J. Membrane Biol. 15, 107–136 (1974)

Permeability of the Bovine Red Cell to Glycerol in Hyperosmotic Solutions at Various Temperatures*

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Received 9 April 1973; revised 27 September 1973

Summary. A central tenet in cryobiology is that low-molecular-weight protective solutes such as glycerol must permeate cells in high concentration in order to protect them from freezing injury. To test this supposition, it is necessary to estimate the amount of solute that has permeated a cell prior to freezing. The amount in bovine red cells was estimated from the flux equation

$$ds/dt = P_\gamma A[(\text{activity external solute}) - (\text{activity internal solute})].$$

Solving the equation required estimates of P_γ, the permeability constant for the solute. Estimates for glycerol in bovine red cells were made in two ways: (1) by measuring the time to 50% hemolysis of red cells suspended in isosmotic or hyperosmotic (1 to 3 M) solutions of glycerol that were hypotonic with respect to NaCl, and (2) by measuring the time required for red cells in hyperosmotic solutions of glycerol in isotonic saline-buffer to become susceptible to osmotic shock upon 10-fold dilution with isotonic saline-buffer. The measurements were made at 0, 10, 15 and 20 °C. The values by the second technique ranged from 2.3×10^{-6} cm/min to 2.7×10^{-6} cm/min at 20 °C, depending on the concentration of glycerol. The values by the first technique were 0 to 30% lower. Both techniques yielded about the same activation energy for permeation between 0 and 20 °C, 21 kcal/mole. This is equivalent to a halving of the permeation rate for every 5° drop in temperature.

Expressing the flux equation in the formulation of irreversible thermodynamics changed the value of P by less than 10%, probably because σ, the reflection coefficient, is 0.95 at 25 °C. Expressing the driving force as the difference in molality or osmolality of glycerol, rather than as the difference in activity, however, had somewhat greater effects on the numerical values of P, but had no effect on the activation energy.

It is concluded that estimates of P based on differences in activities and on the osmotic shock technique are the least subject to error. The use of the usual irreversible thermodynamic equations to express the flux may be a misleading refinement, in that the assumptions underlying them become questionable for concentrations of glycerol as high as 1, 2, or 3 M.

Most mammalian cells survive slow freezing only when a protective solute is present in the surrounding medium (Meryman, 1966). It has been

* Preliminary reports were presented at the 1971 and 1972 meetings of the Biophysical Society and at the 1971 meeting of the Society for Cryobiology.

widely believed that in order to confer this protection the solute must permeate the cell, but recently doubts have arisen about this assumption. For example, sucrose and the macromolecule polyvinylpyrrolidone confer considerable protection during the slow freezing of mouse marrow stem cells and Chinese hamster tissue-culture cells, even though neither solute appears capable of permeating the cell (Mazur, Farrant, Leibo & Chu, 1969; Leibo, Farrant, Mazur, Hanna & Smith, 1970).

As these exceptions have arisen, they have been regarded as just that – exceptions. It is still generally argued that the protective agents *par excellence*, glycerol and dimethyl sulfoxide, must permeate to protect (Meryman, 1971*a*).

This assumption is of central importance to the field of cryobiology, for it has led to certain hypotheses about the modes of protection and injury during freezing (Lovelock, 1953; Meryman, 1971*b*) and to certain rationales in attempts to preserve the complex cellular systems that comprise tissues and organs (Pegg, 1970). If the assumption is wrong, then so too must be portions of the hypotheses.

Because of its importance, we decided to examine in some detail the relation between glycerol permeation and the sensitivity of the bovine red cell to freezing. Our approach was to suspend cells in various concentrations of glycerol at various temperatures for various times and then freeze and thaw them. The next step was to correlate the percentage that survived with the amount of glycerol in the cells prior to freezing.

Although there are considerable data on the kinetics of the permeation of glycerol into bovine red cells suspended in isosmotic glycerol, we could find none for cells suspended at various temperatures in the strongly hyperosmotic solutions (1 to 3 M) used in freezing. [Just prior to our submitting this manuscript, Farmer and Macey (1972) published permeability data for cells in 1 M glycerol at 25 °C.] Accordingly, our first concern was to estimate the amount of glycerol that enters the bovine cell as a function of glycerol concentration, time, and temperature. This is the subject of the present paper. The companion paper (Mazur, Miller & Leibo, 1974) will present the correlative freezing studies.

The chief ways to determine the permeation of solute into red cells are (1) to follow changes in cell volume by hematocrit, by conductivity particle sizers, or by changes in optical density; (2) to measure the amount of glycerol in the cells chemically or isotopically; and (3) to measure the time required for the red cell to hemolyze in hypotonic media. We decided to use the third approach because the calculation of permeability constants from measurements of the time required for hemolysis gives reasonable

agreement with direct chemical analyses (Jacobs, Glassman & Parpart, 1935; LeFevre, 1961), and because direct measurement of intracellular glycerol concentration would require measurements of both the amount of glycerol in the cells and the volume of the cells as functions of time and temperature. These two properties change rapidly during permeation, and as a result estimating them by direct measurement could well introduce as many or more errors than does method (3).

In addition to the usual method of estimating the permeability constant P from the time required to reach a given level of hemolysis (50% here) in hypotonic media, we also used a variant—namely, estimating P from the time required for cells in hyperosmotic solutions of glycerol in *isotonic* saline to undergo osmotic hemolysis when diluted 10-fold with isotonic saline. The assumptions and possible artifacts in these hemolytic methods have been discussed by LeFevre (1961).

