Biochimica et Biophysica Acta, 291 (1973) 165–177

BBA 76159

SOME ASPECTS OF THE OSMOTIC LYSIS OF ERYTHROCYTES
I. A. REEXAMINATION OF THE OSMOTIC LYSIS METHOD

J. M. C. WESSELS, D. T. F. PALS AND J. H. VEERKAMP

Department of Biochemistry, University of Nijmegen, Nijmegen, and Central Laboratory TNO, Delft (The Netherlands)

(Received June 29th, 1972)

SUMMARY

1. A modification of the osmotic lysis method, taking into account differences in osmotic resistance, is introduced. This method allows a more fundamental physicochemical interpretation of the permeability process and the calculation of permeability coefficients.

2. Evidence is presented that the reflection coefficient for glycerol is near to one for human red blood cells as well as for pig red blood cells.

3. The permeability coefficient proved to be independent of differences in lysis behaviour of erythrocytes, which were treated with isotonic solutions of different non-permeants.

4. The osmotic resistance in NaCl solutions is strongly time dependent, whereas in sucrose solutions this parameter is time independent and gives the best approximation of the original osmotic resistance of the red blood cell.

INTRODUCTION

The osmotic lysis method described by Jacobs *et al.*[1] is used frequently in comparative studies of the permeability of erythrocytes[2–4] and other cell types[5,6] for rapidly penetrating substances. Though the time of lysis depends in some way on the permeability, it is clear that the rate of hemolysis may also be affected by the following factors: (a) the surface/volume ratio, which varies with the shape of red blood cells of various mammalian species[7,8]. (b) differences in membrane elasticity, though not observed with human erythrocytes[3]. (c) the loss of osmotic material from the cells during the permeation experiments[9–14]. (d) variations in initial intracellular tonicity[15]. (e) changes in membrane properties induced by the penetrating substances.

Jacobs *et al.*[1], being aware of these factors stated that comparison of hemolysis times obtained with red blood cells from different animals may lead to unreliable permeability data. The importance of these factors has been discussed in a theoretical study of Canham[16] concerning the osmotic fragility of individual human erythrocytes. The aim of our study and the method introduced is to rule out these factors

as far as possible in order to obtain a better estimation of the permeability as such. It proved possible to eliminate the influence of differences in surface/volume ratio on permeability determinations by combining estimations of the osmotic resistance in sucrose solutions used and of the hemolysis rates in solutions of different concentrations of glycerol. Equations are given which describe the lysis process and which can be used to calculate the permeability coefficient. Simplification of the equations by neglecting the reflection coefficient appears to be justified on the basis of the experimental results. It will be shown that the pretreatment of the erythrocytes does not influence markedly the glycerol permeability coefficient, determined by the modified osmotic lysis method, though considerable differences in lysis pattern are observed.

MATERIALS AND METHODS

Erythrocytes were isolated by centrifugation (10 min, 1000 × *g*) from fresh heparinized or defibrinated blood. The buffy coat was removed and the erythrocytes either resuspended in serum, or treated four times with a 5- to 10-fold excess of 300 mM sucrose or 150 mM NaCl solution, both solutions being buffered with 1 mM sodium phosphate to pH 7.5. "Reagent grade" chemicals, obtained from Merck, were used. The osmolarity of the test solutions in mosmoles/l was calculated from the specific gravity and the osmolality estimated from freezing-point depression with an Advanced Instruments osmometer.

Lysis experiments

With a microsyringe 0.05 ml of the erythrocyte suspension, hematocrit 0.4–0.5, was added in duplicate to 10-ml samples of buffered solutions of isotonic or hypotonic NaCl, sucrose or the permeating substance glycerol. Buffer composition and pH were as described above. All solutions were kept at 37 °C. After addition of the erythrocyte suspension the test tubes were shaken immediately and placed again into a bath of 37 °C. After various time intervals hemolysis was stopped by adding 1 ml 1.5 M NaCl, whereafter the non-hemolysed cells were removed by centrifugation (10 min, 1000 × *g*). The absorbance of the supernatant was measured at 540 nm and found to be proportional to the amount of hemoglobin liberated. When in the following the term "50 % hemolysis" is used, this means that the absorbance of the supernatant relative to that of a fully hemolysed preparation (A/A_{max}) is 0.5.

Determination of the mean critical cell volume (V_h)

Per ml erythrocytes resuspended in serum (hematocrit 0.4–0.5) was added 0.05 μCi [*carboxy*-^{14}C]inulin obtained from Radiochemical Center, Amersham, and dissolved in 0.05 ml 300 mM unbuffered sucrose. Thereafter 1 ml unbuffered sucrose of different osmolarity was added to 1-ml samples. After mixing, the samples were centrifuged at once (10 min, 1000 × *g*). The radioactivity was determined in 0.2 ml of the supernatant added to 10 ml Instagel (Packard Instrument Co.), with a Packard Tri-Carb liquid scintillation spectrometer Type 3380, equipped with an Absolute Activity Analyzer Model 544. The activities were compared with the activity of a standard dilution of [^{14}C]inulin. The total cellular volume was calculated from these data. The average erythrocyte volume was calculated from the cell count

made with a Coulter Counter Model B, equipped with a 50-μm aperture tube, and from the total cellular volume. The average erythrocyte volumes were plotted against the corresponding osmolarities determined in a parallel experiment without added [^{14}C]inulin. An approximate value for the average critical cell volume (V_h) was found by extrapolation to the sucrose concentration causing 50% hemolysis.

