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The kinetics of colloid osmotic hemolysis. II. Photohemolysis

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Many of the known features of photohemolysis have been organized in a kinetic model that simulates the lytic time-course in a variety of conditions. The model combines Nernst-Planck flux principles, the osmotic equilibrium model of Freedman and Hoffman, equations relating illumination parameters to ion permeability, and an empirical relation between cell volume and lysis. Model simulations are compared with experiments showing the dependence of lysis kinetics on sensitizer concentration and on the osmotic content of the reaction medium. Additional experiments demonstrate that the inherent osmotic fragility of erythrocytes is not altered by illumination conditions that cause major delayed lysis 23 h later. The successful simulations support the hypothesis that photohemolysis is a colloid osmotic lysis occurring in cells behaving as imperfect osmometers.

Introduction

The preceding paper [1] described a general kinetic model for colloid osmotic hemolysis. In this paper the model is extended to photohemolysis in which erythrocytes are made cation-permeable by illumination with visible light in the presence of photosensitizers.

In essence, the red cell is assumed to be an imperfect osmometer, as described by the Freedman-Hoffman model for osmotic equilibrium [2]. Ions permeate according to Nernst-Planck relations [3], drawing in water osmotically and causing lysis when a critical volume is reached. Available evidence supports the idea that photohemolysis is a colloid osmotic lysis obeying these principles [4,5] but a rigorous test has not been made. The kinetics of photohemolysis are complex. The time-course follows an S-shaped relation not described by any simple mathematical function, making it difficult to relate kinetics to a given illumination protocol. Several investigators have taken the practical expedient of lumping all the kinetics into a single rate parameter and this has proven to be quite useful [6,7]. The dependence of delayed photohemolysis on sensitizer concentration and fluence (light dose) can be expressed empirically by Eqn. 1, where C and τ are sensitizer concentration and illumination duration, respectively, τ_{50} is the total time (illumination plus after-light dark incubation) to 50% lysis, and x and y are dimensionless parameters [7]. By finding a value of 2 for y a so-called 'dose-squared' relation has been identified. This expression is suitable when

$$1/\tau_{50} = kC^x\tau^y \quad (1)$$

the after-light period is long relative to the duration of illumination but becomes ambiguous when much of the lysis occurs during illumination. A bigger drawback is that illumination parameters

are related only to lysis, a complex result of a primary change in the cell membrane, rather than to the primary change itself. Therefore the present model was developed in which illumination parameters are related to changes in membrane permeability, and other influences on lytic rate such as the osmotic content of the bathing medium are expressed independently. The model can be used to predict the results of experimental protocols and then tested rigorously by comparing data with model predictions. Parts of the model resemble Cook's equations used to describe ultraviolet-induced erythrocyte swelling [8,9] but goes further. The present model includes a specific relation between volume and lysis that Cook did not deal with, takes into account the non-ideal osmotic behavior of erythrocytes, and relates illumination parameters to changes in membrane properties.

Theory and Assumptions

It is assumed that the osmotic and lysis behaviors of cells made cation-permeable by illumination are identical to those of normal cells made cation-permeable by other means and that lysis for any level of cation permeability is described by the kinetic equations developed in the preceding paper [1]. Evidence will be given in Results showing that the osmotic fragility of cation-permeable cells is normal. The key element in extending the model to photohemolysis is the relation between illumination and cation permeability. This is expressed in Eqn. 2,

$$p_{Na} = aC^r\tau^s \tag{2}$$

where p_{Na} is the sodium permeability, a is a potency parameter dependent on fluence rate and species of sensitizer, and r and s are dimensionless parameters. Potassium permeability is assumed to bear a fixed ratio to the sodium permeability. Background permeabilities and fluxes associated with active transport are assumed to be negligible relative to passive fluxes through the light-induced pathway. Lysis is computed from Eqn. 3, where L is the percent lysis, V is cell volume, V_0 is the volume at time zero, V_{50} is the volume at which 50% of the cells lyse, and k_1 is a constant.

