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ION TRANSPORT THROUGH HEMOCYANIN CHANNELS IN OXIDIZED CHOLESTEROL ARTIFICIAL BILAYER MEMBRANES

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Summary

Incorporation of molluscan hemocyanins, obtained either from the blood of *Megatura crenulata* or *Paludina vivipara*, into oxidized-cholesterol black lipid films results in the formation of ionic channels. Channel conductance depends on the type of electrolyte present, ranging in our experiments from about 20 to 500 pS. It rises in a non-linear way as the salt concentration is increased, showing a saturation effect. An observed pH dependence of channel conductance suggests that there is a negative fixed charge associated with the pore. We discuss a model based on a simplified form of the Gouy-Chapman theory of the electrified double layer to explain the experimental results.

Introduction

Hemocyanins are large oxygen-transporting copper proteins, occurring freely dissolved in the hemolymph of a number of invertebrates. One of these hemocyanins, extracted from the blood of the giant keyhole limpet *Megatura crenulata*, was shown to be able to induce the formation of ionic channels in black lipid membranes [1,2]. More recently, we could show that also another hemocyanin, obtained from the mollusc, *Paludina vivipara*, has the property of opening channels in artificial membranes which have, however, quite different features [3]. In voltage-clamp experiments, 'opening' and 'closing' of individual conducting channels results in the appearance of current steps. A measure of the amplitude of these steps, in the presence of various ionic solutions, may shed some light on the transport properties of individual *M. crenulata* and *P.*

vivipara hemocyanin ionic channels. The aim of this paper is to investigate, using this technique, the existence of a fixed negative surface charge on the *M. crenulata* hemocyanin channel, as suggested by its almost ideal cation selectivity [4,5]. For this purpose, we tested the saturation properties of both *P. vivipara* and *M. crenulata* hemocyanin channel conductances (by increasing the ionic strength of different metal chlorides) as well as the pH dependence of *M. crenulata* hemocyanin channel conductance. The results of these experiments support the idea that a fixed negative charge influences the ionic properties of the *M. crenulata* hemocyanin channel.

Materials and Methods

Black lipid membranes were obtained by the usual technique [6], using oxidized cholesterol either prepared by us following the procedure of Tien [7] with cholesterin and *n*-octane (Fluka puriss, p.a.), or purchased from P.L. Biochemicals. This was diluted in *n*-octane to a final concentration of 100 mg/ml. The Teflon cell used for bilayer formation had a circular hole in the wall between the two compartments with a diameter of about 2 mm. All the experiments were performed at room temperature ranging from 20 to 23°C.

Electrolytic solutions were prepared using Carlo Erba RPE salts. In the experiments on the pH dependence of channel conductance, solutions were buffered with 25% (mol buffer/mol salt), of (buffer A) Tris (Riedel-De Häen), (buffer B) Bistris (Sigma) or (buffer C) 3-(cyclohexylamino)propanesulfonic acid (Calbiochem). In the experiments on the dependence of the channel conductance on salt concentration, solutions were buffered with 1% Tris-HCl.

The specific conductance of the various salt solutions was measured either with a home-made conductivity cell, or with a conductometer (Helzle & Chelius KG, Neu Isenburg, D). For the electrical measurements, four Ag/AgCl electrodes were used; current was amplified using a virtual grounded operational amplifier (Analog Devices 515-K) with a 10^8 Ω resistor and a 50 pF capacitor in its feed-back loop. Under these conditions, the potential across the membrane with few channels is essentially equal to the source potential, taken from a d.c. voltage supply, and voltage-clamp conditions are attained without external feedback.

Amplified current and voltage signals were monitored on a Tektronix 7613/7A22 and 7A18 storage oscilloscope, and recorded on a chart recorder.

M. crenulata hemocyanin (A grade, in 50% glycerol) was purchased from Calbiochem while *P. vivipara* hemocyanin in about 500% sucrose was a gift from Professor B. Salvato (Center for the Study of Physiology and Biochemistry of Hemocyanins, Padua, Italy). These stock solutions were stored at −20°C. Small amounts of these were diluted to a final concentration ranging from about 0.1 to 10 μg/ml, depending upon electrolytic solution conditions, and added to the bathing solution on one side of the black film suitably for the observation of a few channels.