THEORY

Our studies are based on the assumption that erythrocytes normally behave like perfect osmometers, though this is still questionable[17–19] and on the fact that the exchange of water takes place rapidly[19–22]. Therefore the osmotic equilibrium of erythrocytes with its environment will be established almost instantaneously. Taking account of this, it appeared to be possible to estimate erythrocyte permeability more exactly by combining osmotic resistance and permeability measurements. Outside the erythrocytes the osmolar concentration (further called concentration) of permeant or non-permeant can be regarded as remaining constant due to the low ratio of erythrocyte volume/medium volume, used in our studies. Confining ourselves to the case of one penetrating non-electrolyte, *e.g.* glycerol, being present outside the cell, the concentration of this substance will be denoted by m_p. The total extracellular concentration of non-permeating substance will be assumed to be equivalent to m_n. The sum of concentrations outside the cell is thus:

$$m_p + m_n = m_c \text{ (osmoles/l)} \tag{1}$$

The quantities m_p, m_n and m_c do not depend on time, nor on the condition of the cells. Intracellularly the situation is different. As a result of previous treatments with an isotonic solution of non-permeating substance, all permeating substances have been removed, so that at the start of the experiment only non-permeating components will be present. After a while, however, due to diffusion through the cell membrane, some penetrating substance will have entered, the intracellular concentration of which is denoted as X. Since the erythrocyte membrane is readily permeable to water[19–22], water equilibrium is established within milliseconds. This means that the total osmotic activity on both sides of the membrane can always be taken as equal. Since the strength of the erythrocyte membrane is negligibly small[23] no pressure differences will be built up. So the variable volume of the erythrocyte adapts itself rapidly to each new situation, in such a way that the osmotic activities inside and outside the cell remain equal to each other. In consequence we may write for the concentration inside the cell:

$$m_c = X + \frac{N}{V} \tag{2}$$

with X the concentration of permeating substance in the cell, depending on time; V the volume of the cell depending on time, and N the concentration of non-permeating components in the cell, which can be considered to be constant. N, V and X may be somewhat different for individual erythrocytes of the population due to variations in age and consequently in properties[24,25]. Thus Eqn 2 is only valid for each fraction f of the cell population:

$$m_c = X(f) + \frac{N(f)}{V(f)} \tag{2f}$$

For the sake of simplicity we shall omit the subscript *f* in the following derivations. Assuming that diffusion through the cell membrane obeys the first law of Fick, which states that the amount of transported material is proportional to the difference in concentration, one obtains:

$$\frac{d(XV)}{dt} = P \cdot S\,(m_p - X) \tag{3}$$

in which V and S are the volume and the surface area of the erythrocyte respectively, and P the permeability coefficient.

This equation is, however, overly simplified. On the basis of irreversible thermodynamics, Staverman[26] stated that interactions must take place between solute and solvent permeating through the same channels. These interactions are represented by the reflection coefficient σ, which is defined as the ratio of the actual osmotic pressure to the theoretical van 't Hoff osmotic pressure. Eqn 3 must therefore be replaced by a more general expression, *e.g.* that given by Stein[27]. Using our symbols, this expression reads:

$$\frac{d(XV)}{dt} = S\left\{(1-\sigma)\frac{m_p + X}{2}\cdot\frac{1}{S}\cdot\frac{dV}{dt} + P(m_p - X)\right\} \tag{4}$$

Values for the reflection coefficient of glycerol are only published for human erythrocytes which have a relatively high permeability to glycerol. Goldstein and Solomon[28] found experimentally a σ value of 0.88 by the "zero time method". Stein[27] calculated a σ value of 0.995–0.997 from permeability data which were obtained, however, in the presence of volume flow. When the water permeability of the erythrocyte is high compared to that of the permeant, the permeability coefficient will certainly not differ by an order of magnitude in the presence or absence of water flow[29]. Sha'afi *et al.*[29] obtained experimental evidence that the permeability coefficient of urea in human erythrocyte decreases with increasing solvent flow. Sha'afi *et al.*[30] concluded that there was a surprisingly good agreement between the permeability coefficients computed from hemolysis times[30] and those obtained using rapid reaction techniques. But they argued that it is not possible to combine these data, which differ by an order of magnitude, into a single ratio as was done by Stein[27]. Phenomenologically the permeability coefficient and the reflection coefficient are entirely independent parameters[32]. The frictional treatment[33] cannot be applied to the red blood cell membrane, because the diffusion coefficient in water does not bear a predictable relationship to the solute-water friction in the membrane[29]. The following points make the assumption acceptable that the reflection coefficient may be neglected in our permeability studies:

(1) As will be shown in the results (Table I) the determined permeability coefficient for glycerol is not, or only to a very small degree, dependent on the extracellular glycerol concentration. On theoretical grounds, the reflection coefficient must depend to some extent on the concentration[27,29]. Since the determined permeability coefficient is virtually independent of the concentration, one may take the reflection coefficient to be almost one. Sha'afi *et al.*[29] and Savitz and Solomon[34] also suggested that the effect of solute concentration on the permeability coefficient is relatively unimportant[30].

(2) Since the reflection coefficient is equal to the ratio of the observed osmotic

pressure and the theoretical osmotic pressure, a given concentration of permeant molecules never exerts its full osmotic effect, even at $t = 0$ when no molecules have penetrated the membrane[28]. Extrapolation of our permeation data at various moments to $t = 0$, leads to a concentration of permeant causing a distinct degree of lysis which cannot be distinguished from the concentration of non-permeant causing the same degree of lysis.

(3) Recent investigations of Macey and Farmer[35] suggest that in human erythrocytes water and glycerol do not share the same pathway through the membrane.

We may, therefore, conclude that in our comparative studies of erythrocyte membrane permeability to glycerol σ is about one and Eqn 3 holds.

