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The kinetics of colloid osmotic hemolysis. I. Nystatin-induced lysis

John P. Pooler

Department of Physiology, Emory University School of Medicine, Atlanta, GA 30322 (U.S.A.)

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A kinetic model of colloid osmotic hemolysis for cation-permeable cells has been developed. The model consists of three essential components. The first is a set of flux equations, under the assumption that the membrane potential is equal to the chloride equilibrium potential and that cation fluxes are described by the Goldman flux equation. The second is the osmotic equilibrium model of Freedman and Hoffman that takes into account the non-ideal osmotic behavior of erythrocytes. The third is an empirical relation between hemolysis and cell volume, developed from the lysis behavior in hypoosmotic media. Model simulations are compared with lysis experiments using the antibiotic nystatin to raise cation permeability. The form of the kinetics and inhibition of lysis by sucrose are described well by the model. In additional lysis experiments at different external pH the small pH dependence is accounted for by the model.

Introduction

This paper presents a general kinetic model for colloid osmotic hemolysis of human erythrocytes and compares model predictions with lysis experiments using the channel-forming antibiotic nystatin to raise the permeability to inorganic ions. A companion paper extends the model to the more complex situation of photohemolysis using photosensitizers and light to raise ion permeabilities.

The normal stability of erythrocyte volume is maintained by a near impermeability to sodium and potassium. The very low levels of cation leak are matched by active transport to maintain a steady-state cation content [1]. If the cation permeability is increased, for example by adding channel-forming antibiotics, the leak overwhelms the pump and cells swell and eventually lyse when the volume exceeds a critical level. The lysis observed in a large population of cells follows an S-shaped relation with time, reflecting the time-course of net cation permeation and cell volume, the relation between volume and lysis, and the distribution of cell properties in the population.

In a normal physiological environment, cation-permeable erythrocytes containing hemoglobin can never reach a steady-state volume because an equilibrium distribution of permeable ions can only be reached with a higher osmolarity inside than outside. The addition of an impermeant extracellular solute such as sucrose or another oligosaccharide balances the osmotic drawing power of hemoglobin and a steady-state volume may be obtained even in cation-permeable cells [2]. Freedman and Hoffman [2] found normal cell volumes if 30–35 mM sucrose was added to an otherwise normal ionic environment. Lower sucrose concentrations permit swelling and lysis, but at slower rates than in the absence of sucrose.

Any kinetic model for colloid osmotic lysis must contain several elements. The first is a description of net ion fluxes. In the present case

these are assumed to be driven by the electrochemical gradient as described by the Goldman flux equation [3,4]. The second element is cell volume as a function of solute content. This relation is not simple because the net charge on intracellular hemoglobin is not fixed and because the osmotic coefficient of hemoglobin is a non-linear function of hemoglobin concentration [2]. Fortunately, much of this complexity has been worked out by Freedman and Hoffman [2] who devised empirical equations to describe the net charge and osmotic coefficient of hemoglobin. Their comprehensive model of osmotic equilibria in human erythrocytes has been incorporated into the present kinetic model. The third element of the kinetic model is the relation between cell volume and lysis. This was developed by measuring lysis in hypotonic media as a function of osmolarity, using normal cells. Then the cell volumes expected in these media were calculated from the Freedman-Hoffman model. Finally, the volume calculations and the lysis measurements were combined to yield a simple empirical equation relating lysis to cell volume.

Theory and Assumptions

Sodium flux is assumed to obey the Goldman flux equation [5], Eqns. 1–2, and the membrane potential is assumed to be described by the Goldman constant field equation. Potassium flux is described similarly. It is further assumed that the chloride permeability is significnatly larger than the cation permeability, so that Eqn. 2 reduces to

$$M_{Na} = Ap_{Na}\ln(G)([Na_o]-G[Na_i])/(G-1) \quad (1)$$

$$G = (p_K[K_o]+p_{Na}[Na_o]+p_{Cl}[Cl_i])/(p_K[K_i]+p_{Na}[Na_i]+p_{Cl}[Cl_o]) \quad (2)$$