$$L = 100/(1+\exp((V/V_0 - V_{50}/V_0)/k_1)) \tag{3}$$

Computations

Lysis is calculated by a repetitive numerical integration of the flux equations as described in the preceding paper [1]. For all experiments to be simulated the illumination period, τ, was 1 h. Therefore p_{Na} increased at each cycle of the computation up to 1 h and then remained fixed. In this paper comparisons are made between experimental measurements and model calculations of a parameter called the C_{50}, which is the sensitizer concentration causing 50% lysis 24 h after the beginning of a 1 h period of illumination. Model calculations of the C_{50} are found by an iterative procedure that calculates lysis out to 24 h starting with an arbitrary value for C and adjusts C until the computed lysis just reaches 50% at 24 ± 0.1 h.

The following parameter values were used in the computations: $r = 1.33$, $s = 2$, $V_{50}/V_0 = 1.78$, $k_1 = 8.8$. Different values of a were selected depending on the behavior being modeled.

Materials and Methods

Photohemolysis experiments were performed as described previously [10]. Briefly, human erythrocytes washed three times in Tris-buffered saline were placed in plastic dishes along with appropriate concentrations of sensitizer. The final blood dilution was 800 : 1. The sensitizers were eosin Y or phloxine B, used as obtained from Chem Service Inc., West Chester, PA. Following 30 min dark incubation at room temperature, the dishes were illuminated from below for 1 h by placing them on a clear Lucite platform 1 inch above daylight fluorescent lamps. Following illumination, dishes incubated in the dark at 4°C and were analyzed for percent hemolysis at defined times. Values for the C_{50} were obtained by employing a span of sensitizer concentrations, linearizing the resulting plot of lysis versus sensitizer concentration, and using a regression analysis as described previously [11]. Osmotic fragility experiments followed the same procedures as described in the previous paper [1].

Results and Model behavior

Osmotic fragility

The model assumes that the only light-induced change in cell properties is a rise in cation permea-

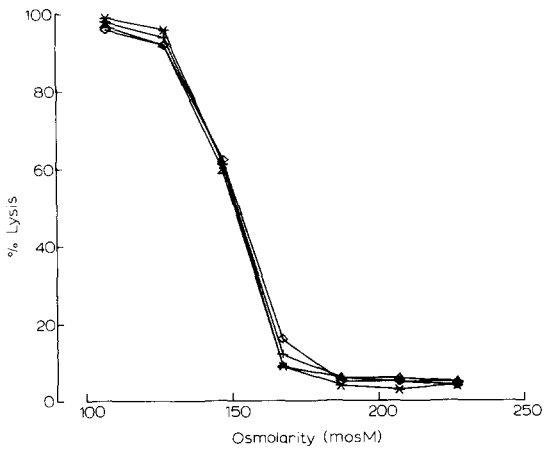

Fig. 1. Osmotic fragility of illuminated and normal cells. Cells were incubated in Tris-buffered saline containing eosin Y at concentrations of 1.5 μM ($\diamond$), 1 μM ($\triangle$), or 0.5 μM (+) at the osmolarity indicated on the ordinate for 0.5 h, illuminated for 1 h and analyzed for percent hemolysis. Controls (*) were treated identically but were not illuminated. In a parallel experiment using a normal osmolarity (302 mosM) and a span of sensitizer concentrations, cells were incubated for an additional 23 h after illumination. From this the C_{50} was found to be 0.387 μM.

bility and that osmotic properties are not altered. This was tested by measuring the osmotic fragility of cells immediately following illumination. Cells were incubated in media of different osmolarities with standard sensitizer concentrations above the C_{50} and illuminated for 1 h. They were then analyzed for percent hemolysis and compared with non-illuminated controls. As illustrated in Fig. 1, there was no change in the osmotic fragility of the cells even though illuminated cells allowed to incubate another 23 h at a normal osmolarity developed complete light-induced lysis at the highest sensitizer concentration. In four experiments the ratio of the osmolarity causing 50% lysis in illuminated samples relative to controls was 1.00 ± 0.01 (mean $\pm$ S.D.), at a sensitizer concentration of 3.82 ± 0.42 times the C_{50}. This demonstrates convincingly that cells which are destined to lyse because of a light-induced change in cation permeability nevertheless retain the osmotic properties of normal cells.