Hemolysis of fraction f of the erythrocyte population occurs, whenever $V(f)$ exceeds a critical value $V_h(f)$, which value may depend on the composition of the solutions on both sides of the membrane. Our experiments indicate that the resistance towards non-permeating sucrose is quite different from that towards non-permeating NaCl. Thus in general the values for $m_n^h(f)$ and $V_h(f)$ — the concentration of non-permeant leading to lysis of fraction f — obtained with NaCl or sucrose, cannot simply be applied to permeation experiments.

We will consider three cases: (a) $m_n \neq 0$, $m_p = 0$; (b) $m_n = 0$, $m_p \neq 0$ and (c) $m_n \neq 0$, $m_p \neq 0$. In our experiments only the first two cases have been studied.

(a) *Presence of only non-permeating substances*

In this case, as a result of $m_p = 0$, also $X = 0$ for all cells, there is no time dependence, a fraction f is either lysed or not. Eqn 2f reads, if fraction f is lysed,

$$m_n^h(f) \leqq \frac{N(f)}{V_h(f)} \tag{5}$$

(b) *Presence of only permeating substance*

Substituting Eqns 2 and 3 and eliminating X we find, with omission of subscript f:

$$V\mathrm{d}V = \frac{P \cdot S \cdot N}{m_p}\,\mathrm{d}t$$

Integration between the limits $t = 0$ and $t = t_h$, where t_h is the time of hemolysis of the fractions considered, gives:

$$V_h^2 - V_0^2 = \frac{2P \cdot S \cdot N}{m_p} \cdot t_h \tag{6}$$

provided that S does not depend on V or on t.

Considering further that at $t = 0$, $X = 0$, with Eqn 2 $V_0 = N/m_c = N/m_p$ and Eqn 5 $N = m_n^h \cdot V_h$ we find:

$$\frac{m_p}{m_n^h} - \frac{m_n^h}{m_p} = 6 \cdot \frac{P}{R_h} \cdot t_h \tag{7}$$

where we have written $S/V_h = 3/R_h$.

Eqn (7) gives a relation between the hemolysis time t_h, and m_p and m_n^h, all for one particular fraction f.

(c) *Presence of permeating and non-permeating substances*

In the same way, by elimination of X from Eqns 2 and 3 and integration, we find for a particulate fraction f:

$$\frac{m_c - m_n^h}{m_n} - \frac{m_n^h \cdot m_c}{m_n^2} \ln \frac{m_n^h(m_c - m_n)}{m_c(m_n^h - m_n)} = 3\frac{P}{R_h} \cdot t_h \qquad (8)$$

We decided to determine the liberation of hemoglobin instead of following turbidity changes, since the last method is criticized frequently[36–38]. The absorbance of the supernatant was determined after removing the intact cells by centrifugation. It should be mentioned here that the individual erythrocytes in the population may differ in their hemoglobin content[39–40]. The consequence may be that the relative absorbance (A/A_{max}) may not be considered as a measure of the percentage of lysed cells. Since we studied the lysis rate of small fractions of the total erythrocyte population, this fact has no important consequences.

Considering Eqn 5 it will be clear that by plotting the relative extinction of the hemoglobin in the supernatant (A/A_{max}) as a function of m_n, we should obtain a curve which shows the total fraction of cells with values of $N/V \geqslant m_n$ as a function of m_n. All cells can be arranged according to the value of N/V_h, so that for a given value of A/A_{max} the same fraction of cells (those with N/V_h values just a little bit larger than the given values) hemolyse. Thus the experimental plot of A/A_{max} *versus* m_n^h is at the same time a plot of percentage of hemoglobin contained in cells with $N/V > m_n^h$. Eqns 7 and 8 show that from measurements of A/A_{max} as a function of time, for systems with and without permeating substances, the constant P/R_h of substances through erythrocyte membranes can be calculated quantitatively. Since R_h can be determined from the critical cell volume, the permeability coefficient can be estimated.

RESULTS AND DISCUSSION

The results of the lysis experiments are presented in the form of graphs in which the relative absorbance (A/A_{max}) of the solutions, obtained after removal

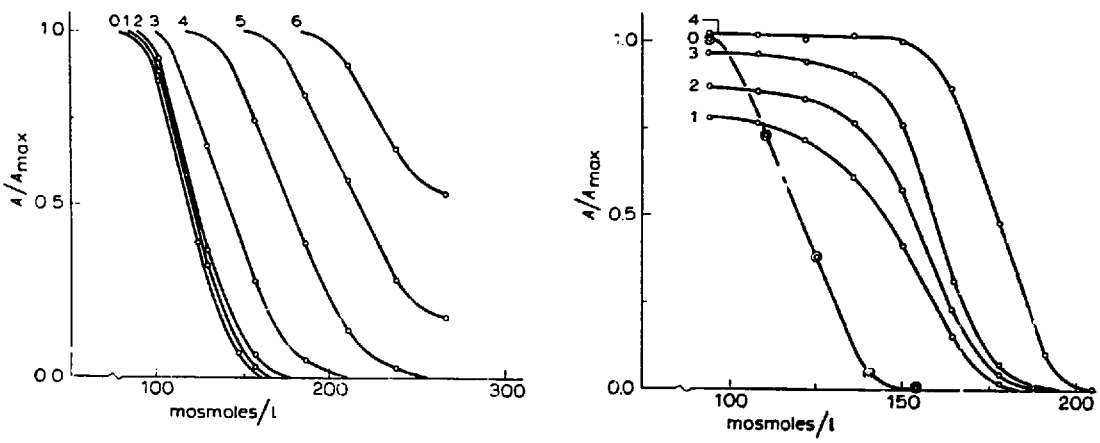
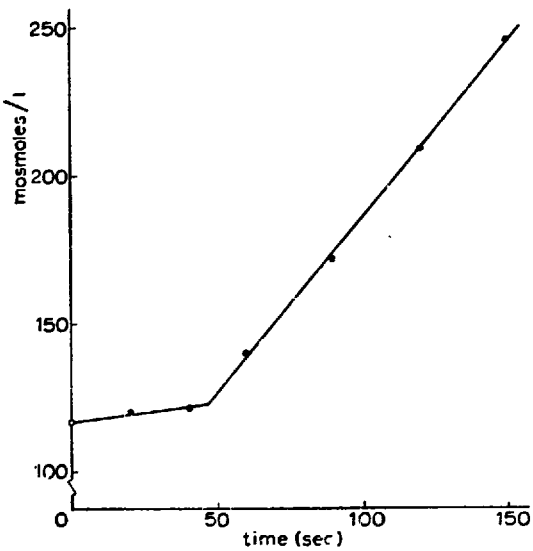


