

HUMAN RED CELL HEMOLYSIS RATES IN THE SUBSECOND TO SECONDS RANGE

AN ANALYSIS

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ABSTRACT Hypoosmotic shock kinetics of the normal human red cell (25°C) were investigated by means of a rapid kinetics apparatus, with a resolving time of about 50 ms. The results are compared with some current models for hemolysis. The fast hemolysis plots are not true symmetric sigmoids, in contrast to results from less stressful conditions, nor can they be simply fitted to an "all or none" process. In the most severe conditions, mixing with neat water, the velocities with which red cells start to hemolyze depend on the rate at which the cell is converted to a swollen sphere (lag phase). Under such conditions, the mean time to rupture and start of leaking is about 0.6 s. The rate of osmotically driven solvent flow is probably the principal controlling factor in the discocyte to sphere transformation. The overall course of hemolysis can be described in terms of two rate processes and a distribution of cell fragilities. The fragilities probably depend on the age of individual cells in the samples. In the low-salt region, the effect of hypotonicity as well as hypoosmolality is discerned. The surface charge on the red cell provides no driving force for rupture above salt concentration 0.10 M, but at 0.05 M salt and below, electrostatic effects may contribute.

The velocities of red cell hemolysis have been studied in many laboratories, including the case of "fast hemolysis." Such a term is relative. In this paper, it is meant to involve resolution times of the order of 10–50 ms. Under those conditions, hemolysis at very low osmolality (shocking with neat water) can be examined and charted.

Although there are numerous analyses of the hemolysis velocities, most are based on plots assumed to be sigmoid in character. Our data, and in fact the data of others, might be assigned as having some sigmoid character. But at best it is a nonsymmetric, or a skewed, sigmoid. The plots referred to are those in which percent hemoglobin leaked by an osmotically shocked cell population is plotted as a function of time after shock.

If in fact one examines the data here, e.g. in Fig. 1, or even in a cursory way that of other authors, it appears that in the short time ranges, the plot is asymptotic with the abscissa, rises as a sigmoid might, but then flattens in a sloping and almost linear fashion, in its progress toward long time intervals. For convenience, we will refer to

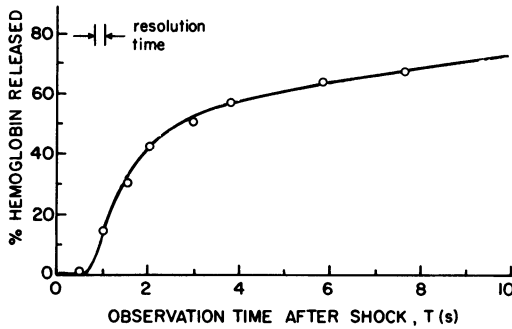


FIGURE 1

FIGURE 1 Hemolysis rates. Points are experimental data from the case of severe shock (addition of neat water, 0.021 osM during shock period). Continuous line: calculated plot from the rate expression for the pooled sample.

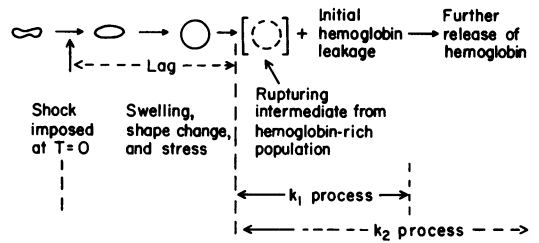


FIGURE 2

FIGURE 2 A model for the principal processes invoked in the analysis for a single cell.

the early part of the plots, where $T =$ observation time, as sigmoid.¹ This is the area of dominance of fast release of hemoglobin, occurring during times $0 < T \leq 2$ s, for normal, unperturbed cells. This initial fast rate of release gives way to a slower rate of appearance of hemoglobin in the later section of the plots, called herein the ramp section. The nature and duration of this ramp section depends, as expected, on the severity and duration of the osmotic shock. But in all cases, at least with normal cells, and even with damaged cells, the ramp is evident and is made apparent if data is taken over an adequate range, $T \geq 4$ s.