Sometimes membranes were formed with the protein already present in one compartment. No apparent difference was observed with both methods. The protein-containing compartment is taken as the reference for the current and voltage sign.

Results

Single-channel conductance

When a steady voltage is applied across the membrane, the addition of small amounts of hemocyanin to one of the two bathing solutions yields a stepwise fluctuation of the current, each step corresponding to a constant variation of membrane conductance. Such fluctuations have been ascribed to the formation of ionic channels through the membrane. *M. crenulata* and *P. vivipara* hemocyanin channels have been studied in 0.1 or 0.2 M KCl [2,3], and some relevant differences have been found between the two. *P. vivipara* hemocyanin channels have apparently only two states, the open and the closed one. The transition rate between the two states and the fraction of time spent in the open and closed states do not depend upon the applied voltage. *M. crenulata* hemocyanin channels have at least four conductive states and the fraction of time spent in each state is strongly voltage dependent. In particular, for negative applied potentials, the channel is always in the most conductive state, called the open state. In Fig. 1 we present some typical step-like current fluctuations obtained

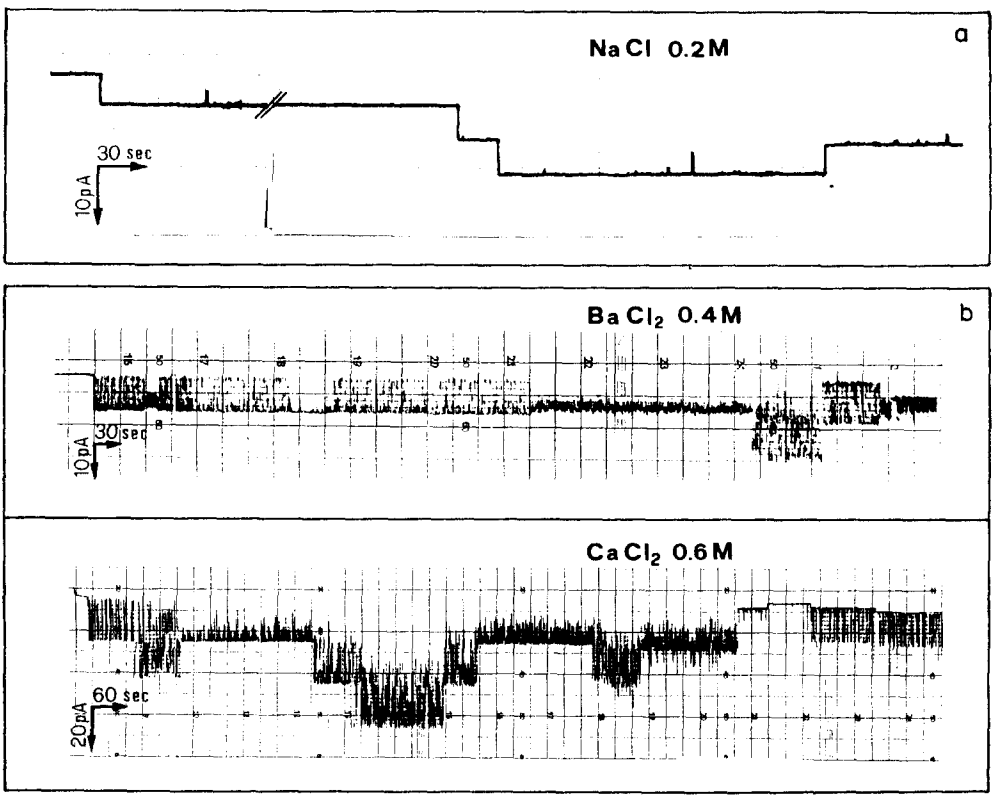

Fig. 1. Current fluctuations during voltage-clamp experiments induced in oxidized cholesterol membranes by *M. crenulata* hemocyanin molecules. Voltage is held at −50 mV and different metal chlorides are used. (a) When monovalent cations are present only formation jumps are observed. (b) When divalent cations are present each formation step is followed by a number of current fluctuations, indicating that the channel jumps between different conductance levels.