Fig. 1. The lysis of untreated pig red blood cells in sucrose and in glycerol. Curve 0 represents the osmotic resistance in sucrose after 300 s, Curves 1–6 the lysis in glycerol after 20, 40, 60, 90, 120 and 150 s, respectively.

Fig. 2. Osmotic resistance of pig erythrocytes. Curve 0: sucrose-treated cells in hypotonic sucrose solutions after 5 and 300 s. Curves 1–4. NaCl-treated cells in hypotonic NaCl solutions after 5, 10, 15 and 300 s, respectively.

of the unhemolysed cells, is plotted against the concentration of the test solution in mosmoles/l. All lysis curves appeared to be S-shaped which means that the erythrocytes were not all identical. It seems reasonable to assume that at each level of absorbance a distinct fraction of the erythrocyte population is lysed[24,25,39]. Fig. 1 shows the lysis curves of erythrocytes, resuspended in serum, when these were added to solutions of sucrose or glycerol. The lysis Curve 0 represents the osmotic resistance of the erythrocyte population in hypotonic sucrose solutions, whereas the Curves 1–6 represent the degree of lysis in glycerol solutions of varying tonicity at various moments. On account of the theoretical considerations it may be assumed that the lysis curves in hypotonic sucrose solutions near $t = 0$ and at $t = 300$ s were the same, since water equilibrium is established almost momentarily[19–22]. Sucrose was chosen as the non-permeant because the permeability experiments were also performed with a non-electrolyte. Moreover the osmotic resistance of pig erythrocytes determined in NaCl solutions appeared to be strongly time-dependent (Fig. 2). This observation agrees with the results of Bowdler and Chan[41], obtained with human red blood cells.

The most striking fact observed in the experiments of Fig. 1 was, that hardly any increase was seen in a rather long period of time after $t = 0$. The osmotic resistance curve in sucrose solutions and the lysis curves in glycerol solutions after 20 and 40 s nearly coincided. The 50% hemolysis values, plotted against time, once more illustrate this observation (Fig. 3). Since it may be assumed that the lysis in hypotonic sucrose would be complete almost instantaneously, the 50% hemolysis value in mosmoles/l for sucrose is plotted at $t = 0$. According to the theoretical considerations an ever-increasing number of erythrocytes should lyse due to a gradual increase of the intracellular glycerol concentration. It was observed that in sublytic glycerol concentrations the erythrocyte population was almost instantaneously lysed to a certain degree. Though it was expected that lysis would continue due to



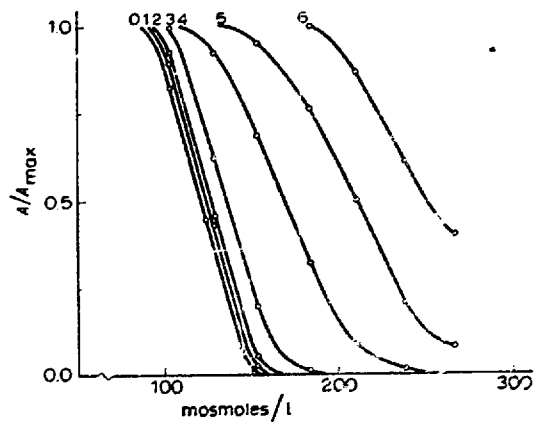


Fig. 3. 50% hemolysis values in mosmoles/l of untreated pig red blood cells in glycerol (●) and in sucrose (○) solutions as a function of time.

Fig. 4. The lysis of NaCl-treated pig erythrocytes in sucrose and glycerol solutions. Curve 0 represents the osmotic resistance in sucrose at $t = 300$ s, Curves 1–6 the lysis in glycerol after 20, 40, 60, 90, 120 and 150 s, respectively.

a gradual increase of the intracellular glycerol concentration, lysis appeared to be retarded considerably, for which phenomenon the term "delayed lysis" is proposed. A possible explanation for the delayed lysis could be the gradual loss of osmotically active substances from the erythrocytes in the beginning of the experiment, compensating for the increase of the intracellular glycerol concentration. This would result in a temporary constancy or decrease of the osmotic activity in the erythrocyte. As soon as the loss of permeating substances from the cells would be complete, the lysis would proceed due to the penetration of glycerol molecules. To check this hypothesis erythrocytes were treated with 150 mM NaCl prior to the lysis experiments in order to remove permeating substances from the cell contents. The results are shown in Fig. 4. Lysis Curve 0 represents the osmotic resistance in sucrose solutions. Curves 1–6 represent lysis in glycerol solutions at various time intervals. The delay of lysis is equal to, or even larger than in the experiment with untreated red blood cells. The 50% hemolysis values as a function of time once more confirm these conclusions.