Materials and Methods

Preparing Suspensions of Blood Cells

Some 50 to 100 ml of blood were collected from the vein of a young steer in a graduated cylinder containing sufficient heparin (Elkins-Sinn, Inc.) to yield a final concentration of 10 U.S.P. units per ml of blood. The same steer was used throughout the course of the experiments. The blood was stored at 4 °C until used, but in no case was it more than 4 days old.

For each experiment, a 5-ml sample of whole blood was washed by three centrifugations at $1{,}400 \times g$ for 10 min at room temperature and resuspended in 5 ml of 0.301 osmolal saline-buffer. This solution, which was defined as isotonic, contained Na_2HPO_4 (0.0061 moles), $NaH_2PO_4 \cdot H_2O$ (0.0039 moles), NaCl (0.149 moles), and glass-distilled water to make 1 liter. The pH was 7.0. The salts were reagent grade.

Hematocrits were run on both fresh blood and washed cells. Samples were drawn up into heparinized capillary tubes and centrifuged for 5 min at $15{,}500 \times g$.

Determining Osmotic Fragility of Washed Cells

The osmotic fragility of each lot of blood was measured by suspending 0.02-ml samples of washed cells in 3.98 ml of saline-buffer held at room temperature (22 to 23 °C). The osmolal concentrations of the 3.98-ml volumes were 0.301, 0.246, 0.221, 0.196, 0.171, 0.146 and 0.121. The concentration of buffer was held constant at 0.01 M (0.0243 osmolal), and the total concentration was varied by varying the concentration of NaCl. The suspensions were mixed on a Vortex mixer, held 15 min, and the extent of hemolysis determined. In addition to these routine measurements at room temperature, fragility experiments were also carried out with solutions held at 20, 15, 10 and 0 °C.

Determining the Time to Hemolysis of Cells Suspended in Glycerol Solutions Hypotonic with Respect to Salt

Washed cells in isotonic saline-buffer were equilibrated either at room temperature or at 0, 10 or 15 °C; and 2 ml were then transferred to 38 ml of 0.27, 1, 2 or 3 M glycerol

in 0.01 M phosphate buffer. The glycerol solutions had previously been equilibrated at 0, 10, 15 or 20 °C for 30 min. At desired intervals, 0.5-ml samples of the cells in glycerol were removed, and the extent of hemolysis was determined.

Determining the Length of Time Required for Cells to Become Susceptible to Osmotic Shock

Washed cells in isotonic saline-buffer were equilibrated at 0, 10, 15 or 20 °C, and a 0.5-ml sample was then transferred to 9.5 ml of 1, 2 or 3 M glycerol in isotonic saline-buffer at those temperatures. At various times, 0.1-ml quantities of the suspension were transferred to separate tubes into each of which was immediately squirted 0.9 ml of isotonic saline-buffer at the same temperature. Mixing was completed on a Vortex mixer, and the percent hemolysis was then determined. A modified procedure was also used in the case of cells in 3 M glycerol (*see* Results).

Times and Temperatures

The bath temperatures of 0, 10, 15 and 20 °C were held to within ± 0.05 °C, and times were measured to 0.1 min.

Determining Percent Hemolysis

Depending on the volumes, treated samples were centrifuged at room temperature either for 2 min at $15{,}500 \times g$ in a microhematocrit centrifuge or for 10 min at $8{,}000 \times g$ in an angle-head centrifuge. For fragility measurements and for the samples subjected to osmotic shock, measured volumes of the supernatants were then mixed with equal volumes of Drabkin's solution (Wintrobe, 1967). In the case of the time-to-hemolysis experiments, 0.1-ml volumes of supernatants were mixed with a solution consisting of 0.9 ml of saline-buffer and 1 ml of Drabkin's. The final dilution of the washed blood was 400 × in all cases. Ten minutes later, the absorbance (A) of the resulting solution of cyanmethemoglobin was read at 540 nm in a Bausch & Lomb Spectronic 20, using a 1:1 mixture of saline-buffer and Drabkin's as a blank. Tests showed that the absorbance was linearly proportional to the concentration of cyanmethemoglobin present, and that it was unaffected by the presence of glycerol in concentrations as high as 3 M. Percent hemolysis was calculated as $100\, A_{exp}/A_{total}$.

To determine total hemolysis, samples were treated in the manner described above, except that samples of washed cells were initially diluted 200-fold (fragility), or 20-fold (time to hemolysis, osmotic shock) in 0.01 M phosphate buffer containing neither glycerol nor NaCl.

Parpart (1931) and Davies, Marsden, Östling and Zade-Oppen (1968) have shown that the percent hemolysis obtained by colorimetric determination of the concentration of released hemoglobin agrees closely with the percentage of individual cells that undergo hemolysis in hypotonic NaCl. In other words, the release of hemoglobin from individual cells is essentially an all-or-none phenomenon (Canham, 1970). Parpart (1931) observed, however, that in the case of hypotonic hemolysis of bovine red cells in isotonic glycerol, the percentage of hemoglobin released is initially about 8% less than the percentage of hemolyzed cells. Nevertheless, because the effect is small, we shall refer to the absorbance ratios as "percent hemolysis" and to 100% minus those values as "percent unhemolyzed" or "percent survival."

Calculation of Concentrations

Table 1 shows the compositions of the various experimental solutions. Those containing glycerol were prepared by adding sufficient saline-buffer or 0.01 M phosphate buffer to 0.27, 1, 2 or 3 moles of the additive to make 1 liter. This procedure kept the molal concentration of salts constant. The concentrations of the experimental mixtures were determined from the known compositions and volumes of the solutions and from the hematocrit of the washed blood. (The calculations ignore the slight dilution that is produced by the water withdrawn osmotically from the cells by the glycerol. This dilution would introduce a maximum error of 0.8%.)