Model behavior

Model predictions of the kinetics of the system are shown in Fig. 2. Cell volume, loss of cellular

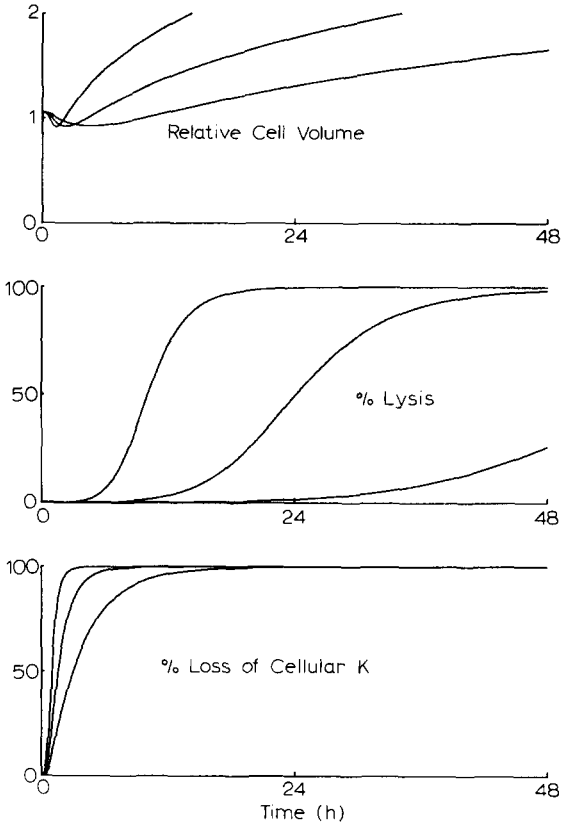

Fig. 2. Model calculations of cell volume, loss of cellular potassium and percent lysis versus time for relative sensitizer concentrations of 1, 2 and 4, with illumination occurring during the first hour.

potassium, and lysis are shown as functions of time for three sensitizer concentrations. The most immediate event is loss of potassium, which responds to the light-induced rise in permeability. Cell volume shows a small initial drop as the potassium leaves, but then rises as sodium from the external medium replaces potassium and eventually exceeds the initial potassium content. Following with an even bigger lag is lysis, which does not reach significant levels until almost all of the potassium has been lost. The general features of this behavior – a loss of potassium preceding lysis and a lag in the development of lysis – have been reported by a number of investigators over the years [4–6]. A direct comparison between model predictions and lysis kinetics at several different rates is shown in Fig. 3. Up to the limit of scatter in the data the agreement is very good.

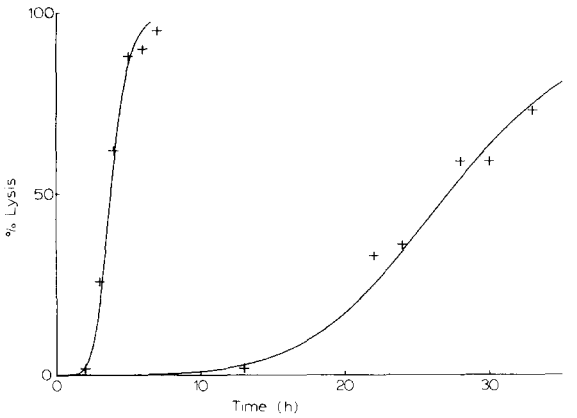

Fig. 3. Model calculations and measurements of lysis kinetics. Cells were illuminated for 1 h in the presence of 30 nM or 150 nM phloxine B in TC plastic dishes and then incubated in the dark for the times indicated on the abscissa and analyzed for percent hemolysis.