the chloride ratio, Eqn. 3. This assumption certainly holds for untreated cells [6], and since nystatin creates anion-selective channels in bilayers [7] and raises the permeability to all monovalent ions in erythrocytes [8] it seems reasonable for cation-permeable cells also. At the temperature of these experiments, 21°C, it is assumed that pump fluxes can be neglected relative to passive leak in cation-permeable cells. Cation content as a function of time is obtained by a numerical integration of Eqn. 1. The chloride content is obtained from electroneutrality considerations, Eqn. 4, in which z is the net charge on impermeant intracellular solutes, expressed in terms of hemoglobin content. In turn, z is a function of intracellular and extracellular pH, temperature and r_{Cl}, as given by Eqns. 5 and 6. As noted, these relations are taken directly from the Freedman-Hoffman model. Erythrocyte volume is the sum of osmotically active and inactive components. At a normal cell volume of 87 μm^3 [9] the osmotically inactive fraction is about 0.35 [10]. The osmotically active volume is given simply by the ratio of osmotic content of the cell to osmolarity of the medium, Eqn. 7,

$$G \cong r_{Cl} = [Cl_i]/[Cl_o] \quad (3)$$

$$Cl_i = K_i + Na_i + zHb_i \quad (4)$$

$$z = -10(pH_i - (7.2 - 0.16t)) \quad (5)$$

$$pH_i = pH_o + \log r_{Cl} - 0.034 \quad (6)$$

$$V = \Sigma\phi_i S_i/\phi_o[S_o] \quad (7)$$

with the assumption that the cell is always in osmotic equilibrium with its environment. The non-ideality of the osmotic behavior is built into the osmotic coefficient of hemoglobin. For the particular solutes used in these experiments, Eqn. 7 expands to Eqn. 8.

$$V = (\phi_{Hb}Hb_i + \phi_S(P_i + Cl_i + Na_i + K))/(\phi_o([Na_o]+[Cl_o]+[Tris_o]+[U_o])) \quad (8)$$

The osmotic coefficient of all solutes other than hemoglobin is assumed to be 0.93, while the osmotic coefficient of hemoglobin is given by Eqn. 9,

$$\phi_{Hb} = 1 + 0.0645\cdot 10^6[Hb_i] + 0.0258\cdot 10^{12}[Hb_i]^2 \quad (9)$$

from the Freedman-Hoffman model. Hemolysis, expressed as a percentage, is related to cell volume by Eqn. 10. The development of this relationship is presented in the results section.

$$L = [(100 - B)/(1 + \exp((V/V_0 - V_{50}/V_0)/k_1))] + B \quad (10)$$

Computations

An explicit expression for lysis as a function of time cannot be written. Instead, a repetitive numerical integratioan of the flux equations for permeant ions is performed, with a calculation of cell pH, net charge on intracellular solute, hemoglobin osmotic coefficient, cell volume, solute concentrations, and lysis at each cycle of computation. Integration steps are chosen that are sufficiently small to permit use of the simple Euler method for numerical integration. To simulate a given experiment, the following procedure is followed. The initial conditions in terms of temperature, pH, and solute content of the environment, cell solute content, area, and volume are defined. Then with cation permeabilities set to zero and variable terms set to arbitrary provisional values, an iterative calculation of Eqns. 3–9 is repeated until convergence of all variables is obtained. For a given set of initial conditions, the variables always converge to the same end point regardless of their provisional settings. At time zero the sucrose concentration in the medium is step-changed to that employed in the experiment to be simulated and the sodium permeability is set to a finite value to simulate the addition of nystatin. The potassium permeability is set to 1.67-times the sodium permeability and the numerical integration is started. As the calculation cycles, the percent lysis versus time is displayed and compared with measured data points. One check on the validity of this computational method is to simulate the conditions employed by Freedman and Hoffman and verify that the computations approach their equilibrium values over time. With 37 mM sucrose and various pH values, Freedman and Hoffman computed equilibrium values of r_{Cl}, ϕ_{Hb}, z, and other quantities in cation-permeable cells (Table V, B in Ref. 2). The present kinetic model proceeds asymptotically to these same values.

Materials and Methods

All experiments were carried out on human blood drawn within the previous 24 h at a hospital blood bank. Each experiment used blood from a separate donor. Cells were washed three times in standard Tris-buffered saline (149 mM NaCl/5 mM Tris (pH 7.4). In colloid osmotic lysis experiments, nystatin (Mycostatin, Calbiochem-Behring Corp., La Jolla, CA) was dissolved in DMSO and diluted in 30 mM sucrose to form a stock solution. The nystatin stock along with concentrated Tris-buffered saline, sucrose, and water were pipetted into plastic petri dishes (Falcon, Oxnard, CA) and then aliquots of cells were added. The final concentrations of NaCl and Tris buffer were always the same as the standard saline. The final concentration of nystatin was 40 mg/l and the final dilution of the blood was 800 : 1. At this dilution the cells settle on the bottom of the dish as a monolayer. Cells incubated at 21°C for various times and were then analyzed for percent hemolysis by centrifuging the cells out and measuring the absorbance of the supernatant at 413.5 nm (the hemoglobin peak). These absorbances were converted to a percentage by scaling to samples completely hemolyzed by freezing.