Osmolalities are defined as $\varphi \nu m$, where φ is the osmotic coefficient, ν is the number of species into which the solute dissociates, and m is the molality. Values of φ for glycerol

Table 1. Composition of experimental solutions

Solution	Conc. units	Components			
		Glycerol	Buffer	NaCl	H_2O
Phosphate buffer	molar		0.010		
	molal [a]		0.010		
	ml/ml				1.00
Saline-buffer	molar		0.010	0.148	
	molal [a]		0.010	0.149	
	ml/ml				0.994
0.27 M glycerol in buffer	molar	0.270	0.010		
	molal [a]	0.276	0.010		
	ml/ml				0.979
1 M glycerol in buffer	molar	1.000	0.009		
	molal [a]	1.078	0.010		
	ml/ml				0.928
1 M glycerol in saline-buffer	molar	1.000	0.009	0.138	
	molal [a]	1.084	0.010	0.149	
	ml/ml				0.922
2 M glycerol in buffer	molar	2.0	0.009		
	molal [a]	2.344	0.010		
	ml/ml				0.853
2 M glycerol in saline-buffer	molar	2.0	0.009	0.127	
	molal [a]	2.348	0.010	0.149	
	ml/ml				0.852
3 M glycerol in buffer	molar	3.0	0.008		
	molar [a]	3.816	0.010		
	ml/ml				0.786
3 M glycerol in saline-buffer	molar	3.0	0.008	0.117	
	molal [a]	3.844	0.010	0.149	
	ml/ml				0.780

[a] Molalities were calculated from the measured molarities and measured densities of the solutions.

were obtained from Scatchard, Hamer and Wood (1938); values of φ or $\varphi\nu$ for salts were obtained from Robinson and Stokes (1959) and the Handbook of Physics and Chemistry (*see* Hodgman, 1963). We assume in calculating osmolalities that the published values of φ for single solutes in water are not changed appreciably when these solutes are in the presence of two or three other components (*see* Appendix).

Theory

When a cell is transferred from one solution to another of higher osmolality containing a permeating solute, it shrinks initially because of the loss in water and then increases in volume as the solute permeates and water re-enters the cell. The permeation of glycerol into the bovine red cell (unlike the human red cell) is by passive diffusion (Hunter, 1947; Macey & Tolberg, 1966). In the following derivation of equations describing that permeation, the superscripts i and e refer to the intracellular and extracellular solutions, respectively, and the subscript s refers to the permeating solute, glycerol. All other solutes are assumed to be nonpermeating and are referred to by the subscript n. Concentrations are expressed as molalities (m) or osmolalities (M); i.e., they are expressed in terms of the water available for solution.

Since the permeation of water into bovine red cells at 20 °C is about 5×10^5-fold faster than the permeation of glycerol [$P_{H_2O}=0.8$ cm/min (Villegas, Barton & Solomon, 1958); $P_{glyc}\approx2\times10^{-6}$ cm/min], we assume as have others (Stein, 1962; Macey & Tolberg, 1966) that water transfer is instantaneous, and that the cells at all times are in osmotic equilibrium with the external medium. The reflection coefficient (σ) at 25 °C for bovine red cells undergoing swelling in glycerol is about 0.95 (Farmer & Macey, 1972). If we assume it to be 1 at 20 °C and below (*see* Discussion), then

$$\pi^i=\pi^e, \tag{1}$$

where π is the osmotic pressure. But $\pi=RT\Sigma\varphi\nu m$ (Robinson & Stokes, 1959), so

$$(\Sigma\varphi\nu m)^i=(\Sigma\varphi\nu m)^e, \tag{2}$$

or, by definition,

$$M^i_{total}=M^e_{total}.$$

Expressed in terms of permeating and nonpermeating solutes, this becomes for any time t

$$[N^i_n+\varphi^i_s(t)s(t)]/V'(t)=(M^e_n+\varphi^e_s m^e_s)/1000, \tag{3}$$

where N^i_n are the osmoles of nonpermeating solutes in the cells, $V'(t)$ is the volume (in cm^3) of solvent water in the cell at time t, and the other symbols

Table 2. Definitions of major symbols

Symbol	Description	Units	Value
P_γ	Permeability constant for glycerol	cm/min	parameter
$\varphi_s^i(t)$	Osmotic coefficient of internal glycerol		variable
$\gamma_s^i(t)$	Activity coefficient of internal glycerol		variable
$s(t)$	Moles of glycerol in cell at time t	moles	variable
$V(t)$	Volume of water in cell at time t relative to the volume in an isotonic cell (*see text*)	$-$ or cm^3	variable
M_{iso}^i	Osmolality of nonpermeating solutes in isotonic cells	osmoles/$liter_{H_2O}$ [a]	0.301
φ_s^e	Osmotic coefficient of external glycerol		parameter
γ_s^e	Activity coefficient of external glycerol		parameter
m_s^e	Molality of external glycerol	moles/$liter_{H_2O}$	parameter
M_n^e	Osmolality of nonpermeating salts in external medium	osmoles/$liter_{H_2O}$	parameter
t	Time in contact with glycerol	min	variable
Δt	Small increment in time	min	parameter
A	Total surface area of the number of isotonic cells required to contain 1 cm^3 H_2O	cm^2	2.25×10^4 [b]
M_d^e	Total osmolality of external medium after dilution	osmoles/$liter_{H_2O}$	parameter

[a] 1 liter H_2O assumed = 1 kg H_2O.

