_2H. The spectra shown in Fig. 4 demonstrate that a gradual increase in the external concentration of $CH_3CO_2NH_4$ resulted in a progressive increase in the absorption, which remained at 485 nm after the photolysis of rhodopsin. In high salt solutions, similar effects were observed when the external pH was

* In the presence of only EDTA or in the presence of both A23187 and either Mg^{2+} or Ca^{2+}, the development of the slow photoproducts was more complex due to the reduction of the chromophore to retinol. Retinol formation appeared to be completely inhibited in the presence of A23187 and either EDTA or Mn^{2+} (see Refs. 9 and 15). For this reason, Mn^{2+} was used as a divalent cation in the present study instead of Ca^{2+} or Mg^{2+}.

increased (Fig. 1). The action of monovalent cations thus parallels that of divalent cations except for the greater effectivity of the latter.

The identification of the compartment where rhodopsin probes the salt effects

In the above experiments, rod outer segments were used which had an intact plasma membrane. Furthermore, the intracellular compartments were made accessible to external electrolyte either by the use of an intrinsically permeant electrolyte or by the application of A23187, which makes all intracellular compartments of rod outer segments accessible to divalent cations [1,3]. Accordingly, it could not be decided from the above experiments from which compartment the rhodopsin molecules in the disk membrane experience the ionic conditions. In order to differentiate between the cytosolic and intradiskal compartments, the experiment shown in Fig. 3 was repeated with rod outer segments with a leaky plasma membrane. A comparison of the photolysis spectra, shown in Fig. 5a and b, respectively, demonstrates that changes of the external concentration of EDTA as well as of Mn^{2+} exerted clear effects on the photolysis spectra of rhodopsin only if the ionophore A23187 was present. Results similar to those shown in Fig. 5 were obtained with rod outer segments, which were made leaky by slow freeze-thawing (not shown). The maximal change in the absorbance at 485 nm observed for leaky rod outer segments (i.e., the difference between spectra 1 and 3 in Fig. 5b)

