Effects of Carbohydrates on the Ion Conductance of the Hemocyanin Channel

Gianfranco Menestrina and Flavia Pasquali

Dipartimento di Fisica, Università degli Studi di Trento, I-38050 Povo (TN), Italy

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The effects of glucose and sucrose on the ionic conductance properties of the channel formed by *Megathura crenulata* hemocyanin in planar lipid bilayers have been studied using membranes of different compositions. It was found that glucose at high concentrations strongly affects the time constants of the current relaxations observed in membranes containing many channels after a step in the voltage clamp from ground to a positive value. At much lower concentrations both sucrose and glucose strengthened the binding of Ba^{2+} to the protein, what in turn has the effect to shift the conductance voltage curve of the pore towards negative potentials. The possible mechanism underlying these effects and the analogies with other studies on the interaction of sugars and alcohols with proteins have been discussed.

Introduction

Carbohydrates are known to interact with biological membranes, sucrose and glucose in particular can inrease membrane stability [1]. It has been shown that sugar have direct effects on the lipid matrix, changing the enthalpy and the entropy of the main phase transition [2], but also that sugars and alcohols can interact with proteins stabilizing them against heat denaturation [3]. Timasheff and coworkers have demonstrated that sucrose [4], lactose and glucose [5] but also glycerol [6] and the alcohol MPD, [7] have a stabilizing effect against protein denaturation by introducing a perturbation of the surface free energy of these macromolecules. In this work we have tested the effects of sucrose and glucose on the electrical properties of the ionic channel formed by hemocyanin, a well studied pore former [8], in planar lipid membranes. Studying the effects of small uncharged molecules on the conductance of an ionic channel is of particular interest in view of the recent finding that alcohols and general anaesthetics increase the permeability of lipid vesicles containing acqueous pores [9], which thing may be relevant to understand the mechanism of general anaesthesia.

We have found that at high concentration, 0.8 M, glucose changes the kinetic properties of the hemocyanin channel, but also that at lower concentrations both sucrose and glucose strongly enhance the binding of Ba^{2+} to the pore, with a consequent shift of its current voltage characteristic of several tens of millivolts. We think that these effects of carbohydrates are due to a direct action on the protein and not on the lipid matrix. These findings are in agreement both with current theories on the mechanism of general anaesthesia, which indicate that sensitive proteins and not membrane lipids are the target of anaesthetic action [10] and with the early report that uncharged molecules such as alcohols and other anaesthetics increase the binding of Ca^{2+} to biological membranes [11].

Materials and Methods

Black lipid membranes were prepared on a 0.5 mm diameter hole in a Teflon sept separating two acqueous solutions, about 4 ml each, by the usual technique [12]. Membranes were comprised either of a mixture 7:3 of PE/PS (both Lipid Products, South Nutfield) or of a mixture 2:1 of PC/PS (P. L. Biochemicals and FIDIA Res. Lab. respectively) or of saturated PC alone (P. L. Biochemicals). PC was more than 99% pure, it gave one spot by TLC on silica gel G. The lipids were dissolved in *n*-decane at a final concentration of 25,

Abbreviations: MPD, 2-methyl-2,4-pentanediol; Hepes, N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid; Bistris, 2-(bis(2-hydroxyethyl)amino)-2-(hydroxymethyl)-1,3-propanediol; EDTA, ethylendiamintetraacetic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine.

Reprint requests to Gianfranco Menestrina.

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47.5 and 25 mg/ml respectively. The electrolytic solutions used were: 100 mM KCl, 5 mM Hepes (Sigma), 1 mM EDTA, pH 7.0, Buffer A; 100 mM KCl, 10 mM Bistris (Calbiochem), 0.5 mM EDTA, pH 7.0, Buffer B. $BaCl_2$, glucose or sucrose (all Carlo Erba RPE) were added when appropriate; Ba^{2+} concentration is given in excess of that of EDTA present.

Megathura crenulata hemocyanin (Calbiochem), dialysed extensively against Buffer A or B and centrifuged at 12 Krpm, was added to one of the two bathing solutions, cis compartment, to a final concentration ranging 5–50 μg/ml. The membrane was connected through Ag-AgCl electrodes to a D.C. variable voltage source and to a virtual ground current to voltage converter (AD 515 K) with 10^8 Ω, 20 pF in the feed-back loop and ground in the cis compartment. The procedure used to measure instantaneous current voltage, I-V, curves of membranes containing many hemocyanin channels has been fully described and discussed elsewhere [13]. Briefly: trains of two or three short pulses, duration ca. 0.5 s, of opposite polarities were applied at first increasing and then decreasing amplitudes, in steps of ten millivolts. Between one train and the other the membrane was grounded. This procedure prevents transitions of the channels to lower levels of conductance [14] and allows corrections for incorporation of new channels during the I-V test. Experiments were run at room temperature.