from *M. crenulata* hemocyanin in different metal chloride solutions. Membrane voltage was held at −50 mV, a value at which the *M. crenulata* hemocyanin channel was found to be always in the open state [2]. Our results show that while this is true in the presence of monovalent cations, when divalent cations are used, the channel behaviour becomes quite different and, even at negative potentials, the pore rapidly fluctuates between different conductance levels. This suggests that the channel undergoes reversible conformational changes during the interaction with divalent cations. Recently, we were able to obtain evidence of this interaction, a kind of block with the finding that both Ca^{2+} and Ba^{2+} when added to the bathing KCl solution can diminish the mean conductance of the *M. crenulata* hemocyanin channel, favouring its lowest conductance states (Menestrina, G. and Antolini, R., unpublished results). Blockage of channels in artificial membranes by divalent cations has already been observed with gramicidin A [8]. Anyway, in the presence of divalent cations, the fraction of time spent by the channel in the open state is long enough to allow us to measure its conductance. The mean conductance and standard deviation of the open hemocyanin channel as presented below are obtained by averaging values from a number (20—100) of different channels.

Salt concentration dependence of the open-channel conductance

The relationship between the single-channel conductance of both *P. vivipara* and *M. crenulata* hemocyanins and salt concentration shows saturation

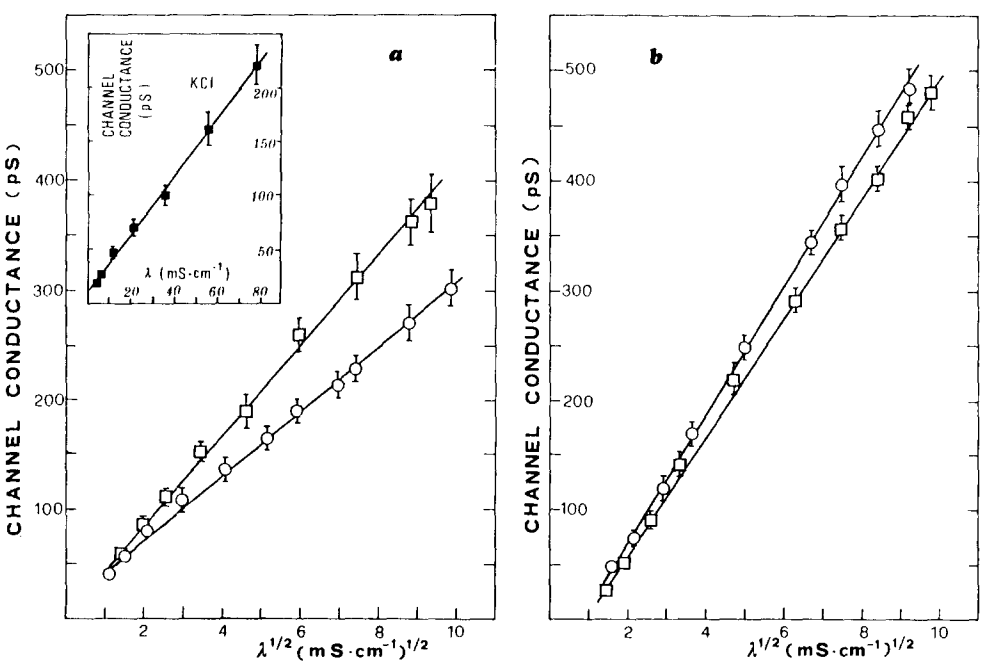

Fig. 2. *M. crenulata* hemocyanin channel conductance as a function of the square root of the specific conductance of different metal chloride solutions. (a and b) Channel conductance in the presence of monovalent and divalent cations, respectively. (Inset) The dependence of *P. vivipara* hemocyanin channel conductance on the solution conductivity. Solutions were buffered with Tris-HCl, pH 7.0. Solid lines are drawn by eye. (a) □, KCl; ○, NaCl. (b) □, $BaCl_2$; ○, $CaCl_2$.