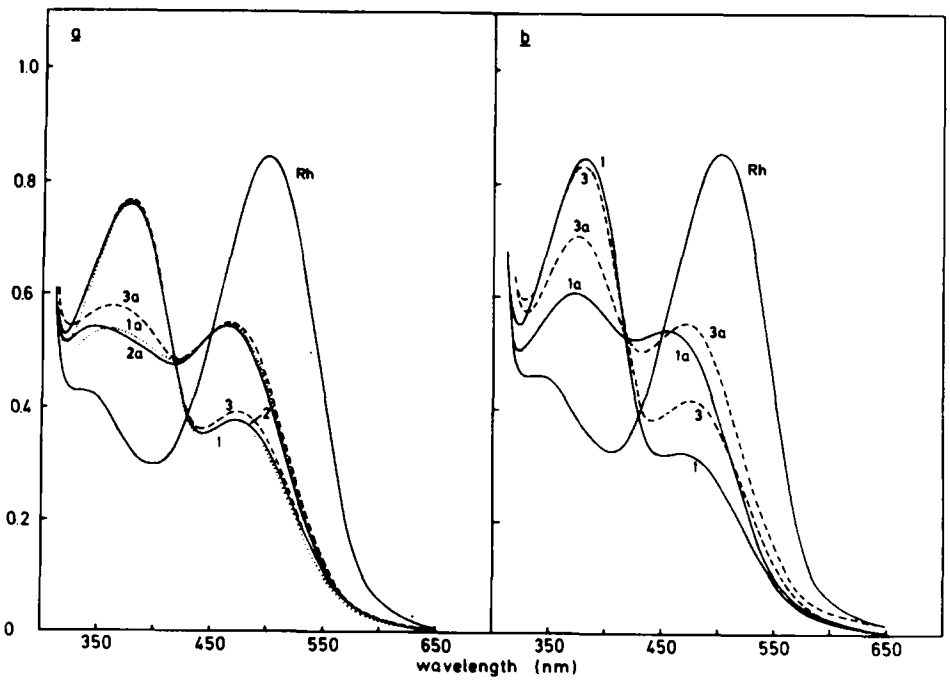


Fig. 5. The effect of EDTA and Mn^{2+} on the photolysis products in leaky rod outer segments. Photolysis spectra of suspensions of leaky rod outer segments in the standard medium were obtained following the same procedure as in Fig. 3. Additions to the standard medium: (a) no A23187; (b) 5 μM A23187; spectra 1 and 1a, 0.5 mM EDTA; spectra 2 and 2a, no further addition; spectra 3 and 3a, 2 mM $MnCl_2$. Spectra 1—3 were recorded 20 s after bleaching, spectra 1a—3a 25 min after bleaching. Spectra marked Rh were recorded before bleaching.

was about 35% smaller than that observed for intact rod outer segments (i.e., the difference between spectra 1 and 3 in Fig. 3). Thus, the greater part of the sensitivity of the photolysis of rhodopsin to the ionic conditions originates from the inner side of the disk membrane. The validity of this conclusion rests on the definition of rod outer segments with a leaky plasma membrane. For both types of leaky rod outer segment, the accessibility of the enzymatic machinery necessary to reduce the chromophore was used. External NADPH could be used by the internal retinol dehydrogenase [9] and the internal enzymes of the pentose phosphate pathway could use externally added substrates such as NADP plus glucose 6-phosphate [9,10].

Adsorption of neutral red at interfaces with a variable surface charge

In the foregoing section the photolytic properties of rhodopsin were investigated under various ionic conditions. We observed that both monovalent and divalent cations which had access to the intracellular compartments of intact rod outer segments affected the photolytic behaviour of rhodopsin as if they changed the pH seen by the rhodopsin molecules in the disk membrane despite the fact that the bulk pH remained constant. To corroborate and extend these results we have used the adsorption of the amphiphilic pH-indicating dye neutral red to rod outer segment membranes. It may be useful first to explore some of the properties of this dye in a more simple amphiphilic environment than the rod outer segment disk membranes.

Following the method described by Fernández and Fromherz [16], the effect of charged and uncharged detergents on the neutral red spectrum was investigated (Fig. 6). The solid and dotted lines in Fig. 6 represent spectra of neutral red in aqueous solutions of various pH values. At the medium pH of 7.4, used in all experiments with rod outer segments reported in this study, the uncharged basic form of the dye predominated in the bulk solution. Upon addition of 1% Triton X-100 to this medium a nearly complete transfer into the basic form was observed. This is explained by a shift of the apparent pK of neutral red adsorbed to Triton X-100 micelles towards a more acidic value (pK_{ap} of about 5.8 as compared to a pK in aqueous solution of 6.6, not shown). This shift is similar to that observed for the pH indicator aminocoumarin upon adsorption to Triton X-100 micelles [16]. Like neutral red this dye has an uncharged basic form. Addition of SDS introduces a negative surface charge into the intrinsically neutral Triton X-100 micelles. We observed that increasing amounts of SDS added to Triton X-100 micelles in the standard medium (pH 7.4) titrated the basic form into the positively charged protonated form. Equal amounts of both forms were present when 6.6 mM SDS was added. This means an apparent pK shift of 1.6 (from 5.8 to 7.4; shifts in the opposite direction were obtained by addition of the positively charged detergent Ammonyx LO to the Triton X-100 micelles, not shown). Finally, replacement of 1% Triton X-100 by 1% SDS (pH in the bulk solution remained at 7.4) yielded the fully protonated form of neutral red (Fig. 6). A prominent feature observed in Fig. 6 is the 10—15 nm red-shift of the absorbance maxima of both forms of neutral red concomitant with a peak-sharpenig and an increase of the molar absorbance, which accompanied the adsorption of neutral red to Triton X-100 and SDS micelles.