Thus a reasonably complete analysis should fit data taken from very severe shock in the subsecond range, on out to relatively long times, several dozen seconds. Both the sigmoid and the ramp sections must be fitted. As often occurs in rate analyses, unequivocal identification of model rate processes is not achieved here. However, some parameters needed to fit the data with our analysis, especially in the severe shock cases, correspond reasonably well to those of Jay and Rowlands (1975). They investigated erythrocyte rapid shock by means of cinematography, observing cell shape changes. It is necessary to invoke a distribution of cell fragilities in our analysis, but that is expected on the basis of varying red cell ages and fragilities. The simplest model for the hemolysis reactions of an individual cell is illustrated in Fig. 2.

The basis of the experimental methods should be kept in mind, both for our work here and that from other laboratories. In some cases, the authors subjected the cell

¹Notation used in this paper: A , fraction of hemoglobin leaked from the k_1 process; H , percent hemoglobin leaked; k_1 and k_2 , rate constants for processes 1 and 2; $N(\tau_i)$, fraction of cells which rupture within the time interval Δt designated by τ_i ; σ , standard deviation; T , time between initiation of hypotonic shock and isotonic restoration, i.e. the interval of shock; \bar{t} , mean time of manifestation of hemoglobin leakage by the cells in the population; Δt , an incremental time for purposes of summation; $\tau_i = i\Delta t$, time elapsed after shock until lysis occurred for a particular subfraction of cells $N(\tau_i)$.

to hypoosmotic shock, centrifuged, and measured hemoglobin in clear supernates. In others, including our own, the shock was followed by restoring osmolality, centrifuging, and measuring hemoglobin by spectrophotometry. In the Jay and Rowlands paper, the aim was not to measure hemolysis as such, but to follow the shape changes of individual cells by rapid moving pictures. Usually shock was imposed by permeants such as glycerol. In still other laboratories, the whole suspension of cells is followed spectrophotometrically. The apparent absorbances in that case depend on some combination of hemoglobin absorbance, cell scattering, and membrane or ghost optical properties, all as a function of time. The complications in that method (spectrophotometry of translucent and scattering materials, or opacimetry in the earlier terminology) have been reviewed by Shibata (1959).

There are two general models for hemoglobin leakage: (a) The cell is not really torn irreversibly; it leaks hemoglobin at variable rates depending on stress gradients. We adhere to this model, by and large, and consider that it is described by the term hemolysis. (b) The cells leak either all their hemoglobin or none of it. This is the "all-or-none" model, discussed by Ponder (1948), and by others since then. In the all-or-none model, the principal time dependencies do not lie in the rates at which hemoglobin leaves the cell, but in the events leading up to rupture.

We believe that some of the time dependency probably depends on finite rates of hemoglobin leakage, at relatively long times after initial membrane disruption. Accordingly we discarded the all-or-none model (massive rupture) in attempting to encompass all the data in the simplest manner, from very short to comparatively long observation times.

METHODS

The apparatus for obtaining the data will be fully described by its inventors, Dr. Olaf Runquist and Rodney Olson of Hamline University, St. Paul, Minn., in a separate publication. For our purpose here, an outline of its function is as follows. The cell suspension, usually 0.50 ml in isotonic solution, is held in test tubes under controlled temperature, 25°C. Feeding into the top of the test tube are two jets. One jet comes from a syringe that injects the shock solution, usually 3.5 ml. The second jet issues from a syringe carrying the restoring solution. The interval of shock time, T , begins when the shock solution is pushed in, and ends when the restoring solution is pushed in. Thus the cells are exposed to an interval of osmotic shock, and analyzed by spectrophotometry at $\lambda = 577$ for the amount of hemoglobin leaked, after restoration and centrifugation.