Results

The results of applying the procedure described in methods to obtain the instantaneous current voltage curve of a hemocyanin doped lipid bilayer is shown in Fig. 1. The bilayer was formed in Buffer B, then hemocyanin was added to the *cis* compartment to a final concentration of 10 μg/ml, which caused the bilayer conductance to increase in discrete steps, representing each the opening of a new channel [8], after about 8 min. When few hundred channels were present the *cis* side was perfused twice with protein free solution, by means of two matched 10 ml

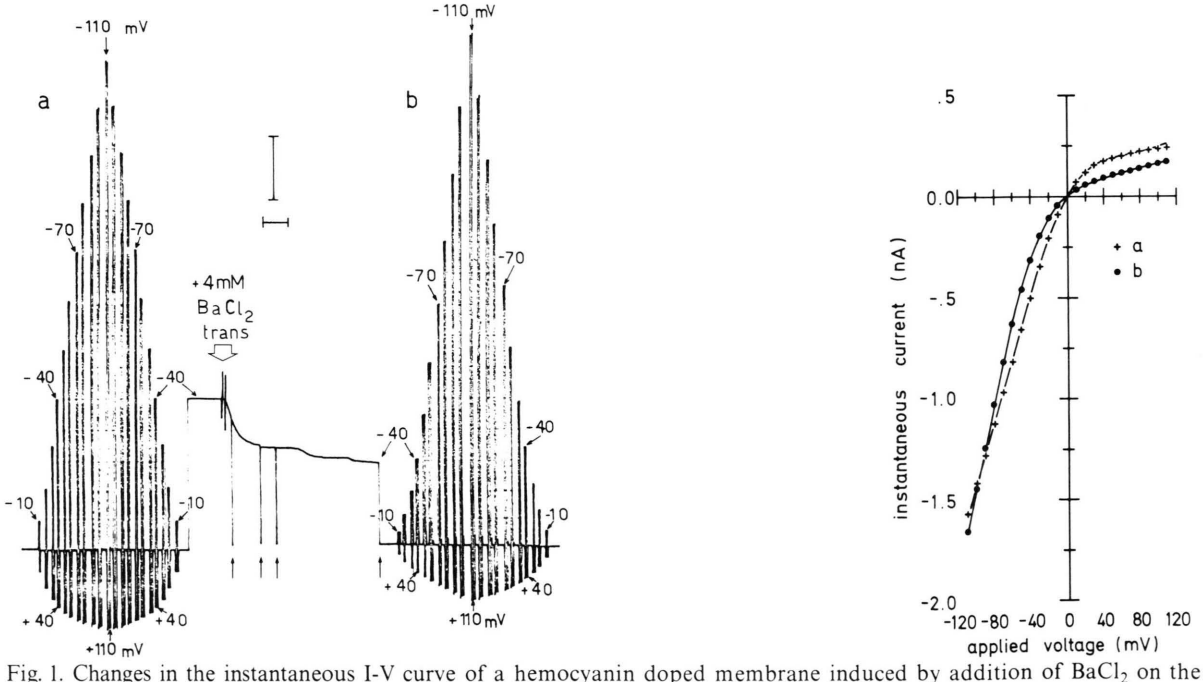

Fig. 1. Changes in the instantaneous I-V curve of a hemocyanin doped membrane induced by addition of $BaCl_2$ on the *trans* side. Patterns a and b were obtained as described under methods. The voltage was increased in steps of then mV and is sometimes indicated. Between curve a and b the voltage was clamped at −40 mV, 4 mM $BaCl_2$ was added on the *trans* side, open arrow, and the conductance checked by returning to 0 mV, arrows. Time scale is 60 s during the −40 mV clamp, whereas it is 12 s during I-V tests a and b. Current scale is 0.2 nA for the whole trace. Membrane comprised of the PE/PS mixture, Buffer B. Extrapolated I-V curves for a and b are shown in the inset of the figure.