effects. More precisely, the channel conductance does not increase linearly with electrolyte concentration, but seems to tend towards an upper limit. More interesting features of these relationships emerge when one considers as independent variable the specific conductance of the solution, λ. Indeed, it seems reasonable that the conductivity rather than the concentration of the solution is the factor determining the number of ions that can enter the channel [9]. When plotted against the variable λ, *P. vivipara* hemocyanin channel conductance values fall on a straight line (inset of Fig. 2a), as one would expect from a passive channel. On the contrary, *M. crenulata* hemocyanin channel conductance values are fitted by straight lines only when plotted against the square root of the solution conductivity (Fig. 2a and b). This finding implies that the *M. crenulata* hemocyanin channel plays an active role in the transport of ions. Tentatively, one may think that this active role together with the large value of conductance of the *M. crenulata* hemocyanin channel compared with that of the *P. vivipara* hemocyanin channel (about 3-times greater in 0.2 M KCl) is due to the effect of some negative charge distributed on the channel, the presence of which is suggested also by the cation selectivity of the channel [4,5] and by the low isoelectric point (approx. pH 4.7) of the hemocyanin molecule [10]. The fixed charge might exert an attraction on the cations that arrive at the

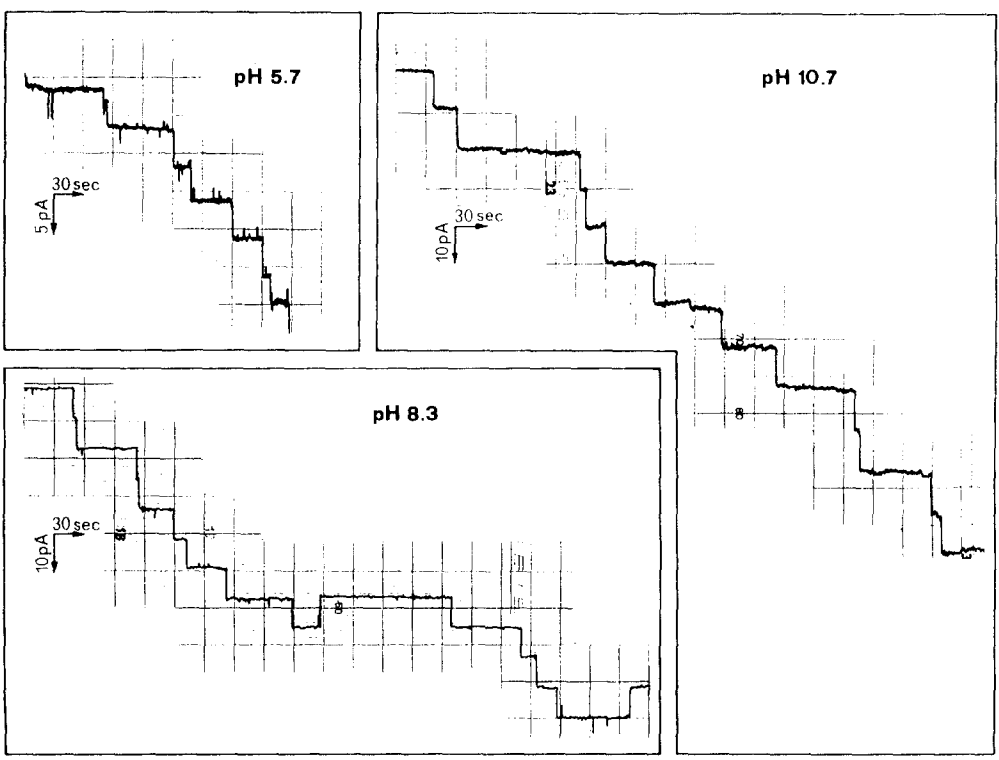

Fig. 3. Stepwise increases of current due to the formation of *M. crenulata* hemocyanin channel in oxidized cholesterol membranes. The three traces refer to different buffered solutions of 0.08 M KCl. Potential was held at −50 mV. It can be seen that the channels remain open virtually all the time after their formation. Mean channel conductance values are different in the three cases.