[b] The area was calculated from data of Villegas *et al.* (1958) as follows: The volume of the isotonic bovine red cell is 58×10^{-12} cm^3. Of this, 71 % (v/v) is water; hence, the volume of water in an isotonic cell is 41×10^{-12} cm^3, and 2.45×10^{10} cells contain 1 cm^3 of H_2O. The surface area of a single bovine red cell is 92×10^{-8} cm^2. Therefore, the surface area of 2.45×10^{10} cells is 2.25×10^4 cm^2.

are as defined in Table 2. We assume that the volume of the external medium is sufficiently large to keep its composition invariant with time (the change in concentration of external glycerol during permeation is less than 0.01 %), and that the permeating solute is a nonelectrolyte, in which case $v_s = 1$.

In an isotonic cell with a volume of water V_{iso} cm^3,

$$N_n^i / V_{iso} = M_{iso}^i / 1000. \tag{4}$$

Substituting Eq. (4) into (3), letting $V_{iso} = 1$ cm^3, and solving for $V(t)$, we obtain

$$V(t) = [M_{iso}^i + 1000\, \varphi_s^i(t)\, s(t)] / (M_n^e + \varphi_s^e\, m_s^e), \tag{5}$$

where $V(t)$ is now the volume of water at time t in that number of cells which in isotonic media would contain 1 cm^3 of H_2O; i.e., $V(t)$ is the relative volume of water in the cell at time t.

The rate at which a solute permeates the cell is ordinarily taken to be a function of the difference in concentrations on the two sides of the membrane. But, because we are treating very concentrated solutions (1 to 3 M glycerol), we express the driving force in terms of the difference in activities of glycerol (Bayliss, 1959) and thus obtain

$$ds/dt = P_y A[(\gamma_s^e m_s^e/1000) - \gamma_s^i(t)\, s(t)/V(t)], \tag{6}$$

again assuming for the moment that $\sigma = 1$. As indicated in Table 2, P_y is the permeability constant, A is the area of the cell surface, and the γ's are activity coefficients.

To approximate a simultaneous solution to Eqs. (5) and (6), we initially assume that at zero time no glycerol has entered the cell [$s(0) = 0$], and that the cell is shrunken so that it contains the volume of water, $V(0)$, given by Eq. (5). We then allow glycerol to flow into the cell for a very short interval of time (Δt), assume that during this short interval $V(t)$ remains constant, and calculate the amount of glycerol in the cell by the integrated version of Eq. (6) (*see below*). Armed with the value of s, we then calculate a new value of $V(t)$ from Eq. (5), allow another short interval of time (Δt) to elapse, calculate a new value for s from the integrated version of Eq. (6), and so forth.

Assuming that s and t are the only variables (*see* Appendix), the solution to Eq. (6) from t to $t+\Delta t$ and from $s(t)$ to $s(t+\Delta t)$ is of the form $\int dx/(a+bx) = (1/b)/\ln(a+bx)$, which in exponential form becomes

$$s(t+\Delta t) = \frac{V(t)\gamma_s^e m_s^e}{1000\gamma_s^i(t)} - \frac{V(t)}{\gamma_s^i(t)}\left[\frac{\gamma_s^e m_s^e}{1000} - \gamma_s^i(t)\frac{s(t)}{V(t)}\right] \cdot \exp[-\gamma_s^i(t) P_{\gamma_i} A \Delta t/V(t)]. \tag{7}$$

The full procedure then was as follows:

1. With $t = 0$ and $s(0) = 0$, $V(0)$ was calculated from Eq. (5).

2. $\gamma_s^i(0)$ was taken to be 1.00.

3. $s(0+\Delta t)$ was calculated from Eq. (7).

4. $\varphi_s^i(0+\Delta t)$ was calculated from the empirical equation $\varphi_s^i(t+\Delta t) = 1.00 + 9.75\, s(t+\Delta t)/V(t)$ fitted to the data of Scatchard *et al.* (1938).

5. The value of $V(0+\Delta t)$ was calculated from Eq. (5), using the values of $s(t)$ and $\varphi_s^i(t)$ calculated in steps 3 and 4 above.

6. A new value of $\gamma_s^i(t)$ was calculated from the empirical equation $\gamma_s^i(t) = 1.00 + 22.3\, s(t)/V(t)$ also derived from the data of Scatchard *et al.* (1938).

7. The value for $s(0+2\Delta t)$ was calculated from Eq. (7) using the values of $V(t)$ and $\gamma_s^i(t)$ calculated in steps 5 and 6 above. And so forth. The iterations described above were carried out on a time-share computer.

The interval of time, Δt, was 1/300 to 1/500 the total time of interest. This was considered sufficiently precise, since reducing the interval 20-fold produced less than 1% change in the calculated values of $V(t)$ and $s(t)$.

The values for the constants M_{iso}^i and A and the parameters m_s^e and M_n^e are known. What is needed is the numerical value of P_γ, the permeability constant for glycerol.

Methods for Estimating P_γ

P_γ was estimated by two methods: (1) by determining the time required for 50% hemolysis of cells placed in isosmotic or hyperosmotic solutions of glycerol that were hypotonic with respect to NaCl, and (2) by determining the time required for cells suspended in solutions that were hyperosmotic with respect to glycerol and isotonic with respect to salt to become susceptible to osmotic shock (i.e. osmotic hemolysis) when suddenly diluted.