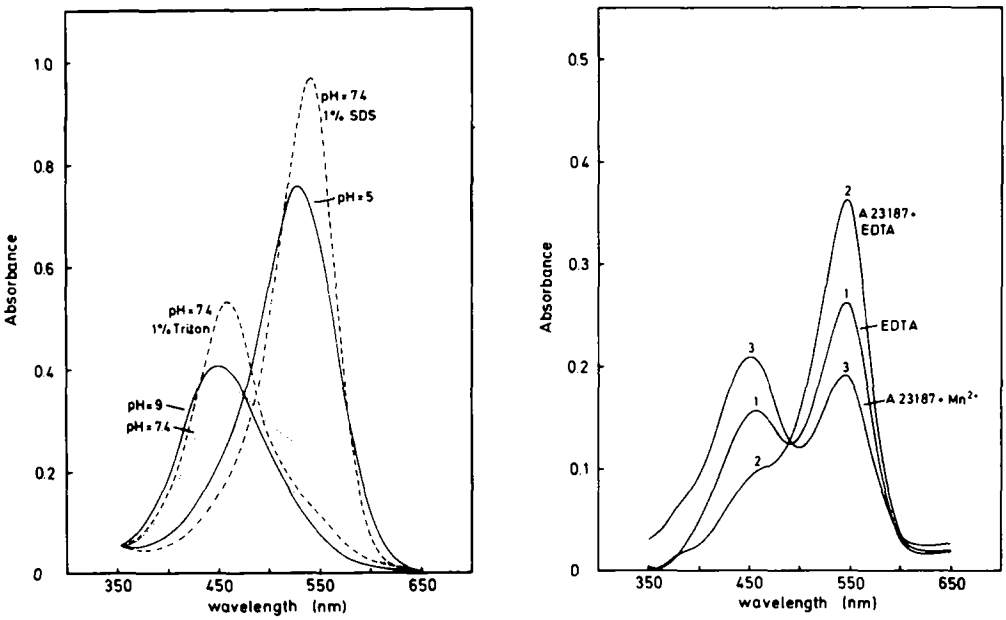


Fig. 6. Spectra of neutral red in aqueous solution and in detergent micelles. Spectra of neutral red (26.7 μM) in different media are shown. (———) 100 mM Mes, adjusted to pH 5 with NaOH or 100 mM Tris, adjusted to pH 9 with HCl (as indicated). (· · · · · ·) 600 mM sucrose, 20 mM Tris-HCl (pH 7.4). (- - - - - -) 600 mM sucrose, 20 mM Tris-HCl (pH 7.4) and 1% Triton X-100 or 1% SDS as indicated.

Fig. 7. Neutral red adsorbed at the disk membranes of intact rod outer segments under different ionic conditions. 20 μM neutral red was added to a suspension of intact rod outer segments in the standard medium containing 32 μM rhodopsin. Spectra were recorded against the same rod outer segment suspension, but without neutral red, in the reference cuvette. In addition to the standard medium: spectrum 1, 0.5 mM EDTA; spectrum 2, 0.5 mM EDTA + 5 μM A23187; spectrum 3, 2 mM $MnCl_2$ + 5 μM A23187. The difference in the absorption at 350 nm between spectrum 1 or 2 and spectrum 3 was due to the complex between A23187 and Mn^{2+}. The same phenomenon can also be observed in Figs. 3 and 5.

Adsorption of neutral red to rod outer segment disk membranes

In the following the contribution of the plasma membrane to the total membrane surface area of rod outer segment membranes and the concomitant contribution to the accumulation of neutral red in rod outer segments will be neglected.

Fig. 7 shows difference spectra of rod outer segment suspensions with (sample) and without (reference) neutral red. Under conditions where all intracellular divalent cations were removed from the rod outer segments (EDTA and A23187 present), the total neutral red spectrum (Fig. 7, spectrum 2; this spectrum is a superposition of aqueous and membrane-incorporated neutral red) looked very similar to that observed in the standard medium containing SDS (Fig. 6). This indicates that the large majority of neutral red was adsorbed at the disk membranes and, moreover, was in the protonated form (cf. neutral red spectrum in the standard medium, Fig. 6, dotted line). In agreement with this, after centrifugation of the rod outer segments, 18%