With normal cells, the steps can be carried out with isotonic solutions throughout, with virtually no hemolysis. Therefore, the very rapid mixing and hydraulic perturbation do not of themselves induce rupture. The volumes of all solutions are reproducible within ± 0.05 ml. The time of injection of solutions is about 50 ms. Since the cells initially awaiting shock are isotonic, shocking with neat water does not bring the salt concentration to zero during shock, but it can be brought down to about 0.02 M. This appears to be low enough such that it is a short extrapolation to zero salt concentration, for obtaining the mean hemolysis time under maximally severe hypotonic shock.

The cells were freshly drawn for the day on which they were used. They were handled and washed as in our previous studies (Lovrien et al., 1975). All the experimental hemolysis rate

plots, as in the circled points in Fig. 1, were redetermined several times on different days with various donors. About 12 individuals provided the cells. The various osmolality-dependent hemolyses were distributed throughout the total period of research, i.e., the data for a particular osmolality were not determined en bloc, nor from a single donor. No particular sequence in osmolality variation was used.

RESULTS

Fitting of the Rate Processes

Representative data for erythrocytes maximally stressed by neat water are shown in Fig. 1. This figure is typical of approximately 25 such plots, with water as the shocking solution. If hypotonic salt is used as the shocking solution, similar plots are obtained with of course a long time lag before leakage starts, as well as a slower rate of leakage. Even with maximum shock, however, there is nevertheless a time lag before hemoglobin can be released. This time lag is considerably longer than the resolving time of the instrument. However, the lag is consistent with the preleakage steps, as noted above, described by other authors, notably Jay and Rowland (1975).

The simplest analysis possible, which fits the data over the complete set of timed observations including the subsecond shock times, appears to have four main aspects. These are illustrated by Fig. 2: (a) There is a finite lag period, taken up by cell swelling and shape change, before any rupture can occur, if the cells start as normal biconcave discocytes in isotonic medium. (b) At some point, there must be rupturing and initial leakage from a stressed cell, with initially a rapid spillage of hemoglobin. (c) This is accompanied by a slower ongoing leakage of hemoglobin from a deflated cell. (d) The pooled cells used in the experiments represent a normal distribution around an average cell, about 60 days old. Individual cells in the pool have somewhat varying hemoglobin concentrations and membrane fragilities.

Fig. 3 shows with solid lines what can be expected from single cells, with various single cells starting to give off hemoglobin rapidly at varying times after initial shock. A slower but continuing leakage of hemoglobin for each cell would be expected to produce a similar overall behavior for the sum of the cells, which we refer to as the "ramp" on Fig. 1. However, the cells are diverse, and have varying fragilities. Accordingly, for individual cells there is a diversity of times at which rupture or initial leakage starts. Thus the effect of having a distribution of cells is likely to govern the early, or k_1 process, at least. In Fig. 3 the dashed line indicates a summation of individual cell hemolysis plots, which takes on the shape of that observed for the pool. Again, it should be noted at this point that hemoglobin leakage for a single cell is not assumed to be an all-or-nothing response.

Fig. 4 outlines a normal distribution of cells, together with the various time intervals that pertain to the analysis. To fit the experimental data, the time interval Δt may be made arbitrarily short. It was made sufficiently small to allow the calculated plot to fit the experimental plot well enough to be congruent within experimental error. The analysis was generated from the following statements.