mouth of the channel, thus increasing the number of cations present in the charged channel with respect to a discharged one. This effect should diminish by increasing the cation concentration in the solution, owing to the screening of the charged surface, in agreement with the experimental results.

pH dependence of the open-channel conductance

Following the above idea of an electrostatic interaction between cations and the charged channel, we expected to find a dependence of the open *M. crenulata* hemocyanin channel conductance on the pH of the bathing solution. In fact, increasing the pH of the solution also increases the negative charge of the protein, and consequently the electrostatic attraction on the cations is expected to rise. In Fig. 3 we present some current fluctuation records from which one can see that even at very different pH values, obtained with different

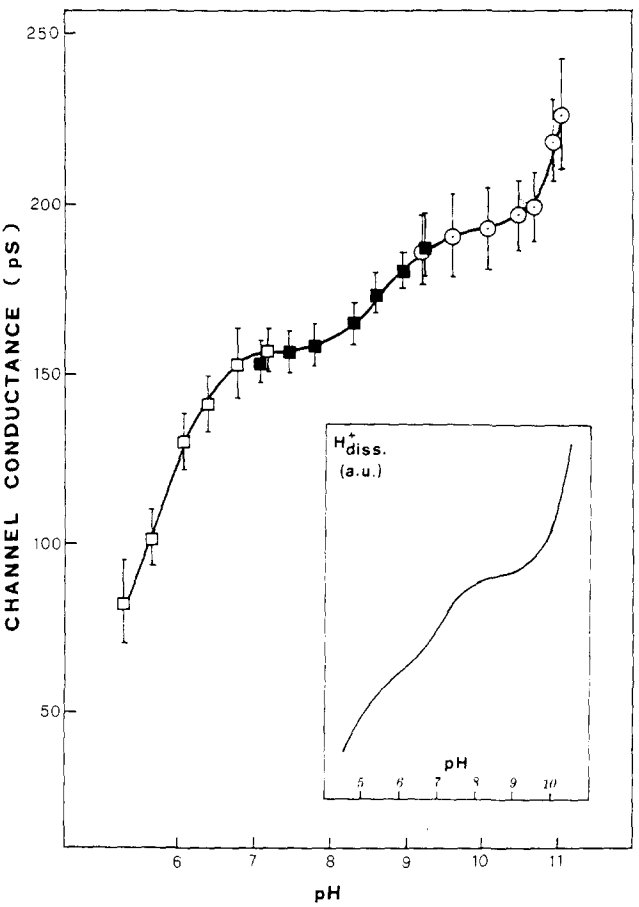

Fig. 4. pH dependence of *M. crenulata* hemocyanin channel conductance in 0.08 M KCl. Different symbols refer to the use of different buffers (■, buffer A; □, buffer B; ○, buffer C; see Materials and Methods). After each conductance measurement, we tested both the pH with an ORION 701 digital pH-meter, and the conductivity of the solution. Points with an asterisk were normalized for small differences in the solution conductivity. Solid line is drawn by eye. In the inset a potentiometric titration curve of *M. crenulata* hemocyanin in 0.5 M KCl by 0.1 M NaOH performed with a PHM-62 pH-meter and a TTT 60 titration unit from Radiometer is shown for comparison.

buffers, channel formation occurs essentially in the same way, the difference being only in the mean value of the single-channel conductance. The pH dependence of *M. crenulata* hemocyanin channel conductance in the pH range 5.0–11.5 is reported in Fig. 4. Outside this range we were not able to observe any clear channel-formation step. The curve obtained indicates that actually channel conductance increases monotonically with the pH. A comparison with the curve of the potentiometric titration of *M. crenulata* hemocyanin in 0.5 M KCl, shown in the inset of Fig. 4, suggests that both carboxyl and histidine groups [10] are involved in determining the charge of the channel. It can be seen that the curves are shifted by about one unit of pH. This may be due to the fact that one is the titration of the protein in the bulk solution, while the other is the titration of the protein bound to a negative surface, the oxidized cholesterol membrane. In this case, the following relationship between the pH in the bulk and the pH at the surface, may be used [11]:

$$pH_{surf} = pH_{bulk} + \frac{\zeta}{60} \tag{1}$$

where ζ is the surface potential expressed in mV. The observed shift of pH would imply a value of $\zeta \simeq -60$ mV which is fairly reasonable for an oxidized cholesterol membrane [12,13]. Titration of acidic groups responsible for different channel properties has recently been performed on both natural [14] and artificial pores [15,16].