syringes, in order to decrease the pore formation rate, *i.e.* the current drift at constant voltage during the experiment [13]. $BaCl_2$ at a concentration 4 mM on the *cis* side and 8 mM on the *trans* side was then added before recording the I-V curve a in Fig. 1. As is apparent from the inset the instantaneous I-V curve of the hemocyanin channel is highly non linear, with the cord conductance decreasing rapidly at positive voltages. Once completed the procedure the voltage was clamped at −40 mV and after about 2 min more 4 mM $BaCl_2$ was added on the *trans* side, as indicated. A fast and large decrease in the conductance was observed which was completed in a few minutes. Jumps to 0 mV applied voltage, indicated by arrows, ensured that the effect observed was due to a real decrease of conductance and not to an offset of the zero current line. Repeating at this time the I-V test showed however that while the conductance was decreased at −40 mV, it was actually slightly increased at −110 mV. In fact a comparison of I-V curves a and b shows that the whole shape of the curve is changed. This is made clearer in Fig. 2a where instantaneous conductances obtained in the course of the same experiment of Fig. 1 and normalized by dividing by the maximum conductance observed are plotted versus applied voltage. This gives conductance voltage, G-V, curves which represent single channel properties and have a characteristic sigmoidal shape [13]. As is apparent in Fig. 2a, addition of 8 mM $BaCl_2$ on the *trans* side of a hemocyanin doped bilayer bathed by a solution containing 4 mM $BaCl_2$ on both sides had the major effect of shifting the G-V curve along the voltage axis towards negative potentials, which is also the reason of the apparent conductance decrease at −40 mV of Fig. 1. The voltage shift was about 80 mV in that experiment. Further addition of 8 mM $BaCl_2$ on the *cis* side had almost no effect, as shown.

To test if the presence of PS, with its negatively charged polar head, in the membrane was crucial for this effect, we performed experiments also with bilayers made of pure PC, a neutral lipid. Also in this case we observed a shift of the G-V curve upon addition of $BaCl_2$ to the bathing solution. In two experiments additions of 4 mM $BaCl_2$ on both *cis* and *trans* side yielded a 46.4 ± 2.3 mV shift towards negative voltages.

The effects of adding glucose in similar experiments to a hemocyanin containing membrane bathed by a solution with 1 mM $BaCl_2$ are shown in Fig. 2b. Addition of 100 mM glucose on the *cis* side had little or no effect, while addition of 100 mM glucose on the *trans* side lead to a relevant shift of the G-V curve of about 50 mV in the negative direction. The combined action of $BaCl_2$ and glucose on the hemocyanin channel G-V curve has been examined in detail using bilayers formed either by PC/PS or by a PE/PS mixture and the results are shown in Fig. 3. Here we report the parameter V_0, *i.e.* the abscissa