First, the profile of hemoglobin leakage^o from a stressed and ruptured cell is considered. The simplest view is consistent with the idea of the red cell having a membrane

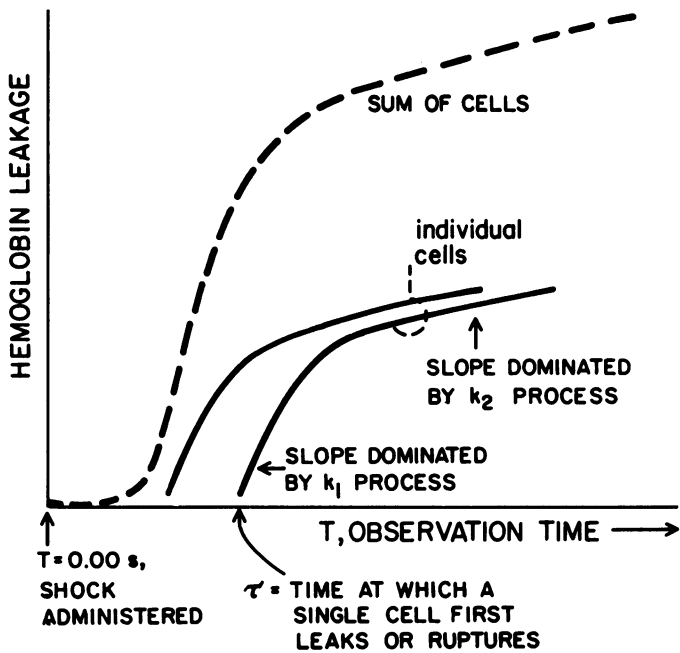


FIGURE 3 Illustration of the role of individual processes for single cells, and the sum of pooled single cells.

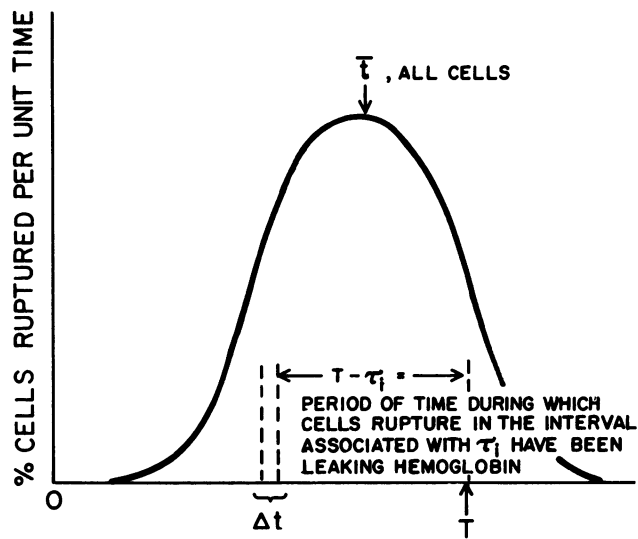


FIGURE 4 Distribution of rupture and hemoglobin leakage appearance, dependent on a population of erythrocytes.

with some amount of tensile strength and possibly elasticity, and a very high concentration of hemoglobin. Hence there are at least two rate processes resulting from two separate driving forces contributing to the hemoglobin leakage. One of these processes with a relatively high rate would be due to the decay upon rupture of hydrostatic pressure built up during the swelling stage. The other contributing rate would relate to the concentration gradient or chemical potential difference of hemoglobin between the inside and outside of the cell. The rate of release of hemoglobin for a single cell in terms of fraction of cell hemoglobin per unit time at an observation time T after shock might then simply be expressed as

$$(dH(\text{single cell})/dt)_{t=T} = f_1(T - \tau) + f_2(T - \tau),$$

where T is the observation time, τ is the time of rupture, and H is the fraction of cell hemoglobin. In the case of a population of cells whose rupture times might be represented by the distribution in Fig. 4, the rate of hemoglobin leakage at the observation time T may be expressed as follows:

$$(dH(\text{population})/dt)_{t=T} = \sum_{i=1}^n N(\tau_i)[f_1(T - \tau_i) + f_2(T - \tau_i)],$$

where $N(\tau_i)$ is the fraction of cells in the population that ruptured during the time interval of duration Δt and designated by τ_i , where $\tau_i = i\Delta t =$ the time elapsed after osmotic shock and $n\Delta t = T$. $N(\tau_i)$ is taken in this analysis to be the area of an interval under a normal distribution of rupture times (Fig. 4). Therefore