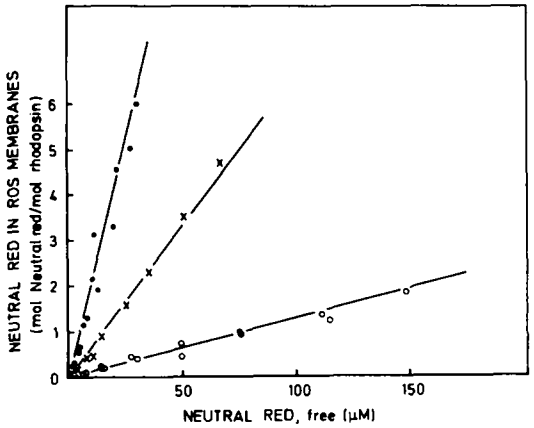


Fig. 8. The distribution of neutral red between the disk membranes in intact rod outer segments (ROS) and the aqueous solution under different ionic conditions. The amount of neutral red present in the intact rod outer segments is plotted against the free neutral red concentration in the external medium. In addition to a suspension of intact rod outer segments in the standard medium were added different neutral red concentrations (total neutral red concentration ranged between 10 and 200 μM) and: (●———●), 0.5 mM EDTA + 5 μM A23187; (X———X), 0.5 mM EDTA; (○———○), 2 mM $MnCl_2$ + 5 μM A23187. Experimental data of four experiments with different rod outer segment preparations were used (rhodopsin concentration ranged between 25 and 35 μM). The slopes of the curves were calculated by the application of linear regression with the additional option that the curves must pass through the origin.

of the total neutral red appeared in the supernatant and, consequently, 82% was present in rod outer segments. When the endogenous divalent cations were still present (EDTA, but no A23817 added), the amount of protonated neutral red was less (Fig. 7, spectrum 1). When the intracellular compartments were made accessible to external Mn^{2+} the amount of protonated neutral red decreased further (Fig. 7, spectrum 3) *. It is noted that in all cases the neutral red in the protonated form showed the red-shifted and sharpened absorption peak (cf. the protonated form of neutral red in aqueous solution in Fig. 6). Centrifugation and measurement of the fraction of neutral red present in the rod outer segments revealed that in the sample with only EDTA (spectrum 1), 57% and in the sample with Mn^{2+} and A23187 (spectrum 3), 32% of the total amount of neutral red resided in the rod outer segment membranes. This result means that the partitioning of neutral red between membranes and aqueous solution depends on the ionic conditions imposed on the membranes. Furthermore, the redistribution of neutral red upon changes of the ionic conditions (as revealed by the partitioning experiment) account for the greater part of the drop, which was observed in the absorbance at 485 nm

* The spectra shown in Fig. 7 result from the superposition of the spectra of aqueous and membrane-adsorbed neutral red. Therefore, these spectra are difficult to interpret. Nevertheless, they allow an estimation of a lower limit for the apparent pK of neutral red adsorbed to disk membranes. Spectrum 1 allows an estimate of the molar absorbance of the protonated form of neutral red adsorbed to disk membranes. Furthermore, in all three cases, shown in Fig. 7, the contribution of neutral red in the external medium to the total spectrum was known (from the distribution experiment). The amount of neutral red, which was adsorbed to the membrane in the protonated form, can now be related to the total amount of neutral red adsorbed to the disk membranes. This yields a lower limit of 7.8 for the pK of neutral red adsorbed to disk membranes for the case in which A23187 and Mn^{2+} were present (Fig. 7, spectrum 3). This value should be compared with an apparent pK of 6.6 in aqueous solution.

in the total spectra (water plus membrane-bound neutral red) of neutral red shown in Fig. 7. From this it can be concluded that the apparent pK of neutral red adsorbed to the disk membranes of intact rod outer segments and under the ionic conditions used in this study is shifted considerably towards the alkaline pH region as compared to the aqueous pK of 6.6.