Cation selectivity

A direct measurement of the p_K/p_{Na} ratio has not been made. However, cation substitution experiments give results that are consistent with model simulations using a p_K/p_{Na} ratio of 1.82. A representative experiment is shown in Fig. 4A. The symbols are measured values of the C_{50} as a function of potassium concentration in the medium (1 for 1 replacement of sodium). Model simulations are shown in Fig. 4B for p_K/p_{Na} ratios of 0.75, 1, and 1.7. While there is no theoretical reason to expect a linear relation between C_{50} and potassium concentration, the deviations from linearity in the simulations are less than the scatter in the experimental data. Therefore slopes of C_{50} versus potassium concentration for a series of p_K/p_{Na} ratios were determined by a regression analysis. By matching the slope from a given experiment with the slopes from the simulations, the p_K/p_{Na} ratio for the experiments could be estimated. In five experiments the p_K/p_{Na} ratios were found to lie between 1.3 and 3.5, with an average of 1.82. This value was used in the subsequent simulations, but essentially the same behavior is obtained over a fairly wide range.

Osmolarity

According to the model, the C_{50} should vary with the osmolarity of the reaction medium (dif-

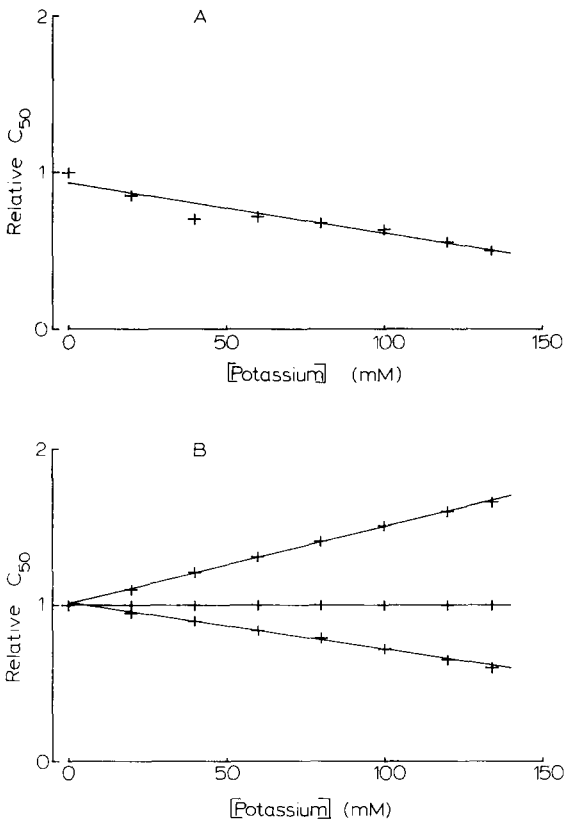

Fig. 4. (A) Potassium concentration dependence of photohemolysis. For each potassium concentration (1 for 1 replacement of sodium), the C_{50} was determined and plotted relative to the C_{50} at zero potassium. The negative slope indicates that lower sensitizer concentrations are required to bring about lysis when potassium replaces sodium. (B) Model simulations of the experiment in Fig. 4A using p_K/p_{Na} ratios of 0.75, 1, and 1.7.