During this set of experiments, we noticed another effect: on lowering the pH of the solution one must add progressively less protein (from 5 to 0.2 μg/ml) in order to obtain few channel recordings. We can understand this fact by remembering that both the protein and the oxidized cholesterol membrane bear a negative charge and therefore electrostatic repulsion must hinder protein incorporation into the black lipid film. Lowering the pH of the solution results in a screening of this charge by H^+, and thus in a facilitated interaction between protein and lipid.

Discussion

The linear dependence that we have found between *P. vivipara* hemocyanin single-channel conductance and electrolyte conductivity can be interpreted by the simple assumption that the open channel is a cylindrical pore filled with the aqueous solution. In this case, the Paludina channel conductance, G_P, is:

$$G_P = g_P \lambda \tag{2}$$

with

$$g_P = \pi r^2 / l \tag{3}$$

where r and l are the channel radius and length, respectively, and λ is the specific conductance of the solution.

In the case of *M. crenulata* hemocyanin, the non-linear dependence of channel conductance on salt conductivity is accompanied by other experimental evidence (cation selectivity and pH control on conductance) that suggests the presence of charged groups on the ionic pathway. We try here to verify whether

fixed charges on the channel can account for the observed relationship between channel conductance and bulk conductivity. To do this, we develop quantitatively the idea that the channel appears to the ions in the solution as a portion of surface A with a certain density σ_f of fixed charge. We may calculate the amount of ionic charge that such a surface coordinates within a distance d. Using the simplified form of the Gouy-Chapman theory of the double layer [12] obtained by the linearized Poisson-Boltzmann equation, we get:

$$Q_{ex} = Q_f(\exp(-\chi d) - 1) \tag{4}$$

where Q_{ex} is the excess counterionic charge coordinated by the surface of the channel within the distance d; $Q_f = \sigma_f A$ is the amount of fixed charge on the channel and χ is the reciprocal of the Debye-Hückel length. Now σ_f at pH 7 may be estimated from the titration curves [10,17] to be about six to eight electronic charges/1000 Å^2. In this case, taking $d = 1$ Å, the co-ionic charge coordinated is so small that Q_{ex} may be practically considered as all the charge present near the channel, i.e.

$$Q_{tot} \simeq Q_{ex} \tag{5}$$

The error introduced by this approximation is always much less than our experimental errors, being about 2% under the most unfavourable conditions. We can calculate now (see Appendix) a local charge density, a local conductivity and finally an expression for the Megatura channel conductance G_M which results in:

$$G_M = g_M \sigma_f \lambda^{1/2} \tag{6}$$

Though oversimplified and limited to the case where $\sigma_f \neq 0$, this model is able to explain all our experimental findings, i.e.

(i) cation selectivity at pH 7 [4]; in fact only counterions, cations in this case, contribute to the channel conductance.

(ii) dependence of channel conductance on the square root of the solution conductivity at pH 7 (Fig. 2).

(iii) dependence of channel conductance on surface charge density, i.e., on the solution pH (Fig. 4).

Furthermore, from the slopes of the straight lines of Fig. 2, we can evaluate on the basis of this model the sequence of the mobilities of the cations into the channel which results in: $K^+ > Ca^{2+} \simeq Ba^{2+} > Na^+$, that is almost the same sequence as in free solution; such a finding confirms the idea that we are still dealing with an aqueous pore.