Method 1. Hypotonic Hemolysis

Fig. 1 shows the percentage of red cells remaining unhemolyzed after various times in the indicated nominal molar concentrations of glycerol at 20 °C. The concentration of NaCl in the medium was approximately 0.04 osmolal (Table 3). Hemolysis occurs when a cell reaches a critical volume. The critical volume (or, more precisely, the critical volume of water) was calculated from the osmotic "fragility" of the cells by determining the concentration of saline-buffer needed to produce 50% hemolysis. Since the internal and external osmolalities are assumed to be equal, then from Eq. (5) the volume of water in the cell will be inversely proportional to the osmolality of the medium. Defining V_c' as the volume of water in a cell exposed to the concentration of NaCl that produces 50% hemolysis ($M_{50\%}^e$), we obtain

$$V_c'/V_{\text{iso}} = M_{\text{iso}}^e/M_{50\%}^e, \tag{8}$$

or in terms of relative volumes (i.e., $V_{\text{iso}} = 1$),

$$V_c = 0.301/M_{50\%}^e. \tag{9}$$

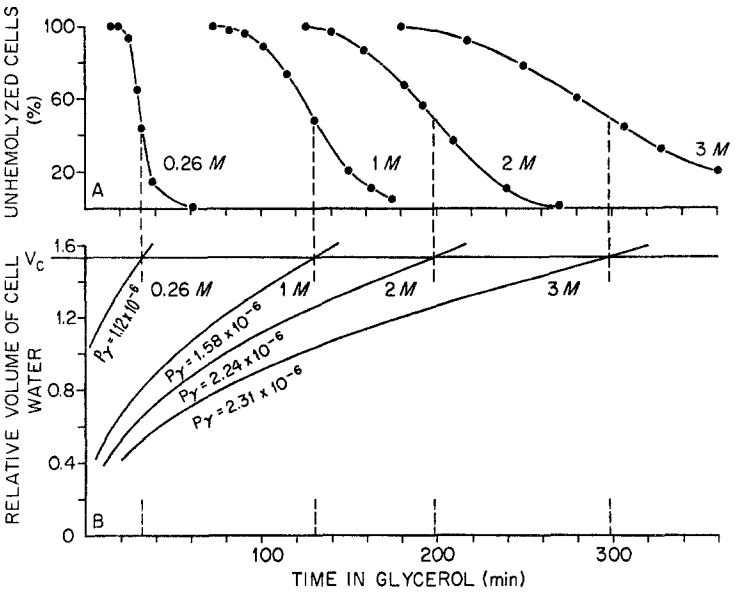

Fig. 1. Estimation of P_γ of bovine red cells in 0.26, 1, 2 and 3 M glycerol at 20 °C from measurements of the time required to produce 50% hemolysis in solutions hypotonic with respect to NaCl (*see text*). (*A*) Experimental determinations of hemolysis as a function of time. (*B*) Calculated volume of water in the cell as a function of time. The curves show the values of P_γ used in the calculations. V_c is the observed critical volume of water

Table 3. Parameters used in calculating P_γ from measured times to 50% hemolysis in solutions of glycerol in hypotonic saline-buffer

Nominal conc. glycerol [a] (molar)	Temp. (°C)	Cell age [b] (days)	V_c	γ_s^e	φ_s^e	m_s^e (molal)	M_n^e (osmolal)	Time to 50% hemolysis (min)	P_γ (cm/min $\times 10^6$)
0.27	20	1	1.54	1.009	1.004	0.266	0.035	32	1.12
	15	3	1.53	1.009	1.004	0.266	0.035	57	0.61
	10	2	1.54	1.009	1.004	0.266	0.035	121	0.30
1	20	1	1.54	1.026	1.013	1.037	0.036	130	1.58
	15	3	1.54	1.026	1.013	1.037	0.036	247	0.83
	10	4	1.54	1.026	1.013	1.038	0.035	485	0.42
	0	1	1.56	1.026	1.013	1.038	0.035	~1760 [c]	~0.12
2	20	1, 4	1.54	1.054	1.025	2.249	0.036	198	2.24
	15	3	1.54	1.054	1.025	2.249	0.036	386	1.15
	10	3	1.54	1.054	1.025	2.252	0.036	705	0.63
3	20	2	1.54	1.084	1.037	3.645	0.038	298	2.31
	15	4	1.60	1.084	1.037	3.650	0.037	641	1.17

[a] Concentration of glycerol to which cells were added. The molar concentrations of glycerol in the resulting suspensions were 0.26, 0.965, 1.93, and 2.90 M, respectively.
[b] Time stored at 4 °C prior to use.
[c] By linear interpolation between 10% hemolysis at 1470 min and 64% hemolysis at 1860 min.

The next step was to find the value of P_γ that is required in Eq. (7) to make Eqs. (5) and (7) yield a value of $V(t)=V_c$ at the observed time for 50% hemolysis. The procedure is illustrated in Fig. 1*B*. V_c in these experiments was 1.54. We see, for example, that for cells in 1 M glycerol, a value of 1.58×10^{-6} cm/min for P_γ gives the desired value, $V(t)=1.54$, at the observed 50% hemolysis time of 130 min.

Method 2. Osmotic Shock

The second method for estimating P_γ depended on the fact that a cell held in a hyperosmotic glycerol solution for a given time and then abruptly transferred to isotonic saline-buffer will swell to an extent that is dependent on the amount of glycerol that has penetrated prior to the transfer and on the extent to which the external glycerol is diluted as a result of the transfer. If enough glycerol permeates the cell, it will swell beyond its critical volume and hemolyze. Fig. 2 shows the percentage of cells remaining unhemolyzed as a function of time in 1 M glycerol prior to dilution with isotonic saline-buffer. Results are shown for several temperatures.

By our primary assumption of instantaneous water transfer and Eqs. (2) and (3), the total osmolal concentration of glycerol and nonpermeating solutes in the cell equals the total concentration outside the cell at all times; i.e., the total osmolality is $M_n^e+\varphi_s^e m_s^e$.