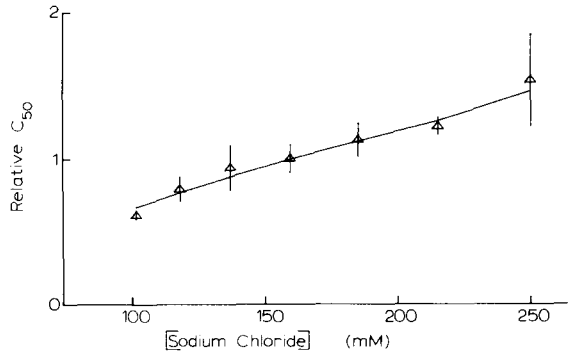

Fig. 5. Relative C_{50} for eosin Y versus sodium chloride concentration in the external medium. The points are means (±S.E.) of four experiments normalized to the value at 157 mM. The continuous line is the model prediction.

ferent NaCl concentrations). This influence is manifested both by changes in the driving force for ion flux and by changes in the initial cell volume. The latter influence dominates so that the C_{50} rises as the osmolarity rises. Fig. 5 shows model predictions of the behavior and experimental measurements of C_{50} versus sodium chloride concentration. The C_{50} values are normalized to those at a sodium chloride concentration of 157 mM. The agreement is good and supports the idea that lysis is related to cation permeability as described in the model.

Discussion

The model presented here is a synthesis of the osmotic equilibrium model of Freedman and Hoffman [2], Nernst-Planck flux principles [3], the concentration and fluence dependence for photohemolysis reported by Valenzeno and Pooler [7], and an empirical function relating lysis to cell volume. It successfully mimics the general features of photohemolysis behavior, specifically including the kinetics of lysis, the relation between percent lysis and sensitizer concentration measured at a fixed time after illumination, and the response to alterations in the osmotic content of the external medium.

The connection between photon absorption and modification of membrane properties is expressed by the potency parameters a and the concentration and illumination time parameter r and s. It is not known whether all sensitizers act to produce the same photooxidative events but the similarity in photohemolysis behavior observed when a variety of fluorescein sensitizers is employed suggests that differences between membranes of the fluoresceins can be accounted for simply by scaling the value of a [10].

The parameters r and s of the model are analogous to x and y of the empirical relation (Eq. 1). Model simulations using given values of r and s can be analyzed according to Eqn. 1. The x and y values so found are close to (but always slightly less than) r and s. Cook and Blum [6] first reported a dose squared relation between fluence and lysis rate. Valenzeno and Pooler [7] confirmed this by showing that y in Eqn. 1 had a value of 2. Therefore the fluence dependence was incorporated into the model by setting s to 2. The concentration dependence is less firm. Valenzeno and Pooler reported a value for x of 1.4. Subsequent experiments (unpublished) using tissue culture-treated plastic reaction vessels to avoid sorption of sensitizer to the plastic (see Ref. 11) yield a value for x of 1.27. A value for s of 1.33 was chosen for the present calculations because it gives the best simulation of the most recent kinetic data. It remains for future experiments to see whether this value is appropriate for all sensitizers.

One should expect lysis to show the same squared dependence on sensitizer concentration as it does on photon dose. Valenzeno [12] has shown that uptake of sensitizer by erythrocyte ghost membranes bears a power relationship to sensitizer concentration in the medium, with sensitizer in the membrane being proportional to sensitizer in the medium raised to the 0.614 power. If this power dependence of membrane-located sensitizer is combined with Eqn. 2, the dependence of membrane permeability on sensitizer in the membrane is 2.16, a number not far from 2. Allowing for uncertainties in the experiments it therefore seems quite possible that fundamental events in the membrane bear a squared relation to both light dose and sensitizer concentration.

In a normal state the anion selectivity of erythrocytes justifies the assumption that the membrane potential follows the chloride equilibrium potential. Normal chloride permeability is about $2.5 \cdot 10^{-8}$ cm/s [13]. To simulate the faster lysis experiment of Fig. 3 a potassium permeability of $5.6 \cdot 10^{-8}$ cm/s is required, meaning that cation concentrations may also have a substantial influence on the membrane potential. Simulations using the approximation that membrane potential is equal to the chloride equilibrium potential duplicate the experiments rather well, but simulations using the full Goldman equation, with p_{Cl} set to $2.5 \cdot 10^{-8}$ cm/s, give too slow a time-course for lysis reaching 50% in less than 15 h. This implies that chloride permeability is raised by illumination as well as cation permeability. As yet there is no direct evidence on this point. Photodynamic effects on band 3 protein (the anion exchanger) using protoporphyrin as sensitizer show a decreased transport rate for sulfate [14]. Whether there are effects on the conductive chloride pathway is not known.