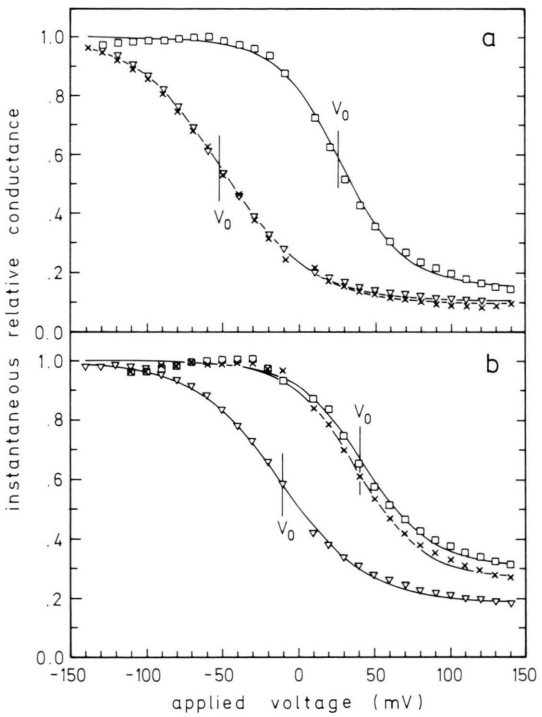

Fig. 2. Effects of Ba^{2+} and glucose on the normalized G-V curve of hemocyanin channels. a: squares, G-V curve in presence of 4 mM $BaCl_2$ symmetrically added after incorporation of hemocyanin in the bilayer, followed by perfusion of the *cis* compartment with protein free solution; crosses, same experiment after addition of 8 mM $BaCl_2$ on the *trans* side; triangles, same experiment after addition of 8 mM $BaCl_2$ also on the *cis* side. Solid lines are best fit to the experimental points using Eqn. (1) with the following parameters: $V_0 = 26.6, -50.5, -51.6$ mV; $Q = 1.2$, 0.9, 0.9 electron charges; $B = 0.15$, 0.11, 0.10, all given in the same order as above. b: squares, G-V curve in presence of 1 mM $BaCl_2$ on both sides; crosses, same experiment after addition of 0.1 M glucose on the *cis* side; triangles, same experiment after addition of 0.1 M glucose also on the *trans* side. Solid lines as above with the following parameters: $V_0 = 42.1$, 39.1, −11.1 mV; $Q = 1.2$, 1.2, 0.9 electron charges; $B = 0.31$, 0.27, 0.19. Membranes comprised of the PE/PS mixture, Buffer B.

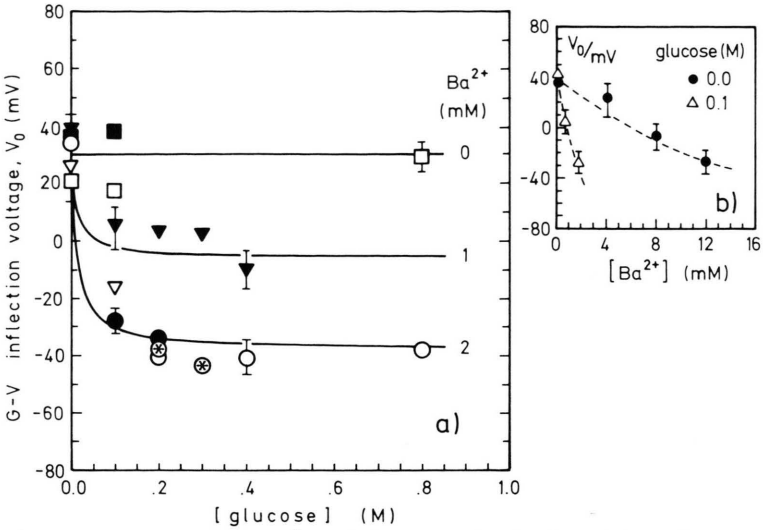

Fig. 3. Combined action of Ba^{2+} and glucose on the abscissa of the inflection point of the hemocyanin channel G-V curve. a) V_0 is plotted as a function of glucose concentration on the *trans* side of the bilayer, that on the *cis* side being equal or less, at three different Ba^{2+} concentrations: squares, 0 mM; triangles, 1 mM; circles, 2 mM. Open symbols refer to membranes comprised of PC/PS, Buffer A, whereas full symbols refer to PE/PS bilayers, Buffer B. The points bearing an asterisk inside were obtained using sucrose instead of glucose. Results with up to six different membranes, and a mean of 2.7, have been averaged to give the experimental points; error bars are twice the standard deviation. Points without error bar are single determinations. Repeated I-V tests during the same experiment gave V_0 values which were within the symbol dimensions. Solid lines are drawn according to Eqn. (2) using V_0(free) = 30 mV, $K = 80$ M^{-1} and $V_0(Ba^{2+}) = 30$, -6, -38 mV from top to bottom. b) V_0 is plotted as a function of Ba^{2+} concentration with and without glucose added. Dashed lines are drawn by eye, a theoretical interpretation of the divalent cation induced change of V_0 is given in Ref. 16. Membranes comprised of PE/PS, other conditions as above.

of the inflection point of the G-V curve, in different conditions. It is apparent that glucose alone, in the absence of $BaCl_2$, could not change the parameter V_0 even at concentrations as high as 0.8 M. Furthermore low concentrations of $BaCl_2$, up to 2 mM, did not affect V_0 in the absence of glucose. When however glucose was added to solutions which contained 1 or 2 mM $BaCl_2$ a large shift of V_0 towards negative potentials could be observed. Inspection of the figure shows that for concentrations of glucose larger than 0.2 M the parameter V_0 was no more dependent on glucose concentration being effectively determined only by $BaCl_2$ concentration.