For the three different ionic conditions used in the experiment shown in Fig. 7, the partition between behaviour of neutral red was studied as a function of the neutral red concentration in the aqueous solution. In all three cases a linear plot was observed (Fig. 8). This means that the partition coefficient between the membrane phase and aqueous solution in these cases was independent of the external neutral red concentration. Furthermore, addition of gramicidin (3 μM) did not change the partition coefficient of the neutral red distribution for the cases in which A23187 and either EDTA or Mn^+ were present.

Discussion

In this study we have made a first attempt to assess the interfacial potentials at the disk membranes of intact isolated cattle rod outer segments by spectroscopic methods. We have investigated the effects of the ionic conditions on the interfacial potential at disk membranes, in particular the concentration of divalent cations was varied, which results in different occupation states of the internal binding capacity for divalent cations. The use of intact rod outer segments instead of the more accessible disk membranes of fragmented rod outer segments was motivated by previous observations that Ca^{2+} transport, Ca^{2+} storage and a rapid light-induced change of Ca^{2+} binding behave significantly differently in intact rod outer segments as compared to the various more fragmented preparations [1,3].

In the presence of the divalent cation ionophore A23187, the disk membranes can be exposed to two rather different ionic conditions. Addition of EDTA results in complete removal of endogenous divalent cations, which, for the greater part, were bound to the internal binding capacity. On the other hand, addition of 1—2 mM Ca^{2+} results in a saturation of this binding capacity [1]. In this study Mn^{2+} was used rather than Ca^{2+}, since in its presence (but not with Ca^{2+} or Mg^{2+}, see also Ref. 15) the reduction of the chromophore to retinol is blocked. Accordingly, an undisturbed metarhodopsin II to metarhodopsin III transition could be determined spectroscopically (see Figs. 3 and 5). Furthermore, Mn^{2+} shows binding parameters very similar to those of Ca^{2+} (Furrer, R. and Kaupp, U.B., unpublished results). Finally, we observed that Ca^{2+}, Mg^{2+} and Mn^{2+} had similar effects on the photolysis products of rhodopsin or on the distribution of neutral red (the affinity decreased in the order $Mn^{2+} > Ca^{2+} > Mg^{2+}$; with Mg^{2+} the effects were about 33% smaller than with Mn^{2+}).

Rhodopsin as pH indicator at the surface of the disk membrane

In this study, we have attributed the differences in the photolysis products of rhodopsin under the various ionic conditions to differences in the local pH near the disk membrane surface. In the absence of divalent cations at the

internal binding sites in intact rod outer segments, the local pH at the disk membrane surface appeared to more acidic by about 1 pH unit as compared to the bulk pH (Fig. 3). Addition of Mn^{2+} at a concentration which saturates the internal binding sites resulted in an apparent shift to alkaline pH values of the interfacial pH by 1.0—1.1 pH units to a value close to the bulk pH (Fig. 3, spectra 1—3). Thus, we assume that the ionic conditions only affect the photolysis products of rhodopsin by their effect on the interfacial pH. The interfacial pH at the disk membrane is related to the interfacial potential at the disk membrane according to a Boltzman distribution, which can be rewritten as $\Delta pH = \Delta\Psi/59$, where $\Delta\Psi$ is the change of the interfacial potential at room temperature expressed in millivolts.

The distribution of neutral red as indicator for the interfacial potential

The amphiphilic pH-indicating dye neutral red was greatly enriched in intact rod outer segments under all conditions used (Figs. 7 and 8). Furthermore, the apparent pK_m of neutral red adsorbed to the disk membranes was shifted far towards alkalinity as compared to the pK_w of neutral red in aqueous solution. From the data shown in Fig. 7, a lower limit of 7.8 was estimated for pK_m. This means that at the external pH of 7.4 used in our experiments, the basic uncharged form predominates in the external medium, whereas the protonated positively charged form predominates in the membrane phase. Under these conditions, the change of the partition coefficient of neutral red between membranes and aqueous solution is, to a reasonable approximation, an indicator of the change of the interfacial potential at the disk membranes (a quantitative treatment is given in Appendix).

Table I summarizes the changes of the interfacial potential obtained by the pH-indicating properties of rhodopsin as well as from the distribution of neutral red. Both methods give reasonably comparable results, although they have a quite different physicochemical basis.