Since the delay of lysis in glycerol solutions was not only detected with untreated erythrocytes but also, and even more pronounced, with erythrocytes treated with 150 mM NaCl, this phenomenon cannot simply be explained from a gradual loss of osmotically active substances from the red blood cell. It seemed likely that intracellular osmotically active electrolytes originating from serum or NaCl solution, acting in or at the cell membrane, contribute to the delay of lysis. In order to test this assumption the osmotic resistance in sucrose and the lysis rate in glycerol solutions were determined for erythrocytes previously treated with 300 mM sucrose (Fig. 5). The delay of lysis was absent under these conditions, indicating that an ever increasing number of red blood cells lysed due to the gradual increase of the intracellular glycerol concentration. The 50% hemolysis values plotted against time, confirm the foregoing statement (Fig. 6). The values in glycerol showed a

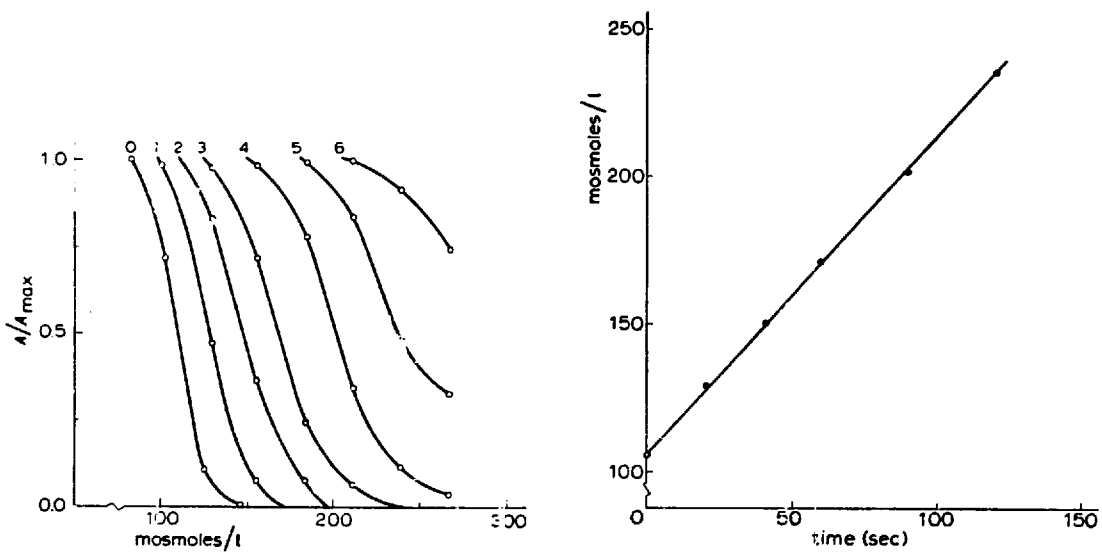


Fig. 5. The lysis of pig erythrocytes treated with 300 mM sucrose in sucrose and glycerol solutions, respectively. Curve 0: osmotic resistance in sucrose at t = 300 s. Curves 1–6: lysis in glycerol after 20, 40, 60, 90, 120 and 150 s, respectively.

Fig. 6. 50% hemolysis values in mosmoles/l of pig erythrocytes treated with 300 mM sucrose in glycerol (●) and in sucrose (○) solutions as a function of time.

straight line relationship. Extrapolation to $t = 0$ gives an m_p value (the concentration of permeating glycerol causing, due to water flow only, 50% hemolysis at about $t = 0$) fitting very well with the experimental m_n^h value (the concentration of non-permeating sucrose causing 50% hemolysis instantaneously due to water flow only). This justifies the conclusion that the reflection coefficient for glycerol, penetrating into pig erythrocytes is very near to one and may be neglected in the mathematical considerations.

Pig erythrocytes, however, are rather poorly permeable to glycerol in contrast to those of man, rat and rabbit. Under comparable circumstances the lysis behaviour of human red blood cells is in agreement with theoretical predictions and the reflection coefficient does not deviate very much from one. Human erythrocytes were treated with an excess of buffered sucrose. The pretreatment was not repeated, since it was observed that human red blood cells loose cations and anions in non-electrolyte solutions[9–13,42,43]. The lysis behaviour of these pretreated erythrocytes was studied in buffered sucrose and glycerol solutions (Fig. 7). Lysis Curve 0 represents the osmotic resistance of the erythrocyte population in sucrose solutions, whereas Curves 1–7 represent the degree of lysis in glycerol solutions. Obviously no lysis delay takes place, as was also observed for pig red blood cells (Fig. 5). The 50% hemolysis values plotted *versus* time confirm this statement (Fig. 8). In this case all essential conditions for a mathematical analysis of the lysis data appeared to be fullfilled.