When cells are transferred to a more dilute medium of total osmolality M_d^e, water flows into the cell to reduce its osmolality from $M_n^e+\varphi_s^e m_s^e$ to

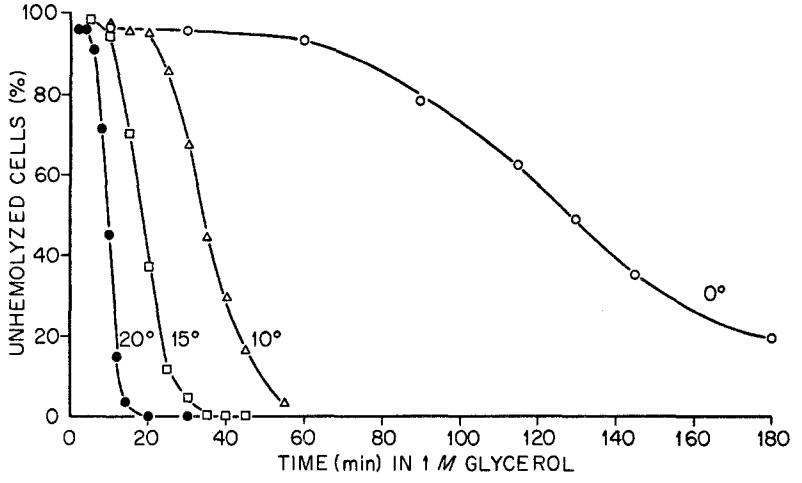

Fig. 2. Percentage of bovine red cells remaining unhemolyzed after suspension in a 1-M solution of glycerol in isotonic saline-buffer at 0, 10, 15 and 20 °C for various times and then abruptly diluted 10-fold with isotonic saline-buffer

M_d^e, and the volume of water in the cell, $V_d(t)$, becomes

$$V_d(t)=(M_n^e+\varphi_s^e m_s^e)\,V(t)/M_d^e. \tag{10}$$

As before we assume that osmotic equilibration after dilution results only from the movement of water and not of glycerol (i.e., $\sigma=1$).

The permeability constant P_γ was estimated by determining the value that was required in Eq. (7) to make the calculated volume of water after dilution, $V_d(t)$, equal to the critical volume V_c at the observed time for 50% hemolysis. For example, for cells in 1 M glycerol at 20 °C, $V_d(t)$ becomes equal to the observed critical volume (1.50) at the observed 50% hemolysis time (9.8 min) when the value of P_γ is taken to be 2.21×10^{-6} cm/min. The average difference in the times to hemolysis in duplicate runs with the two procedures was 5%.

Experimental Results

Determination of the Critical Volume of Cell Water from Osmotic Fragility

Values for the volume of water in the cells at the time of 50% hemolysis (V_c) are required in both methods of calculating the permeability constant for glycerol. The mean value of V_c at 20 °C was 1.55 for 22 determinations. The values for individual lots of blood ranged from 1.50 to 1.62, and these individual values were used in the calculation of P_γ in the corresponding experiments.

The osmolal concentration of saline-buffer that produced 50% hemolysis in the fragility test was slightly temperature-dependent, the values at 0, 10 and 15 °C being 105, 104, and 103% of that at 20 °C. Seeman, Sauks, Argent and Kwant (1969) have found a similar temperature dependence in human red cells, but they also found that the critical *volume* of the cells as measured directly by hematocrit is independent of temperature. The discrepancy is apparently due to the fact that the cells undergo a greater prelytic loss of K^+ at higher temperatures. If the critical volume of the cells is independent of temperature, then so too is V_c, the critical volume of water.

We assume that the same situation holds for bovine red cells, so we did not attempt to correct V_c for temperature. Even if our assumption is faulty it would not be serious, for from Eq. (9) the temperature-corrected values of V_c at 0, 10 and 15 °C would be 95, 96 and 97% of the values at 20 °C. The effect of using these temperature-corrected estimates of V_c would be to reduce P_γ by some 10 to 15% at the lower temperatures.

Determination of P_γ by the Hypotonic Hemolysis Method

The kinetics of hemolysis for cells in 0.26, 1, 2 and 3 M glycerol at 20 °C are shown in Fig. 1. The kinetics at 15 and 10 °C are shown in Fig. 3. The time for hemolysis in 1 M glycerol at 0 °C was very long – 1760 min (29 hr). Presumably it would have been much longer in 2 and 3 M glycerol at 0 °C if the measurements had been attempted. Table 3 shows both the values of the parameters used in the computer calculations and also the values of P_γ that gave the best fit. Finally, Fig. 4 shows Arrhenius plots of $\log P_\gamma$ *vs.* $1/T$ and lists the calculated activation energies for permeation. Although P_γ is somewhat dependent on the concentration of glycerol, the activation energy for permeation is not.

Determination of P_γ by the Osmotic Shock Method

Fig. 2 shows the times of exposure in 1 M glycerol at 0, 10, 15 and 20 °C required to produce hemolysis after dilution. Figs. 5 and 6*A* show the analogous data for cells in 2 and 3 M glycerol. It will be noted that as the concentration of glycerol was increased and the temperature lowered, there was an increasing percentage of hemolysis at even the shortest exposure times. In 1 M glycerol, the effect is undetectable. In 2 M glycerol (Fig. 5), the initial survival drops from 97% at 20 °C to 84% at 0 °C. In 3 M glycerol (Fig. 6*A*), the initial survival is depressed, and the kinetics become peculiar in that survival first drops, then rises, and finally drops again. In this last