Osmotic fragility determinations were made by pipetting aliquots of cells into dishes of different osmolarity without nystatin, all with 4.1 mM Tris buffer. After 1 h the degree of lysis was determined as above.

Results and Model behavior

Osmotic fragility

A representative osmotic fragility experiment is shown in Fig. 1. At osmolarities higher than 180 mosM/l or so there is no lysis beyond a small background level, while lower osmolarities cause increasing lysis, leveling at 100%. The relation between lysis and osmolarity is described quite well by Eqn. 11.

$$L = [(100 - B)/(1 + \exp((O - O_{50})/k_2))] + B \quad (11)$$

There was considerable variation in osmotic fragility from blood sample to blood sample. In 12 samples the average osmolarity causing 50% lysis (O_{50}) was 166.1 ± 7.3 (mean ± S.D., $n = 12$), the range being 150.3 to 178.6. The steepness of lysis with osmolarity varied also. The constant k_2 in Eqn. 11 averaged 6.92 ± 0.70 (mean ± S.D., $n = 12$) with a range of 5.86 to 8.08.

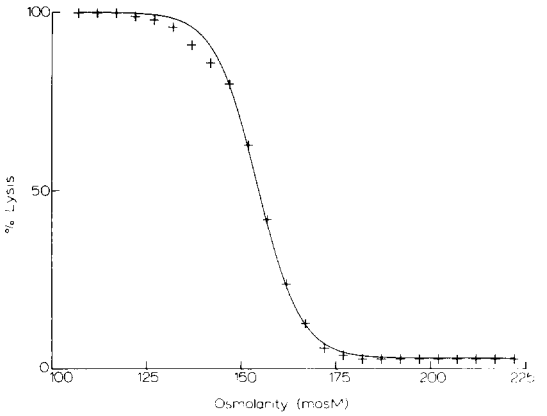

Fig. 1. Osmotic fragility. Cells incubated in indicated osmolarity for 1 h in plastic dishes at 21°C. Osmolarity was varied by changing the NaCl concentration at constant Tris buffer concentration of 4.1 mM titrated to pH 7.4. The + symbols are data points. The continuous curve is calculated according to Eqn. 11 with the following parameters: $O_{50} = 154.4$, $k_2 = 5.62$, $B = 3$.

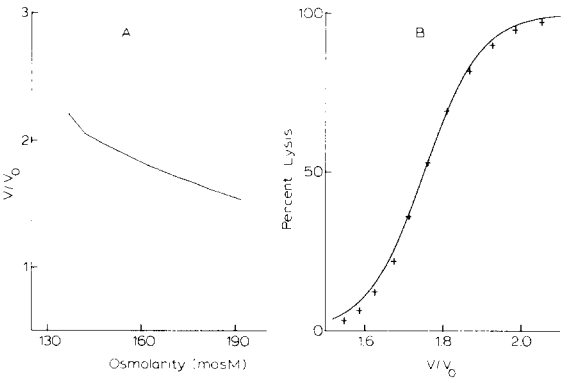

Fig. 2. (A) Relative cell volume versus osmolarity calculated from Eqns. 3–9 as described in the text for external conditions as in Fig. 1. (B) Percent lysis versus relative cell volume. For a set of osmolarity values, relative cell volume was calculated from Eqns. 3–9 and percent lysis was calculated from Eqn. 11, using the average values for O_{50} and k_2 given in the text. The resulting set of points is plotted as + symbols. The continuous curve is calculated according to Eqn. 10 with the following parameters: $V_{50}/V_0 = 1.78$, $k_1 = -13.62$, $B = 0$.

Several experiments were performed in which 50 mosM/l of the osmolarity of the medium was replaced with sucrose. With sucrose present the osmotic fragility was considerably less. The osmolarity causing 50% lysis was reduced by 20 mosM/l when 50 mosM/l of the medium was sucrose. About 6 mosM/l of this shift is predicted by the Freedman-Hoffman model, because at equal osmolarities the lower chloride concentrations in the sucrose-containing media lead to lower cell volumes. About 1 mosM/l of the shift is accounted for by the higher molar volume of sucrose versus sodium chloride. The rest is apparently a direct influence of sucrose on cell properties.