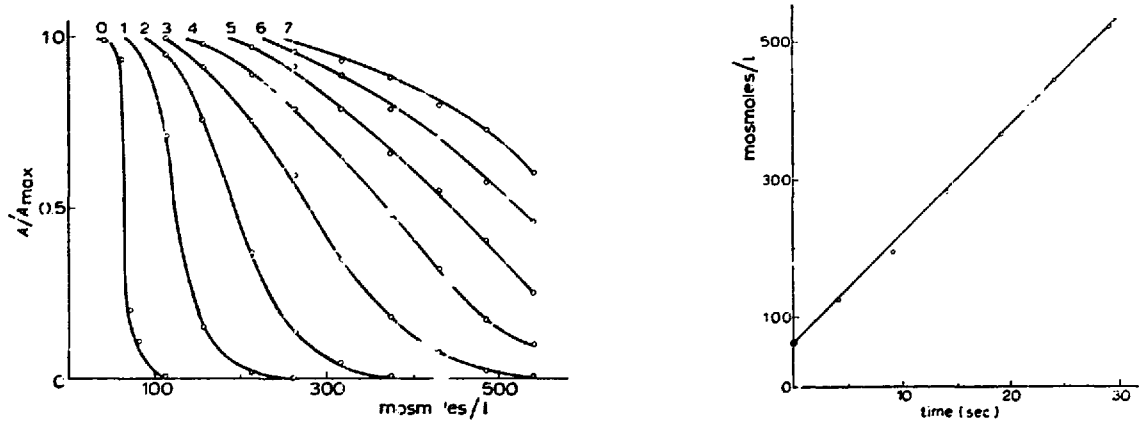


Fig. 7. Lysis of sucrose-treated human red blood cells in sucrose and in glycerol solutions. Human erythrocytes were pretreated once with a 10-fold volume of 300 mM sucrose, buffered with 5 mM sodium phosphate to pH 7.5. Curve 0 represents the osmotic resistance in sucrose after 300 s, Curves 1–7 the lysis in glycerol after 4, 9, 14, 19, 24, 29 and 34 s, respectively.

Fig. 8. 50% hemolysis values in mosmoles/l of human erythrocytes treated with 300 mM sucrose in glycerol (○) and in sucrose (●) solutions as a function of time.

COMPARISON OF THEORY AND RESULTS

If the assumption is true that at each absorbance level a distinct fraction of the erythrocyte population is lysed, the theoretical Eqn 7;

$$\frac{m_p}{m_n^h} - \frac{m_n^h}{m_p} = 6\,\frac{P}{R_h}\cdot t_h$$

must be valid for each absorbance level representing a uniform fraction of red blood cells. Therefore the Curves 1–6 of Fig. 5, referring to lysis in glycerol solutions of pig erythrocytes treated with 300 mM sucrose, may be described by this equation, since glycerol is the only solute present extracellularly. The osmotic contribution of 1 mM sodium phosphate buffer may be neglected. Another consequence of the above assumption is that the difference in glycerol concentration between the interior of the cells and the surrounding solution must be equal to m_n^h at the moment of lysis. This concentration difference must be the same for all time curves at a distinct absorbance level, provided that the same red blood cell suspension is used.

In order to compare theory and experimental data, pairs of points of different time curves representing the same relative absorbance, were selected from Fig. 5. The following equation can be derived for such pairs from Eqn 7 when $t_2 = n \cdot t_1$:

$$n\left(\frac{m_{p_1}}{m_n^h} - \frac{m_n^h}{m_{p_1}}\right) = \left(\frac{m_{p_2}}{m_n^h} - \frac{m_n^h}{m_{p_2}}\right) \text{ and thus; } m_n^h = \left(\frac{n m_{p_1} - m_{p_2}}{\frac{n}{m_{p_1}} - \frac{1}{m_{p_2}}}\right)^{\frac{1}{2}} \qquad (9)$$

Pairing the data of all six curves permits the calculation of a mean value for m_n^h (actually the value of m_p at $t = 0$) for each fraction corresponding to a given relative absorbance. The results of such a calculation are given in Table I. The fact that the

TABLE I

COMPARISON OF THE OSMOTIC RESISTANCE IN SUCROSE AND THE CALCULATED m_p VALUES IN GLYCEROL AT ZERO TIME

The m_p (± S.D.) values in glycerol solutions at $t = 0$ were calculated by means of Eqn 9 at three absorbance levels. These values and the experimentally found critical sucrose tonicities (m_n^h) are given in mosmoles/l.

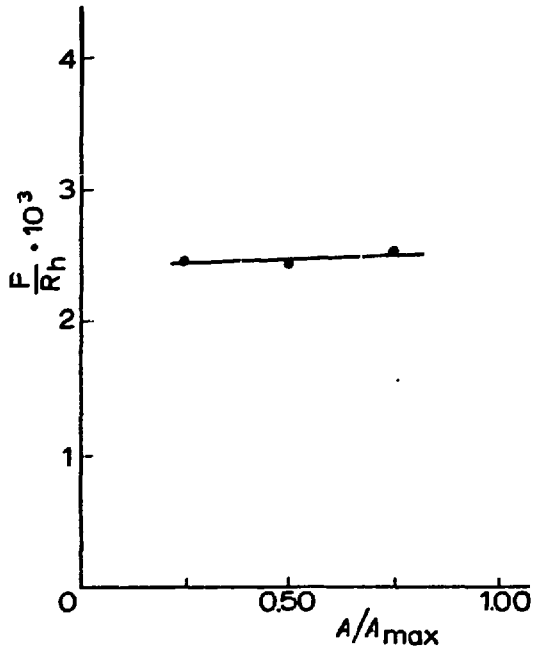
A/A_{max}	m_p at $t(x)$ s				mean m_p at $t = 0$	m_n^h
	20	40	60	90	(calc.)	(exp.)
0.25	138	161	184	222	119.8 ± 1.9	119
0.50	128	147	170	204	110.8 ± 1.9	110
0.75	116	136	155	187	100.7 ± 2.9	100

calculated m_p values at $t = 0$ and the experimental values for m_n^h, obtained from resistance measurements in sucrose fit the same curve, renders additional support to the theory. Extrapolation of the 50% hemolysis values in glycerol at various times to $t = 0$ (Fig. 6), also confirms this statement.