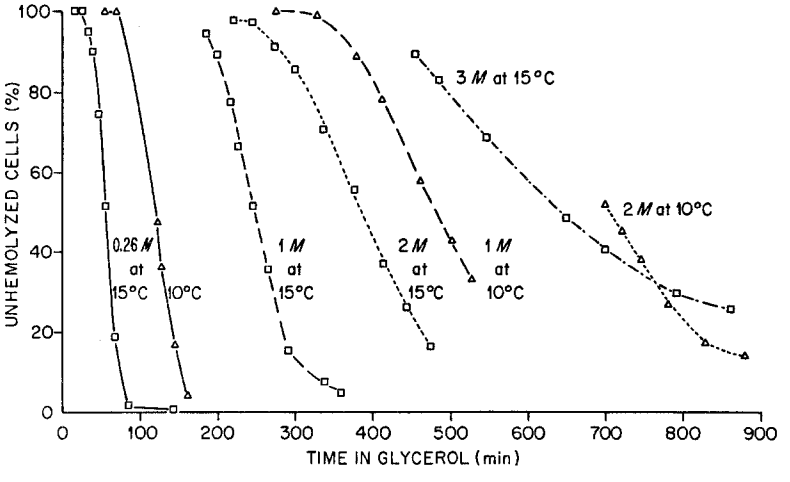

Fig. 3. Percentage of bovine red cells remaining unhemolyzed as a function of time in various concentrations of glycerol at 10 and 15 °C. The solutions were hypotonic with respect to NaCl (0.04 M)

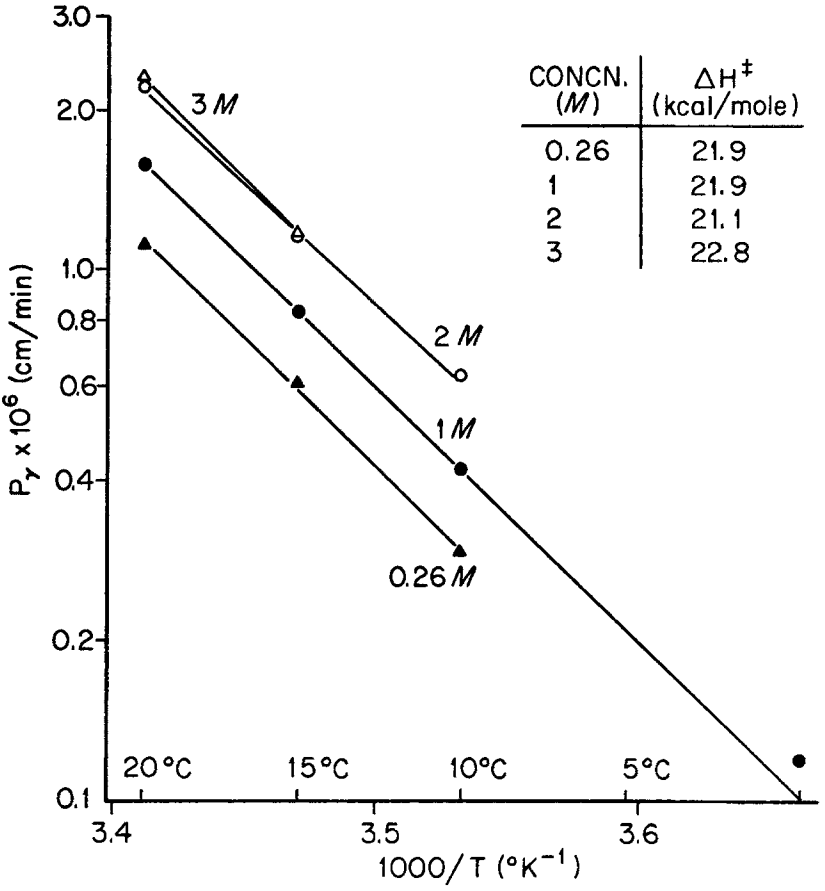

Fig. 4. Arrhenius plot of P_f for bovine red cells in 0.26, 1, 2 and 3 M glycerol. The values of P_f are those calculated by the hypotonic hemolysis method

case, both the initial depression and the peculiarity of the kinetics become especially exaggerated at the lower temperatures.

The initial lowered survival, when present, reflects some deleterious factor other than osmotic shock. Thus, cells suspended in the nonpermeating solute sucrose (Bishop, 1964) at a concentration isosmotic with 2 M glycerol and then diluted 10-fold with isotonic saline show the lowered initial survival but do not show any second drop.

The experiments that yielded the results in Figs, 2, 5, and 6*A* involved the use of a syringe to transfer samples of cells in hyperosmotic glycerol. The experiments with 3 M glycerol were repeated using a modified procedure, which avoided expelling cells through a syringe. First, 0.1 ml of cells in saline-buffer at room temperature was pipetted into test tubes and equilibrated at the desired temperature. Then at zero time, 1.9 ml of temperature-

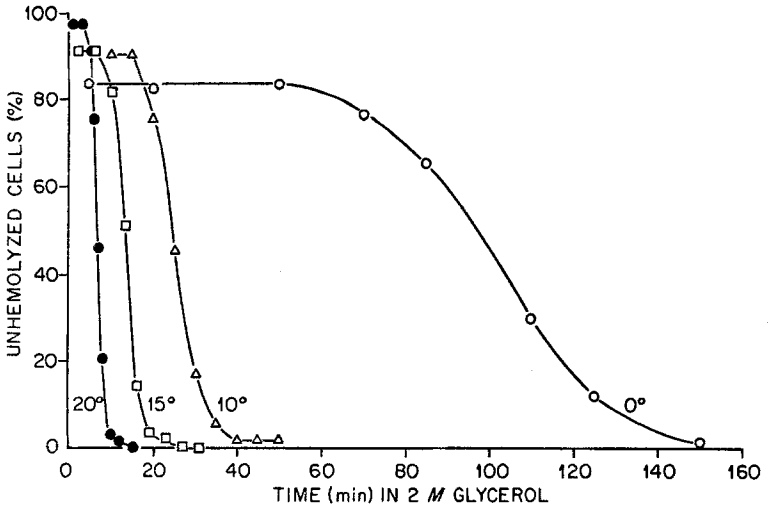

Fig. 5. Percentage of bovine red cells remaining unhemolyzed after suspension in a solution of 2 M glycerol in isotonic saline-buffer at 0, 10, 15 and 20 °C for various times and then abruptly diluted 10-fold with isotonic saline-buffer