Cook [8] analyzed cation fluxes in erythrocytes induced by ultraviolet illumination without sensitizer using the flux equations of Tosteson and Hoffman [15] to describe volume changes following a rise in cation permeability. Although there is no a priori reason to expect ultraviolet illumination to alter erythrocytes in the same way as visible light in the presence of sensitizer, there does seem to be a remarkable similarity in cellular response. Both follow a dose squared relationship when the fluence is varied and both behave as if the sodium permeability were between one-half to two-thirds of the potassium permeability. It is not known whether the cation permeabilities in response to these two treatments have a common molecular basis.

The osmotic fragility experiments establish unambiguously that lysis behavior in response to a hypotonic challenge is not altered by illumination doses sufficient to cause a vast majority of cells to lyse in isoosmotic media 24 h later. Some reports in the literature are in seeming conflict with this finding and describe an increase in osmotic fragility immediately following illumination [16–18]. There are several possible reasons for the difference in results. One relates simply to the degree of osmotic swelling at the time of measurement. Cells which are already swollen due to cation influx cannot tolerate the same low osmolarities as cells with a normal volume. When osmotic fragility is assessed at times short relative to the τ_{50} (as in the present experiments) there should be essentially no change in cell volume and no change in osmotic fragility. But when swelling occurs much faster and osmotic fragility is measured after a significant fraction of the τ_{50} has elapsed, there should be a considerable increase in osmotic fragility. This is the case for studies cited above. DeGoeij and Van Steveninck [19] showed that osmotic fragility does not change until the majority of the potassium has left the cells. Since potassium loss precedes volume change this further supports the view that the light-induced rise in osmotic fragility reported by others reflects the fact that cells were already swollen at the time of measurement.

It is also possible that the sensitizers employed by others have a greater influence on the inherent cell tolerance to a rise in volume than do the fluorescein sensitizers used here. The studies reporting an increase in osmotic fragility used protoporphyrin or blood from patients with erythropoietic protoporphyria [16–18]. While it appears that sensitizers of different classes lead to similar kinds of lysis behavior, it has not been shown that the underlying photooxidative events are the same. Cell membranes contain multiple molecular targets for sensitized attack and the relative susceptibility may vary with the species of sensitizer. All sensitizers apparently affect cation permeability but there may be differential effects on the moieties which govern cell tolerance to an increase in volume.

A third possibility is that target susceptibility may vary with the potency of illumination conditions (fluence rate and sensitizer concentration). It is possible that mild conditions affect only cation permeability while highly potent conditions simultaneously affect other cell properties. If other sensitizers or more severe illumination conditions induce changes in osmotic properties beyond a rise in cation permeability then this can be revealed in future tests of the model under different conditions.

According to the model, potassium permeability is not influenced by swelling, but it is well known from other work that there is a prelytic loss of potassium as the critical volume is approached [20,21]. While the loss is greater at slower swelling rates, the critical hemolytic volume is not influenced by swelling rate [17]. Inclusion of a prelytic volume-induced rise in potassium permeability in the model does not have a large influence on model behavior.

In conclusion, the model presented here successfully mimics many of the known features of photohemolysis. It is a simple matter to simulate a variety of experimental protocols, such as changing illumination parameters, solute concentrations, temperature, and pH. While a number of the underlying assumptions do not yet have firm experimental support the model can serve as a working hypothesis for their test. The predictions of the model should serve as useful tool in the exploration of membrane photosensitization.

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

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