$$N(\tau_i) = \left[\frac{1}{\sigma(2\pi)^{1/2}} \exp \frac{-1}{2} \left(\frac{\tau_i - \bar{t}}{\sigma} \right)^2 \right] \Delta t,$$

where Δt is the size of the interval of interest and \bar{t} is the mean rupture time. If the rate expressions are then integrated from τ_i to T for all members of the cell population, the amount of hemoglobin leaked before the observation time T can be obtained. The expression then becomes

$$H_{\text{population}} = \sum_{i=1}^n \frac{\Delta t}{\sigma(2\pi)^{1/2}} \left[\int_{\tau_i}^T f_1(T - X) dX + \int_{\tau_i}^T f_2(T - X) dX \right] \cdot \exp \frac{-1}{2} \left(\frac{\tau_i - \bar{t}}{\sigma} \right)^2,$$

where $X =$ an integration variable between T and τ_i . It was found in attempts to fit the experimental data that exponential expressions were adequate for f_1 and f_2 . Substituting and integrating gives, for the fraction of cell hemoglobin leaked by time T

$$H = \sum_{i=1}^n \frac{\Delta t}{\sigma(2\pi)^{1/2}} \{ [A(1 - \exp[-k_1(T - \tau_i)])] + [(1 - A)(1 - \exp[-k_2(T - \tau_i)])] \} \cdot \exp \frac{-1}{2} \left(\frac{\tau_i - \bar{t}}{\sigma} \right)^2,$$

where k_1 and k_2 are the rate constants for the two processes and A is a normalization factor such that at large τ_i the fraction of hemoglobin leaked approaches unity. Thus A and $1 - A$ are the fractions corresponding to the contributions of each rate process to the total hemoglobin leakage. Under experimental conditions the summed fractions should approach 0.98 at equilibrium. This final expression was then used to fit the experimental data, taking the time intervals Δt small enough that any further decrease caused no observable change in the plot. The parameters $\bar{\tau}$, k_1 , k_2 , and A were then determined for a number of shocking osmotic strengths with normal red blood cells. A calculated plot is shown in Fig. 1, with the solid line as the best fit from the foregoing equation compared to the experimental points.

Parameters from the Fitting Process, as a Function of Osmolarity during Shock.

These are summarized by Figs. 5–8. The error bars are a consequence of the number of repeat experiments (4–12) carried out for each salt concentration. The trends seen are not likely to originate from artifacts which somehow occur because all the data was gathered at once for a single shock osmolarity, nor are the data from that of a particular blood source, as noted above.

Fig. 5, a plot of A vs. osmolarity, represents roughly the percentage of hemoglobin leaked from the k_1 process. Likewise $(100-A)$ is the percentage of leakage associated with the k_2 process. In severe shock, osmolarity ≤ 0.06 , about half the hemoglobin leaks as a result of the fast process, the k_1 process. As one might have expected, a markedly larger fraction is leaked via the k_2 process at the higher osmolarities. In any case both processes have to be accounted for in the analysis over most of the osmolarity range for even semiquantitative accounting. Figs. 6 and 7 plot the values for k_1 and k_2 , respectively. As expected, k_1 is considerably larger than k_2 , sometimes by an order of magnitude. (But it is necessary nevertheless to invoke the k_2 process in all cases,