TABLE I

CHANGES OF THE INTERFACIAL POTENTIAL AT ROD DISC MEMBRANES

Changes of the interfacial potential are expressed in mV. All data are presented as the mean ± S.D. (number of observations in parentheses). In the presence of both EDTA and A23187, the absolute interfacial potential amounted to about −60 mV (first row). The change of the interfacial pH is related to the change of the interfacial potential according to $pH = \Delta\psi/59$, where $\Delta\psi$ is expressed in mV. Changes of the interfacial potential were obtained from changes in the partition coefficient of neutral red between membranes and water by using Eqn. 9 in the Appendix. The partition coefficients p (membranes/water) were calculated from the data shown in Fig. 8 using a rhodopsin concentration of 10 mM within the disk membrane. The standard deviations of the partition coefficients were obtained from a regression analysis on the data shown in Fig. 8.

Addition to the standard medium	Method			
	Rhodopsin as pH indicator		Neutral red distribution	
	ΔpH	$\Delta\psi$	p	$\Delta\psi$
5 μM A23187 + 500 μM EDTA	≡0	≡0	1880 ± 80	≡0
500 μM EDTA	0.59 ± 0.11 (9)	35 ± 6 (9)	670 ± 20	26 ± 1
5 μM A23187 + 2 mM $MnCl_2$	1.06 ± 0.10 (7)	63 ± 6 (7)	110 ± 4	72 ± 4

The interfacial potential at the inner side of the disk membrane

The observed difference between the interfacial pH at the disk membrane surface and the pH in the bulk medium was not the result of an electrochemical H^+ gradient, which could have been generated by the exchange of divalent cations and H^+ mediated by the ionophore A23187. This possibility is ruled out by the observation that addition of H^+ ionophores (FCCP, gramicidin) did not change the interfacial pH (as assessed via the photolysis products of rhodopsin). Also, the distribution of neutral red between disk membranes and aqueous solution was not affected by the addition of gramicidin. With the interfacial H^+ in electrochemical equilibrium with those in the bulk, the observed changes of the interfacial H^+ concentration are caused by changes of the interfacial (i.e., electrostatic) potential. This interfacial potential is sensed by rhodopsin and exists in a compartment which is not directly accessible to the external medium in rod outer segments, both with a leaky as well as with an intact plasma membrane. Therefore, this compartment is most likely identical with the intradiskal space. The presence of a fixed net negative charge at the intradiskal membrane interface will generate a negative electrostatic potential inside disks, which may account for the observed changes of the interfacial pH upon changes of the ionic conditions. In the presence of the appropriate ionophores (A23187, gramicidin) or upon application of intrinsically permeant electrolytes ($CH_3CO_2NH_4$), the disk membrane may be considered as a semipermeable membrane and a Donnan equilibrium will be established across the disk membrane. On the time scale of our experiments and in the presence of A23187, H^+ appears to be distributed according to this electrostatic potential. Addition of Mn^{2+} may reduce the Donnan potential either by binding to the fixed negative charges (compensation) or by increasing the electrolyte concentration between the fixed charges (screening). The lowering of the Donnan potential will result in a reduction of the free H^+ concentration inside disks as observed.

If the fixed negative charges inside disks are not distributed uniformly, but restricted to the membrane surface, the theory of the diffuse double layer and the application of the Gouy-Chapman equation may be considered [17]. However, the Gouy-Chapman equation is derived from the Poisson-Boltzmann equation on the assumption that the diffuse double layer stretches out into an infinite bulk phase. The latter is clearly not the case inside disks. On the contrary, the close apposition of the opposite intradiskal membrane surfaces (about 20—40 Å, see Refs. 18 and 19) impedes the development of an extended diffuse double layer and due to the comparatively large thickness of the latter under the conditions prevailing inside disks in our experiments the intradiskal space is approximately equipotential. Therefore, the electrostatic interface potential creates a transmembrane potential between the intradiskal space and the bulk solution. The superposition of the interfacial potentials from the opposite intradiskal membrane surfaces will cause a transbilayer asymmetry of interfacial potentials even if the membrane itself is perfectly symmetrical.