Membranes of different lipid composition gave essentially the same results even if PC/PS membranes, open symbols, had usually values of V_0 slightly lower than PE/PS membranes, full symbols. Moreover in some experiments we used sucrose instead of glucose, again with the same results. Finally in all experiments in which *cis* and *trans* concentrations of glucose were varied independently we noticed that only *trans* glucose was effective in changing V_0. For this reason the glucose concentration given in Fig. 3 is the *trans* one, the *cis* being always equal or less to the *trans*.

In Fig. 3 we have shown that sugars affect the G-V curve of hemocyanin channel only in presence of $BaCl_2$. Yet at very high concentrations, 0.8 M, we could observe a direct effect of glucose on the kinetic properties of the channel. It is known that application of long lasting voltage pulses of positive values to a hemocyanin doped membrane bathed by a 0.1 M KCl solution yields current decreases which can be fitted by the sum of different exponentials [13, 14]. In Fig. 4 we have reported in a half logarithmic scale the digitization of the current transient obtained after stepping the voltage clamping a hemocyanin containing bilayer from 0 to +40 mV, in a 0.1 M KCl solution with or without 0.8 M glucose added on both sides. One can see that in both cases the transient can be fitted reasonably well by the sum of two exponentials, the faster, filled symbols, being replotted after the subtraction of the slower one [14]. What is also evident, note the

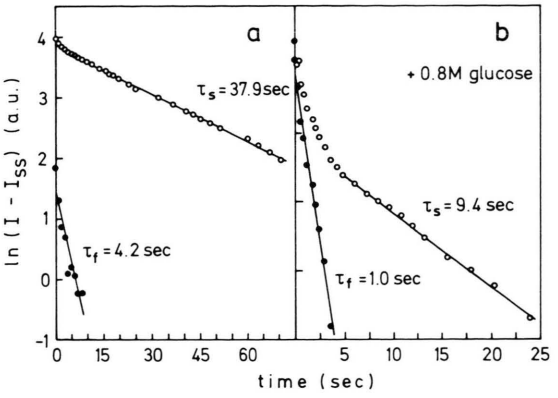

Fig. 4. Half logarithmic plot of the current transient $I - I_{SS}$, where I and I_{SS} are the actual and steady state current respectively, *versus* the time elapsed from a step change of the clamp voltage from 0 to +40 mV. a, Buffer A; b, Buffer A plus 0.8 M glucose. In both cases the relaxation could be fitted by the sum of two exponentials, the faster, full symbols, being calculated after subtraction of the slower one. Solid lines are the regression ones through the points of the relevant interval which gave the time constants indicated. Fast and slow time constant, τ_f and τ_s respectively. Membranes comprised of the PC/PS mixture.

different time scale used, is that both the time constants, fast and slow, were decreased roughly by a factor of 4 by addition of 0.8 M glucose.

Discussion

Instantaneous I-V curves of membranes containing many hemocyanin channels are non linear [13, 15], in particular the derived G-V curves have a sigmoidal shape. This can be explained on the basis of a two state model in which the pore is in equilibrium between two configurations, A and B, with different conductances, G_{max} and G_{min} respectively, and the movement of one charged group under the applied electric field can trigger the transition betwen the two states [13]. In this case the relative conductance, $G_{rel} = G/G_{max}$, can be written:

$$G_{rel} = B + (1 - B)/(1 + \exp Q(V - V_0)/kT) \qquad (1)$$

in which B is the ratio G_{min}/G_{max}, Q is the moving charge, V_0 is the potential at which half channels are in configuration A, and k and T have their usual meaning.

Figs. 1 and 2a demonstrate that addition of $BaCl_2$ either on the *trans* side or on both sides of a hemocyanin doped bilayer has the same effect, *i.e.* a shift of the G-V curve towards negative potentials and a slight decrease of the gating charge Q. Both these effects are expected since they have been already observed and studied in detail in the case of another divalent cation, Ca^{2+}, and of its vicariant Tb^{3+}, when added to hemocyanin doped PC bilayers [16]. Both effects were ascribed to a specific binding of the cations to the pore forming protein and could be explained by a perturbation of the equilibrium between the two states A and B due to a different affinity of these for the binding cation [16]. Several arguments indicated that the protein and not the lipid matrix is the target of cation action. The strongest were: first, that symmetrical addition of plurivalent cations should affect in the same way the surface potentials on both sides of the membrane and hence should be unable to produce the observed voltage shift; second, that the estimated apparent binding constants for both Ca^{2+} and Tb^{3+} to the channel correlated quite well with those measured with hemocyanin in free solution [16–18] while exceeded by more than two orders of magnitude those measured for the binding of the same cations to PC bilayers [19–21]. In addition to the above arguments we have shown in this paper that similar shifts can be observed at equivalent concentrations of $BaCl_2$ both in the case of PE/PS and pure PC membranes. Given that the apparent binding constant of Ba^{2+} to PS containing membranes, due to their negative surface potentials, may be estimated to be more than two orders of magnitude larger than that to neutral PC bilayers [21–23], the equivalent effect observed with the two types of membranes indicates that the protein forming the channel is the site of action of the cation. For this reasons we think that binding of Ba^{2+} from the *trans* side to the hemocyanin pore can explain the experimental results in Figs. 1, 2a and that V_0 is a suitable parameter to quantitate this effect.