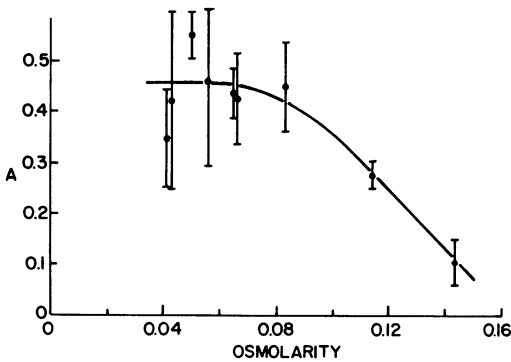


FIGURE 5

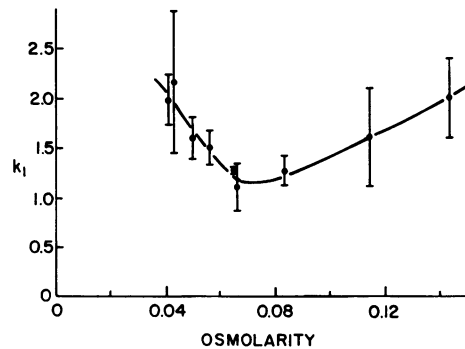


FIGURE 6

FIGURE 5 Course of behavior of the parameter A as a function of osmolarity, 25°C.

FIGURE 6 The k_1 parameters needed to fit the data. Points with smaller error bars represent averages of 6–12 separate experiments and calculations; larger error bars, 4–8 experiments.

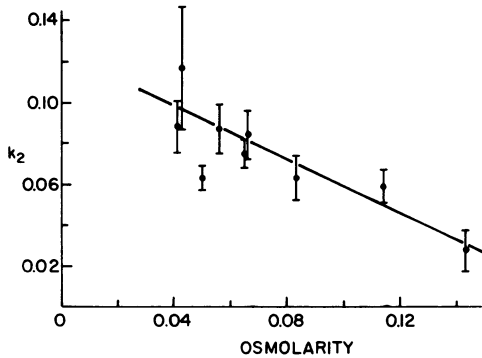


FIGURE 7

FIGURE 7 The slower process, k_2 parameters, consistent with the data.

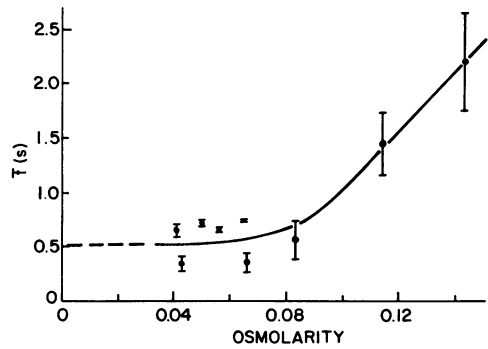


FIGURE 8

FIGURE 8 Mean hemolysis times level out at lower osmolarities of shocking, indicating a limiting rate at which cells can be made to leak, regardless of the severity of shock.

particularly at longer hemolysis times). The biphasic nature of the k_1 parameter is discussed below.

The mean time to rupture, \bar{t} , seems roughly constant from about 0.08 osM, as Fig. 8 shows, and is extrapolated to 0 osM, corresponding to the case wherein the cell is simply shocked with water. (The first cells which leak do release some salt, so that strictly, the salt concentration is not zero throughout, but this effect is small). The \bar{t} value obtained in the limit of zero external salt is about 0.6 s. This is of considerable interest to compare with the related values from Jay and Rowlands' laboratory (1975).

Resealing Rates

An assumption both convenient and necessary is that resealing is very rapid. After the restoring solution is injected, it is desirable that hemolysis stop, i.e., resealing occur, within a time comparable to the instrument resolution time. The literature is quite imprecise about this, with no data for the quite short time frames used here. Although it is difficult to prove that resealing is adequately rapid, the question was approached by the rationale outlined in Fig. 9. Two means of dealing with the problem were used.

First (upper branch of Fig. 9), cells were stressed by low salt in conventional experiments using ordinary equipment, i.e. without the fast hemolysis apparatus. These stressed cells, brought to salt concentrations in the range of 0.071–0.081, were returned to isotonic solution, washed, and then subjected to severe shock with the fast kinetics method.

Second (lower branch of Fig. 9), cells were given a rapid, severe hypotonic shock, and restored with a fairly wide range of salt concentrations, from 0.10 to 0.20. At the lower concentrations of salt in the restoring solution, 0.10–0.14, there is an ongoing but small leakage after restoration. By working rapidly, that effect can be minimized.