Lacking a more appropriate theory, we have used the Gouy-Chapman equation and, alternatively, the Donnan equation (cf. Ref. 20, pp. 80—84) to estimate the net density of the fixed negative charges at the intradiskal mem-

brane surface from the data shown in Fig. 4. It is assumed that NH_4^+ does not bind to the fixed charges (this assumption will most likely not hold for Mn^{2+}, see Ref. 17, pp. 85—86). The changes of the intradiskal potential upon increasing the concentration of $CH_3CO_2NH_4$ were obtained from the changes of the intradiskal pH (see legend of Fig. 4). With both equations a relatively unambiguous and accurate fit could be obtained. In both equations the same two parameters were fitted, namely the charge density and the amount of 1 : 1 electrolyte already present inside disks (divalent cations were completely removed in this experiment). The values for these parameters obtained with both methods are in reasonable agreement. The latter parameter ranged between 3 and 4 mM (expressed as bulk concentration; this means an intradiskal concentration of 21—22 mM at the potentials indicated below). The charge density ranged between 0.5 (Donnan) and 1.5 (Gouy-Chapman, corrected for the superposition of surface potentials) electronic charges/rhodopsin molecule or about 1 electronic charge/4400 Å^2. Using the Gouy-Chapman equation this yields an interfacial potential of —52 mV under conditions where all endogenous divalent cations were remoed from rod outer segments and no $CH_3CO_2NH_4$ was added. Application of the Donnan equation yields a potential inside disks of —42 mV. These values for the (interfacial) potential inside disks agree reasonably well with the observed acidification by about 1 pH unit observed under these conditions (Fig. 3), and, therefore the above estimate for the charge density at the intradiskal membrane surface does not seem to be greatly in error. A similar low charge density can be inferred from the consideration that disks possess a stable geometry with the opposite intradiskal membrane interfaces approaching each other to 20—40 Å [18,19]. A quantitative analysis of the attractive (van der Waals) and repulsive (electrostatic and hydrostructural) forces for a similar case (thylakoid membrane stacking) has been carried out recently by Sculley et al. [21]. This study infers that the net charge density at the membrane surfaces involved must not exceed 1 electronic charge/3000—6000 Å^2, if the repulsive forces are not to exceed the van der Waals' attraction.

Concluding Remarks

The major assumptions underlying both methods described in this study of determining the interfacial potential inside rod outer segment disks seem to be reasonable, since both methods yield comparable results. Furthermore, the absolute values of the interfacial potentials observed and the charge density at the intradiskal membrane surface inferred from these potentials appear to be reasonably consistent. In our present experiments, we obtained evidence for a negative potential at the intradiskal membrane surface. In view of the fact that rhodopsin is asymmetrically located in the disk membrane and protrudes from both sides of that membrane [22], it may be expected that it will sense the interfacial pH and, therewith, the interfacial potential at only one of both sides of the disk membrane (i.e., the intradiskal side). On the other hand, neutral red molecules pass membranes at least in the uncharged form and, therefore, have no a priori preference for either intradiskal membrane interface. Nevertheless, preliminary experiments showed that the changes of the

neutral red distribution that occurred upon changes of the concentration of divalent cations were much larger (at least 4-fold) in the presence than in the absence of the ionophore A23187. The above result was also observed when rod outer segments with a leaky plasma membrane were used. This indicates a strong asymmetry of the disk membrane in preparations with the original disk stacking, and could indicate that the interfacial potential at the outside of disks is much less than that at the inside. However, we cannot exclude the possibility that neutral red molecules are very asymmetrically distributed over both sides of the disk membrane, also in the absence of interfacial potentials.