These facts can be used to interpret the experimental results shown in Figs. 2b, 3. We have found that addition of a carbohydrate into the acqueous solution at the *trans* side of the hemocyanin doped bilayer can change the parameter V_0 of the G-V curve but only if Ba^{2+} cations are present. Addition on the *cis* side, on the other hand, has almost no effect. This can be understood simply assuming that the sugar facilitates the binding of Ba^{2+} from the *trans* side to the channel. The idea is reinforced by the observation that V_0 at sugar concentrations

larger than 0.2 M is solely dependent on the Ba^{2+} concentration in solution. It is worth noting at this point that a mutual interaction between one small uncharged molecule, urea, and one anion, SO_4^{2-}, in determining the conformation of hemocyanin has been described recently [24], and also that both sucrose and glucose were shown to be able to change the quaternary structure of this protein [25]. Whether the observed reinforcing action of sugars on the binding of Ba^{2+} to the hemocyanin channel is due to an effect on the conformational equilibria of the protein or, for example, to a reduction in the water structuring inside the channel, as proposed for the effects of ethanol and other anaesthetics on some ionic channels [9], is still open to debate.

Following the recent proposal that anaesthetics can develop their action by directly binding to sensitive proteins [10] we can use a simple binding model to explain the variation of V_0 shown in Fig. 3. We assume an equilibrium between unoccupied pores [P], free sugar [S] and occupied pores [PS] given by a constant $K = [PS]/[P][S]$, and that unoccupied pores have a G-V curve with $V_0 = V_0$ (free) and occupied channels have a $V_0 = V_0$ (Ba^{2+}), solely determined by Ba^{2+} concentration, then:

$$V_0 = V_0(Ba^{2+}) + [V_0(\text{free}) - V_0(Ba^{2+})]/(1 + K[S]) \quad (2)$$

Eqn. (2) fits to the experimental points of Fig. 3 using V_0 (free) = 30 mV, $V_0(Ba^{2+}) = 30, -6, -38$ mV for $Ba^{2+} = 0$, 1, 2 mM respectively and with a K ranging $60 \div 100\ M^{-1}$. The high value of K estimated indicates that sugars are effective at very low concentrations, similar to those required by anaesthetic action [9, 10], but considerably lower to those necessary for major conformational effects on proteins [4–7].

Finally Fig. 4 demonstrates that glucose at high concentration, 0.8 M, can by itself alter the kinetic properties of the hemocyanin channel fastening by a factor of 4 both of the two time constants observed in the current relaxation which follows a step from 0 to +40 mV in the voltage clamping a hemocyanin doped membrane. It has been shown that current relaxations induced in hemocyanin channels by long lasting positive voltages are due to conformational transitions of each channel through several configurations with different conductances [14]. This fact suggest a straightforward interpretation for glucose effect *i.e.* that this sugar at high concentrations can alter the free energy differences between the various configurations of the channel.

In conclusion we have found that glucose at high concentrations, similar to those known to alter the surface free energy of proteins [3–7], affects the kinetic properties of the hemocyanin channel, probably by changing the equilibria between the different states of this pore. At much lower concentrations, similar to those at which general anaesthetics are effective [9, 10], carbohydrates strongly increase the affinity of the pore for Ba^{2+} cations, which in turn modulates its conductance. We think that it is appropriate at this point to remember that a number of alcohols and other anaesthetics were found to greatly enhance, even at millimolar concentrations, the binding of Ca^{2+} to erythrocyte membranes. Proteins were probably involved in this effect, which was suggested to be one of the possible molecular mechanisms underlying general anaesthesia [11].

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