The values of P/R_h calculated from Curves 0–6 in Fig. 5 with Eqn 7 are plotted in Fig. 9 as a function of the relative absorbance. The P/R_h ratios did not appear to differ considerably for the different fractions of the red blood cell population. An average value for R_h can be calculated from the determined average value of V_h. The value for R_h amounted to $2.85 \cdot 10^{-4}$ cm. The average glycerol permeability coefficient at pH 7.5 and 37 °C of pig erythrocytes amounts to $6.60 \cdot 10^{-7}$ cm·s^{-1} (S.D. ± $0.66 \cdot 10^{-7}$ cm·s^{-1}, n = 8) at the 50% lysis level.

The lysis behaviour of untreated red blood cells or red blood cells, treated with 150 mM NaCl, was more complex. During the first 40–60 s the lysis in hypotonic glycerol solutions proceeded very slowly (Figs 1 and 4). Thereafter, however, lysis

proceeded at nearly the same rate as that observed with 300 mM sucrose-treated erythrocytes (Fig. 10). Since after 60 s lysis in glycerol solutions proceeded almost independently of the pretreatment, these observations suggest that erythrocyte membrane permeability to glycerol in our circumstances hardly depends on the



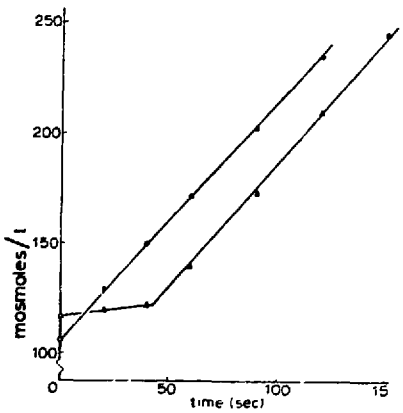


Fig. 9. Ratio of permeability coefficient and critical radius of sucrose-treated pig erythrocytes, *versus* the relative absorbance.

Fig. 10. 50% hemolysis values in mosmoles/l of untreated and 300 mM sucrose-treated pig red blood cells in sucrose (△ and ○, respectively) and glycerol solutions (▲ and ●, respectively) as a function of time.

pretreatment. This statement is, as will be shown, supported by the permeability coefficients calculated from experimental data.

From the m_p values, obtained at $t \geq 60$ s, the m_p value at $t = 0$ can be calculated with Eqn 9, or estimated by extrapolation to $t = 0$. In the ideal case the m_p value should be almost equal to m_n^h, as found for sucrose-treated red blood cells. This does not always hold true, as shown by Fig. 10. One cause of the difference between the experimentally found m_n^h value and the m_p value at $t = 0$ might be, that a marked substitution of chloride by phosphate has taken place in erythrocytes which were treated with buffered 300 mM sucrose whereas, in glycerol permeation experiments at time "zero", chloride has only exchanged from erythrocytes against hydroxyl. In the next paper[44] the lysis delay will be explained from the exchange of intracellular chloride with extracellular hydroxyl ions coupled with the buffering action of hemoglobin. This phenomenon results in a decrease of the intracellular osmotic activity and hence in mean cell volume and increase of osmotic resistance. Upon extensive and prolonged treatment of erythrocytes with 300 mM sucrose buffered with 10 mM sodium phosphate (pH 7.5), intracellular chloride ions are principally replaced by phosphate and only to some extent by hydroxyl ions. Intracellular osmotic activity, therefore, will be decreased, though less than in the case of complete chloride–hydroxyl ion exchange. Experimental evidence was obtained

by measuring the osmotic resistance of erythrocytes which were treated with citrate-buffered 300 mM sucrose. In this case only chloride–hydroxyl ion exchange takes place due to the impermeability of the red cell membrane to citrate. The osmotic resistance of these erythrocytes was markedly higher than that of erythrocytes treated with phosphate-buffered sucrose but was still not quite equal to the value of m_p found by extrapolation to $t = 0$.

The difference between m_n^h and m_p at $t = 0$ did not appear to affect significantly the permeability data. The glycerol permeability coefficient was determined in comparative studies to check the validity of the mathematical description in the case of untreated, 300 mM sucrose and 150 mM NaCl-treated pig blood cells. From the lysis curves after 60 s and longer, the m_p value in mosmoles/l at $t = 0$ was calculated with Eqn 9. With this m_p value at $t = 0$ and the m_p values after different periods of time, the permeability coefficient to critical radius ratio can be calculated with Eqn 7. The critical radius amounted to $2.85 \cdot 10^{-4}$ cm. Obviously only small variations in glycerol permeability coefficients were found after the different pretreatments (Table II). These results show that it is justified to simplify the permeability determinations, since after a certain moment lysis proceeds independently of the pretreatment. Therefore, the determinations of the glycerol permeability coefficient for red blood cells from different mammalian species were performed with untreated red blood cells[45].

TABLE II

COMPARISON OF GLYCEROL PERMEABILITY COEFFICIENTS OBTAINED WITH DIFFERENTLY TREATED PIG RED BLOOD CELLS

Pig red blood cells untreated or treated with 300 mM sucrose or 150 mM NaCl were added to glycerol solutons. All solutions were buffered with 1 mM sodium phosphate to pH 7.5. The permeability was determined at 37 °C. The glycerol permeability coefficient is given in $cm \cdot s^{-1} \cdot 10^6$.

Expt	*Treatment*		
No.	*Untreated*	*300 mM sucrose*	*150 mM NaCl*
1	—	0.63	0.67
2	0.68	0.65	0.68
3	0.72	0.68	0.70
4	0.55	0.55	0.51
5	0.72	—	0.70

ACKNOWLEDGEMENTS

The theoretical part of this study was performed at the Central Laboratory TNO, Delft, and was sponsored by Abbott Laboratories, North Chicago, Illinois. We are grateful for obtaining permission for publication. The excellent technical assistance of Mrs A. van Bakel-Negrijn, Mrs Th. E. van Kesteren-Tijdeman and Mrs J. Slotboom-Luyendijk was very appreciated.