An important aspect of the use of charges probes to sense the interfacial potential is that the probe itself makes no contribution to this potential. This is not self-evident in the case of neutral red. In Fig. 8 it is shown that up to 6 mol of neutral red/mol rhodopsin may adsorbed to disk membranes under conditions where virtually all neutral red molecules carry a positive charge. This would introduce a positive surface charge density, which exceeds the intrinsic negative charge density of the membrane. Nevertheless, the linear curves observed in Fig. 8 indicate that the adsorption of neutral red to disk membranes does not change the mechanism underlying this adsorption. In the presence of EDTA and A23187, a negative interfacial potential most likely occurs at the disk membrane surface and, therewith, the partition coefficient of neutral red between the disk membrane and the bulk water will be dependent on this interfacial potential (see Appendix). However, the partitioning behaviour of neutral red observed under these conditions (Fig. 8) was independent of the amount of neutral red adsorbed to the disk membrane. Consequently, the adsorption of neutral red to disk membrane most likely did not change the interfacial potential at the disk membrane and, therefore, was unlikely to change the charge density. One should bear in mind that the ratio of membrane surface to aqueous volume inside rod outer segments and, in particular, inside disks is very high. Neutral red molecules most likely pass the disk membrane in the uncharged form and become protonated upon adsorption to the diskal membrane surface. This H^+ may originate from buffer groups at the membrane surface, and, consequently, a protonation-deprotonatin at the membrane surface could have taken place without a change of the net charge density. A quantitative account of the potential- and pH-indicating properties of the partition behaviour of neutral red in rod outer segments is currently in progress.

Appendix

We derive an expression for the partition coefficient of neutral red molecules between the membrane phase and the external aqueous phase. The following symbols are used: NR^+ and NR, concentration of the acidic and basic forms, respectively, of neutral red; H^+, free concentration of H^+; V_m and V_w, volume of the membrane phase and external aqueous phase, respectively; k, Boltzmann constant; e, charge on H^+; T, absolute temperature; Ψ, interfacial potential; subscripts w and m refer to the bulk water phase and the membrane interface, respectively.

The following relationships are defined:

$$NR_w \cdot H_w^+/NR_w^+ = K_w \quad (1)$$

$$NR_m \cdot H_m^+/NR_m^+ = K_m \quad (2)$$

$$NR_m/NR_w = B \quad (3)$$

$$H_m^+ = H_w^+ \cdot \exp(-\Psi e/kT) \quad (4)$$

Dividing Eqn. 1 by Eqn. 2 yields:

$$NR_m^+/NR_w^+ = (K_w/K_m)B \cdot \exp(-\Psi e/kT) \quad (5)$$

The observed parameter is the partition coefficient p:

$$p = (NR_m + NR_m^+) \cdot V_m/[(NR_w + NR_w^+)V_w]$$

Assuming that the volume of the intracellular aqueous compartments can be neglected with respect to the volume of the external medium, substitution yields:

$$p = \frac{K_w/K_m + K_w/[H_w^+ \cdot \exp(-\Psi e/kT)]}{1 + (K_w/H_w^+)} \cdot \frac{V_m}{V_w} \cdot B \cdot \exp(-\Psi e/kT) \quad (6)$$

Under conditions where $K_w/K_m >> K_w/[H_w^+ \cdot \exp(-\Psi e/kT)]$, Eqn. 6 reduces to:

$$p = \frac{(K_w/K_m)B}{1 + (K_w/H_w^+)} \cdot \frac{V_m}{V_w} \cdot \exp(-\Psi e/kT) \quad (7)$$

For different ionic conditions, the partition coefficients p_1 and p_2 are observed and with Eqn. 7 the ratio of the partition coefficients is related to the change of the interfacial potential according to:

$$\log(p_1/p_2) = -(\Psi_1 - \Psi_2)/59 \quad (9)$$

where the interfacial potentials are expressed in millivolts. Application of Eqn. 7 instead of Eqn. 6 results in an underestimation of the change of the interfacial potential. With pK_w being 6.6 and using $pK_m = 7.8$ as a lower limit (estimated from the data in Fig. 7, see footnote on p. 223), the errors in the determination of the changes of the interfacial potential would amount to 3.5% for $\Psi_1 = -60$ mV and $\Psi_2 = -30$ mV, and 13% for $\Psi_1 = -60$ mV and $\Psi_2 = 0$ mV.

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