Calculated volume in hypoosmotic media

The expected steady-state cell volumes of cation-impermeable cells in the media used in the osmotic fragility experiments can be calculated from Eqns. 3–9, assuming that sodium and potassium contents remain unchanged. The results of this calculation are shown in Fig. 2A. There is a nearly linear rise in volume with decreasing osmolarity over the range shown. From the previously measured relation between lysis and osmolarity and the volume-osmolarity calculation, one may construct a plot of lysis versus volume, as shown in Fig. 2B. This relation is described fairly well by Eqn. 10. The direct sucrose influence on osmotic fragility translates into an increase in the relative cell volume at which 50% lysis occurs (V_{50}/V_0) of 0.003 per millimolar sucrose.

Nystatin-induced lysis

Kinetics. The presence of nystatin in media of normal or elevated osmolarity caused cells to lyse over time, with kinetics and final level dependent on the sucrose concentration. Fig. 3 shows raw data (symbols connected by straight line segments) along with model calculations (curvilinear lines). The number beside each curve indicates the concentration of sucrose. The nystatin concentration was the same throughout.

Clearly the main features of the cellular behavior are described well by the model. Increasing concentrations of sucrose inhibit the maximum amount of lysis because cells swell to progressively smaller volumes, and hence a progressively smaller fraction of cells reach their critical lytic volume. A small amount of the inhibition is attributable to a direct sucrose influence on cell fragility. The kinetics are also slowed. Curves normalized to their degree of lysis at infinite time do not superimpose.

The influence of pH. . Changes in pH had a

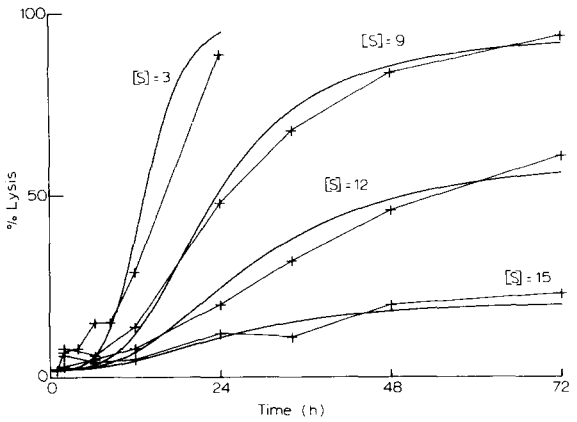

Fig. 3. Kinetics of nystatin-induced lysis at different sucrose concentrations. Dishes contained identical Tris-buffered saline and nystatin concentrations as described in Methods and incubated at pH 7.4, 21°C. The + symbols connected by linear segments are data points. The smooth curves are calculated from the model with the following parameter values: $p_{Na} = 0.6 \cdot 10^{-8}$ cm/s, $V_{50}/V_0 = 1.55$, $k_1 = -10$, $B = 2$.

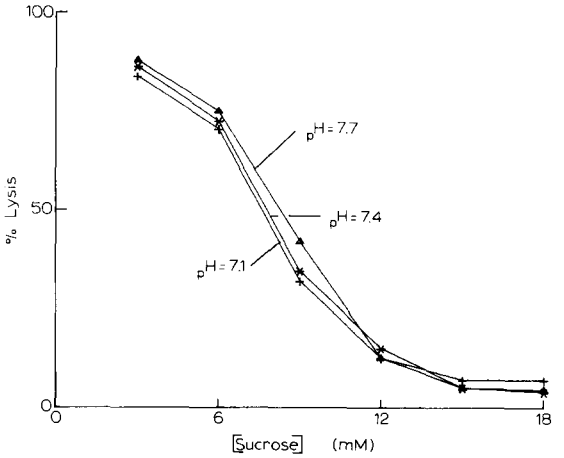

Fig. 4. Nystatin-induced lysis as a function of sucrose concentration at three pH values. Cells incubated in Tris-buffered saline at the indicated pH and sucrose concentration at 21°C for 24 h.

small but consistent influence on nystatin-induced lysis. Fig. 4 shows a family of lysis versus sucrose concentration curves at three different pH values. At any given sucrose concentration lysis was higher at a higher pH. The concentration of sucrose at which 50% lysis occurred (U_{50}) was increased by 1.1 mM per pH unit in these experiments. The pH effect is predicted by the model. Simulations of U_{50} at different pH values give a non-linear increase with pH. The average slope in the range pH 7.1 to 7.7 is 2.8 mM per pH unit. While the model shows a somewhat steeper pH dependence than found in the data, the direction is the same. At higher pH the model predicts smaller initial chloride contents and cell volumes, but also more negative membrane potentials initially and throughout the development of lysis. The higher degree of lysis at higher pH is accounted for by the higher net cation influx driven by the more negative membrane potential.