If resealing is a major rate-limiting process, variance in the treatment of the mem-

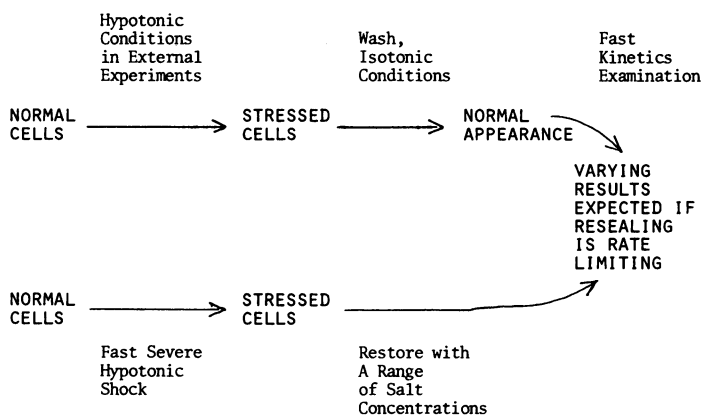


FIGURE 9 Outline of steps used in the resealing rate question.

brane, both before the fast shocking step (upper branch), or after it (lower branch), is expected to give varying final results in a parallel fashion. Since the latter were not observed, our position is that resealing rate variation, or a resealing limitation, is not a major contributor to our overall results.

DISCUSSION

As noted in the introduction, “sigmoid kinetics,” in the sense of a symmetric sigmoid representation for a sample of nonfractionated red cells, probably does not apply.

The often used sequence: $A \xrightarrow{k_1} B \xrightarrow{k_2} C$, with A the intact cell, C the product, and B a hemolyzing intermediate, will not really fit the data, even if the only objection was that A should actually be replaced by a population or distribution of A species (old and young cells). We regard several analyses as having fitted the sigmoid section of the plots (usually when considerably less than maximum stress was imposed on the cells), but the same analyses seldom fit what we call the ramp section. Of course, given the weaknesses and unknowns in any model for such rate processes, we make no claim to have settled the matter.

However, the main features should be compared to values such as mean hemolysis times, observed in other laboratories by means of independent techniques. Jay and Rowlands (1975) carried out cinematography of individual red cells in several hundred cases after shock with various permeants, repeating the work with water only. In the case of neat water, at 22°C, Jay and Rowlands arrived at an average swelling time, T_{sw} , of about 1.2 s. Their swelling time refers to a model for hemolysis quite similar to that invoked here. That is, their swelling time roughly corresponds to our lag time in the case of water shock, and probably also our extrapolated mean hemolysis time from Fig. 8, about 0.6 s. From Fig. 1, the average lag time from simple inspection is also seen to be in the neighborhood of 0.5–1 s, with maximum (neat water) shock.

The 0.5–1.0-s interval compares rather well with an estimate derived from combining the osmotic gradient flux, J_v , with the volume needed to convert the cell from a discocyte to a sphere. The authors are indebted to one of the referees for making note of this. The notation, and the phenomenological coefficient values, are those used by Stein (1967). The volume flow rate caused by the salt concentration difference ΔCs is $J_v = L_p \rho RT \Delta Cs$. The value of $L_p \rho$ for the human red cell is $1.25 \times 10^{-2} \text{ cm s}^{-1}$, or $7.2 \times 10^{-7} \text{ cm}^3 \text{ of flow (cm}^2\text{)}^{-1} \text{ (cm H}_2\text{O pressure)}^{-1} \text{ s}^{-1}$. Upon shocking with neat water under our conditions, the $RT\Delta Cs$ value will build to about $6.6 \times 10^6 \text{ dyn cm}^{-2}$, yielding $J_v \sim 6.2 \times 10^{-5} \text{ cm}^3 \text{ flow (cm}^{-2} \text{ of area) s}^{-1}$. As an approximation, presume that this volume flow might be maintained up to the point of critical hemolytic volume, $1.6 \times$ initial volume, according to Whittam (1964). Combining the foregoing value of J_v with the area of the normal discocyte, about 0.4–0.5 s would elapse before critical volume. Probably J_v will decelerate during swelling, and the elapsed time will be somewhat longer than that. In any case, much of the lag can be assigned to the limitation on the rate at which solvent can get into the cell, assuming $L_p \rho$ is about constant during the discocyte to sphere stage.