REFERENCES

1 M. H. Jacobs, H. N. Glasmann and A. K. Parpart, *J. Exp. Zool.*, 113 (1950) 277.
2 J. de Gier, L. L. M. van Deenen and K. G. van Senden, *Experientia*, 22 (1966) 20.
3 T. J. Moore, *J. Lipid Res.*, 9 (1968) 542.

4 J. G. Szelényi and R. J. Hollan, in E. Deutsch, E. Gerlach and K. Moser, *Stoffwechsel und Membranpermeabilität von Erythrozyten and Thrombozyten*, G. Thieme Verlag, Berlin, 1968, p. 440.
5 W. E. Love, *Biol. Bull.*, 105 (1953) 128.
6 B. Lucké, H. S. Hempling and J. Makler, *J. Cell. Comp. Physiol.*, 47 (1956) 1070.
7 M. H. Jacobs and S. A. Corson, *Biol. Bull.*, 67 (1934) 325.
8 R. Whittam, *Transport and Diffusion in Red Blood Cells*, Arnold Ltd, London, 1964, p. 2.
9 M. H. Jacobs and A. K. Parpart, *Biol. Bull.*, 65 (1933) 512.
10 M. H. Jacobs, A. K. Parpart and S. A. Corson, *J. Cell. Comp. Physiol.*, 9 (1937) 177.
11 W. Wilbrandt, *Arch. Ges. Physiol.*, 243 (1940) 537.
12 P. La Celle and A. Rothstein, *J. Gen. Physiol.*, 50 (1966) 171.
13 J. A. Donlon and A. Rothstein, *J. Membrane Biol.*, 1 (1969) 37.
14 D. Cotterell and R. Whittam, *J. Physiol.*, 214 (1971) 509.
15 E. G. Olmstead, *Mammalian Cell Water. Physiologic and Clinical Aspects*, Lea and Febiger, Philadelphia, 1966, p. 55.
16 P. B. Canham, *J. Cell Physiol.*, 74 (1969) 203.
17 R. Ege, *Biochem. J.*, 21 (1927) 967.
18 H. L. White and D. Rolf, *Am. J. Physiol.*, 202 (1962) 1195.
19 D. A. T. Dick, in E. E. Bittar, *Membranes and Ion Transport*, Vol. 3, John Wiley and Sons, London, 1971, p. 21.
20 C. V. Paganelli and A. K. Solomon, *J. Gen. Physiol.*, 41 (1957) 259.
21 R. M. Blum and R. E. Forster, *Biochim. Biophys. Acta*, 203 (1970) 410.
22 R. E. L. Farmer and R. I. Macey, *Biochim. Biophys. Acta*, 196 (1970) 53.
23 R. P. Rand, *Biophys. J.*, 4 (1964) 303.
24 D. J. O'Connell, C. J. Caruso and M. D. Sass, *Clin. Chem.*, 11 (1965) 771.
25 R. J. Weed and J. A. Bowdler, *Blood*, 29 (1967) 297.
26 A. J. Staverman, *Rec. Trav. Chim.*, 70 (1951) 344.
27 W. D. Stein, *The Movement of Molecules across Membranes*, Academic Press, New York, 1967, p. 47.
28 D. A. Goldstein and A. K. Solomon, *J. Gen. Physiol.*, 44 (1960) 1.
29 R. I. Sha'afi, G. T. Rich, D. C. Mikulecky and A. K. Solomon, *J. Gen. Physiol.*, 55 (1970) 427.
30 R. I. Sha'afi, C. M. Gary-Bobo and A. K. Solomon, *J. Gen. Physiol.*, 58 (1971) 238.
31 R. Höber and S. L. Ørskov, *Pflügers Arch. Ges. Physiol.*, 231 (1933) 599.
32 R. Di Polo, R. I. Sha'afi and A. K. Solomon, *J. Gen. Physiol.*, 55 (1970) 63.
33 A. Katchalsky and P. F. Curran, *Non-Equilibrium Thermodynamics in Biophysics*, University Press, Cambridge, Mass., 1965, p. 113.
34 D. Savitz and A. K. Solomon, *J. Gen. Physiol.*, 58 (1971) 259.
35 R. I. Macey and R. E. L. Farmer, *Biochim. Biophys. Acta*, 211 (1970) 104.
36 P. Latimer, D. M. Moore and F. Bryant, *J. Theor. Biol.*, 21 (1968) 348.
37 F. D. Bryant, P. Latimer and B. A. Seiber, *Arch. Biochem. Biophys.*, 135 (1969) 109.
38 A. L. Koch, *Anal. Biochem.*, 38 (1970) 252.
39 P. A. Marks and A. B. Johnson, *J. Clin. Invest.*, 37 (1958) 1542.
40 A. F. W. Morselt and J. James, *Blut*, 23 (1971) 25.
41 A. J. Bowdler and T. K. Chan, *J. Physiol.*, 201 (1969) 437
42 M. Maizels, *Biochem. J.*, 29 (1935) 1970.
43 H. Davson, *Biochem. J.*, 33 (1939) 389.
44 J. M. C. Wessels and J. H. Veerkamp, *Biochim. Biophys. Acta*, 291 (1973) 178.
45 J. M. C. Wessels and J. H. Veerkamp, *Biochim. Biophys. Acta*, 291 (1973) 190.