Discussion

Most observers agree on the general principles governing colloid osmotic lysis as they were proposed many years ago [11], but these principles have not been previously incorporated into a set of equations with which to calculate the time-course of lysis. In essence the present kinetic model starts with the osmotic equilibrium model of Freedman

GLOSSARY OF SYMBOLS USED IN EQUATIONS

A	area per cell (cm^2)
B	background lysis (percent)
Cl	chloride (moles)
G	ratio of terms in Goldman equation
Hb	hemoglobin (moles)
K	potassium (moles)
k_1	steepness factor in Eqn. 10
k_2	steepness factor in Eqn. 11
L	lysis (percent)
M	net flux (mol/s)
Na	sodium (moles)
O	osmolarity (osmole/cm^3)
p	permeability (cm/s)
P	impermeant cellular anions other than Hb (moles)
r_{Cl}	ratio of cellular to outside chloride concentrations
S	solute (moles)
t	temperature (degrees Celsius)
U	sucrose (moles)
V	cell volume (cm^3)
V_0	cell volume at time zero (cm^3)
V_{50}	volume at which 50% lysis occurs, or critical lytic volume (cm^3)
ϕ_{Hb}	osmotic coefficient of hemoglobin
ϕ_S	osmotic coefficient of all other solutes
[ ]	concentration (mol/cm^3)
i	subscript denoting cellular medium
o	subscript denoting external medium

and Hoffman, adds Nernst-Planck flux equations, and develops a cell volume-lysis relation based on the degree of lysis in hypotonic media. The key element of the Freedman-Hoffman model is the concentration dependence of the hemoglobin osmotic coefficient, ϕ_{Hb}. At a normal cell volume, ϕ_{Hb} is 2.85 and falls to about half that value at the critical lytic volume. In the lysis simulations the initial cell volume change is a shrinkage, followed by swelling, and ϕ_{Hb} transiently rises to greater than 3 before falling during cell swelling. This means that the effective osmotic contribution of hemoglobin varies from more than 25 mosmol/l at minimum cell volume down to less than 5 mosmol/l at significant swelling. With sucrose in the external medium, the kinetics of lysis become highly sensitive to the osmotic contribution of impermeant intracellular solute and the concentration dependence of ϕ_{Hb} is therefore critical to a kinetic description of lysis.

Many other quantities in the model also influence lysis kinetics, specifically including the initial cell contents of hemoglobin and other impermeable solutes. And clearly there are significant variations from blood sample to blood sample in lysis kinetics. To be perfectly legitimate a simulation of a given experiment should begin with initial solute values specific for the blood sample used. For simplicity the values of internal solute chosen for simulations were always those used by Freedman and Hoffman. The external concentrations, of course, were those of the actual experiments. The hypotonic lysis experiments also demonstrated clear sample-to-sample variations in osmotic fragility. This variation translates in the model into variations in the critical lytic volume and steepness of the lysis-volume relation. How much of this represents true variation in cell tolerance to swelling as opposed to different contents of impermeable cellular solute is not known.

In the simulations there were three adjustable parameters that could be varied to achieve a fit. These were the critical lytic volume, the steepness of the lysis-volume relation, and the sodium permeability. Fits were achieved with sodium permeabilities in the range of 10^{-8} cm/s. The potassium permeability was always fixed at 1.67-times the sodium permeability. Since the assumption is made that the membrane potential follows the chloride ratio a specific value for chloride permeability does not appear in the calculations. The true potassium/sodium selectivity is not known and the ratio chosen for the model is in line with the ratio of their conductances in aqueous solution [12]. The kinetics vary with sodium permeability but depend very little on the selectivity ratio.

In Ponder's classic work on hemolysis [13] he found that lysis behavior varied with the means used to bring it about. It remains to be determined whether the lysis-volume relation under conditions of water entry without solute entry in hypotonic media is identical to that under conditions of parallel solute and water entry in isoosmotic media.

A computer program in Basic to calculate lysis versus time will be gladly furnished on request.

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