A model in which the discocyte to sphere transformation is very rapid, but in which the apparent lag time is set by a hemoglobin diffusion limitation, is quite unlikely. We use diffusion from a sphere as an approximate model and calculate the amount of hemoglobin remaining, via the expression (6.30) from Crank's treatise (1956). In this, we assume r is about $3.5 \times 10^{-4} \text{ cm}$, the diffusion coefficient D is about $7 \times 10^{-7} \text{ cm}^2/\text{s}$ for hemoglobin, the external solution is well stirred, and ratio of volume of solvent to that of the spheres after mixing is about 4. Various time intervals with the amounts of hemoglobin leaked from an open sphere are: 0.001 s, 30%; 0.01 s, 70%; 0.1 s, 99.9%. If the cell has a lower equivalent "porosity" than the open model, leakage would not proceed with such velocities. But it is apparent that any reasonable hemoglobin diffusion model falls considerably short of describing two aspects of the hemolysis curves. That is, the general rates of hemolysis, and more important, the shape of the curves, in fact are not simply exponential.

The behavior of the parameters with decreasing salt concentration is nonlinear, and plots of these parameters characterizing the red cell are often not monotonic: for example, take Fig. 6. Both Fig. 5 and Fig. 6 indicate that in the region of 0.05 M salt, more than one process or phenomenon may occur. Without doubt, as salt concentration decreases from the 0.05 M region, osmotic shock, or hypoosmotic stress, becomes more severe. But there is a good possibility that electrostatic consequences of decreasing salt, i.e. hypotonic stress, from lowered ionic strength, are likely to become manifest. Seaman (1975) gives estimates of the red cell isoelectric pH (and its rather large variation with electrolyte), and also an average value for the separation of charged groups on the red cell surface. The dominant population (97%) of charged groups are anions, with a mean separation of 25–35 Å at physiologic pH. In what is seen from Figs. 5 and 6, where the region 0.05 M salt marks a kind of transition or departure from linearity, one can estimate the reciprocal Debye radius to give a distance at which charges begin to become unshielded. Under our conditions, we have:

$1/\kappa = (3 \times 10^{-8})/\sqrt{c}$, with $1/\kappa$ the Debye distance and c = salt concentration. At 0.05 M salt, the distance is about 13 Å. Although this is smaller than the mean separation given by Seaman, it should be kept in mind that many pairs or groups of charges might be considerably closer than the average separation and that also, the Debye radius is merely a rough average estimate. Summarizing, it seems reasonable that as salt concentration drops, the ionic strength consequences become an important addition to the osmotic consequences, given the electrically charged nature of the red cell surface. The electrostatic effects are probably minor, relative to osmotic effects, at salt concentrations above about 0.10. But around $c = 0.05$ or so, they probably become important, and govern part of the hemolysis rate behavior via membrane stress.

In another paper, we will show how the rate processes change when we start with a spherocyte from patients with hereditary spherocytosis, and from spherocytes produced by means of certain drugs.

The authors are indebted to Drs. Olaf Runquist and Rodney Olson of Hamline University, St. Paul, Minn., for use of the apparatus, and to Gary Hart for considerable technical aid. The advice and correspondence of Dr. A. W. L. Jay was especially helpful to us.

This research was supported by National Institutes of Health grant GM 18807 and a University of Minnesota grant in aid.

Received for publication 13 May 1977.

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