Appendix

Relationship between M. crenulata hemocyanin channel conductance and solution conductivity

The solution conductance is related to the ion mobility by [18]:

$$\lambda = \Sigma_i n_i z_i e_0 \mu_i \tag{1A}$$

where n_i, $z_i e_0$ and μ_i are the number per unit volume, charge and mobility of

ions of species i in the solution, respectively. The Debye-Hückel coefficient, χ, is defined [12]:

$$\chi = \left(\frac{1}{\epsilon kT} \Sigma_i n_i z_i^2 e_0^2\right)^{1/2} \tag{2A}$$

where ϵ is the dielectric constant of water and k, T have their usual meaning. Combining Eqns. 1A and 2A we obtain:

$$\chi = \left(\frac{1}{\epsilon kT} \cdot \frac{ae_0\lambda}{\Sigma_i u_i}\right)^{1/2} \tag{3A}$$

where a is an integer that is 2 for a 1 : 1 salt and 3 for a 2 : 1 salt. From Eqn. 5 and under the limits stated there, we can evaluate a local charge density n_{+loc}:

$$n_{+loc} = Q_{ex}/Ad \tag{4A}$$

As d is very small we can assume $\chi d << 1$, obtaining from Eqn. 4:

$$Q_{ex} = -Q_f \chi d \tag{5A}$$

and finally:

$$n_{+loc} = -\sigma_f \chi \tag{6A}$$

The local conductivity is:

$$\lambda_{loc} = n_{+loc} w_+ \tag{7A}$$

where w_+ is the cation mobility into the channel. In this case we can express the channel conductance as:

$$G_M = (\pi r^2/l)\lambda_{loc} \tag{8A}$$

where r and l are the channel radius and length, respectively. Combining Eqns. 3A and 6A–8A we obtain:

$$G_M = g_M \sigma_f \lambda^{1/2} \tag{9A}$$

where:

$$g_M = -\frac{\pi r^2}{l}\left(\frac{1}{\epsilon kT} \cdot \frac{ae_0}{\Sigma_i u_i}\right)^{1/2} w_+ \tag{10A}$$

Eqn. 9A is the relationship discussed in the text for the *M. crenulata* hemocyanin channel conductance.

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References

1 Pant, M.C. and Conran, P. (1972) J. Membrane Biol. 8, 357–362
2 Latorre, R., Alvarez, O., Ehrenstein, G., Espinoza, M. and Reyes, J. (1975) J. Membrane Biol. 25, 163–182

3 Menestrina, G. and Antolini, R. (1979) Biochem. Biophys. Res. Commun. 88, 433—439
4 Antolini, R. and Menestrina, G. (1979) FEBS Lett. 100, 377—381
5 Alvarez, O., Reyes, J. and Latorre, R. (1977) Biophys. J. 17, 214a
6 Szabo, G., Eisenmann, G. and Ciani, S. (1969) J. Membrane Biol. 1, 346—382
7 Tien, H.T. (1974) Bilayer Lipid Membrane; Theory and Practice, Marcel Dekker, New York
8 Bamberg, E. and Läuger, P. (1977) J. Membrane Biol., 35, 351—375
9 Latorre, R., Ehrenstein, G. and Lecar, H. (1972) J. Gen. Physiol. 60, 72—85
10 Salvato, B., Ghiretti Magaldi, A. and Ghiretti, F. (1974) Biochemistry 13, 4778—4783
11 Rubery, P.M. and Sheldrake, A.R. (1973) Nat. New Biol. 244, 285—288
12 Aveyard, R. and Haydon, D.A. (1973) An Introduction to the Principles of Surface Chemistry, pp. 40—47, University Press, Cambridge
13 Neumcke, B. (1976) J. Electrochem. Soc. 123, 1331—1334
14 Wanke, E., Carbone, E. and Testa, P.L. (1980) Nature 287, 62—63
15 Benz, R., Janko, K. and Läuger, P. (1979) Biochim. Biophys. Acta 551, 238—247
16 Varanda, W. and Finkelstein, A. (1980) J. Membrane Biol. 55, 203—211
17 Van Holde, K.E. and van Bruggen, E.F.J. (1971) in Biological Macromolecules Series (Timasheff, S.N and Fasman, G.D., eds.), Vol. 5, pp. 1—53, Marcel Dekker, New York
18 Bockris, J.O.M. and Reddy, A.K.N. (1970) Modern Electrochemistry, Vol. 1, p. 373, Plenum Press New York