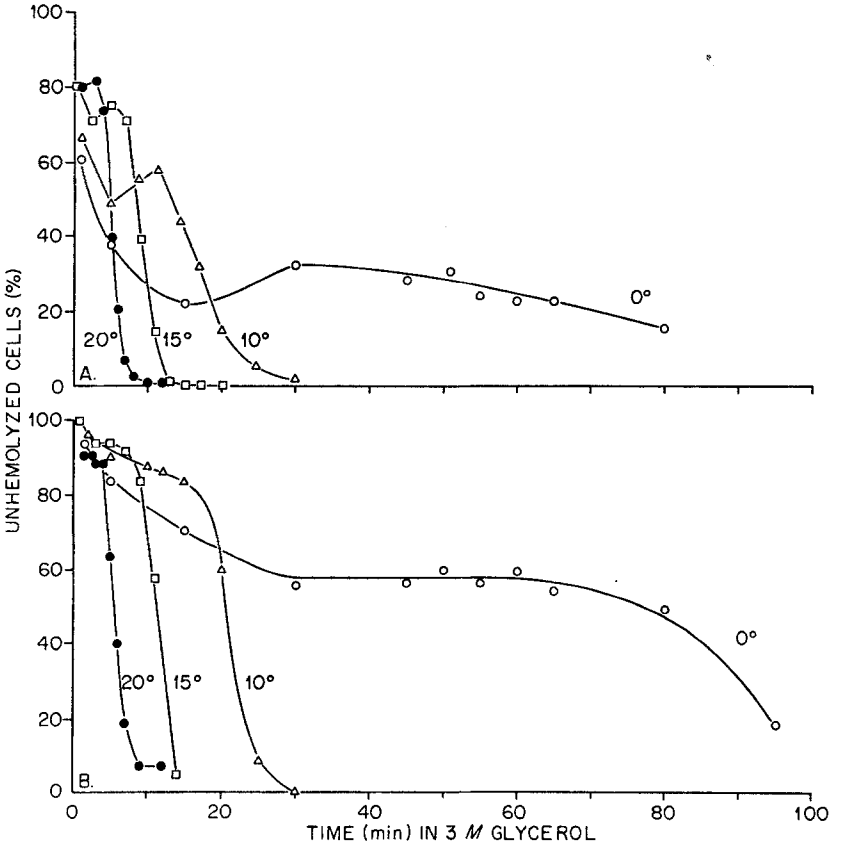

Fig. 6. Time required for bovine red cells in a solution of 3 M glycerol in isotonic saline-buffer at 0, 10, 15 and 20 °C to undergo hemolysis when diluted 10-fold with isotonic saline-buffer. (*A*) Original procedure (*see* Methods). (*B*) Modified procedure (*see* Results)

Table 4. Parameters used in calculating P_γ from time to susceptibility to osmotic shock upon dilution

Nominal conc. glycerol [a] (M)	Temp. (°C)	Cell age [b] (days)	V_c	γ_s^e	φ_s^e	m_s^e (molal)	M_n^e (osmolal)	M_d^e (osmolal)	Normalized time to 50% hemolysis [c] (min)	P_γ (cm/mi $\times 10^6$)
1	20	2	1.50	1.026	1.013	1.045	0.302	0.398	9.8	2.21
	15	1	1.50	1.026	1.013	1.045	0.302	0.398	18.2	1.18
	10	3	1.50	1.026	1.013	1.045	0.302	0.398	34.5	0.63
	0	3	1.58	1.026	1.013	1.048	0.302	0.398	134	0.19
2	20	2	1.50	1.053	1.025	2.256	0.302	0.497	6.9	2.29
	15	1	1.50	1.053	1.025	2.258	0.302	0.496	13.2	1.19
	10	3	1.50	1.053	1.025	2.256	0.302	0.497	25.0	0.63
	0	3	1.57	1.054	1.026	2.264	0.302	0.497	102.8	0.17
3	20	2	1.59	1.085	1.037	3.683	0.302	0.596	5.8	2.71
	15	2	1.62	1.085	1.037	3.683	0.302	0.596	11.6	1.42
	10	2	1.62	1.085	1.037	3.683	0.302	0.596	21.0	0.78
	0	1, 3	1.62	1.085	1.037	3.680	0.302	0.596	79.5	0.21

[a] Concentration of glycerol added to cells. The concentrations in the suspensions were 0.97, 1.93, and 2.90 M.
[b] Time stored at 4 °C prior to use.
[c] *See* Results.

equilibrated glycerol-saline was added to each tube. Finally, at given times the cells in a given tube were shocked by the rapid addition of 18 ml of isotonic saline-buffer. Fig. 6*B* shows the results. The curves for 3 M now appear much more like those for 1 and 2 M. Apparently, chilled cells in 3 M glycerol are highly susceptible to hemolysis when subjected to passage through a syringe and to rapid volume changes. This matter has mechanistic implications and is currently the subject of a separate study (Leibo & Mazur, *in preparation*).

Since in certain instances the osmotic shock procedures produced some hemolysis even in the absence of osmotic shock, we defined the highest survival value as 100% and then defined the time to hemolysis due to osmotic shock as the time required for the survival to fall to one-half of the highest value.

These normalized times to 50% hemolysis are listed in Table 4 along with the values of the several parameters needed to calculate P_γ and the values of P_γ that give the best fit. Comparison of this table with Figs. 2, 5, and 6*B* shows that the differences between the absolute and normalized

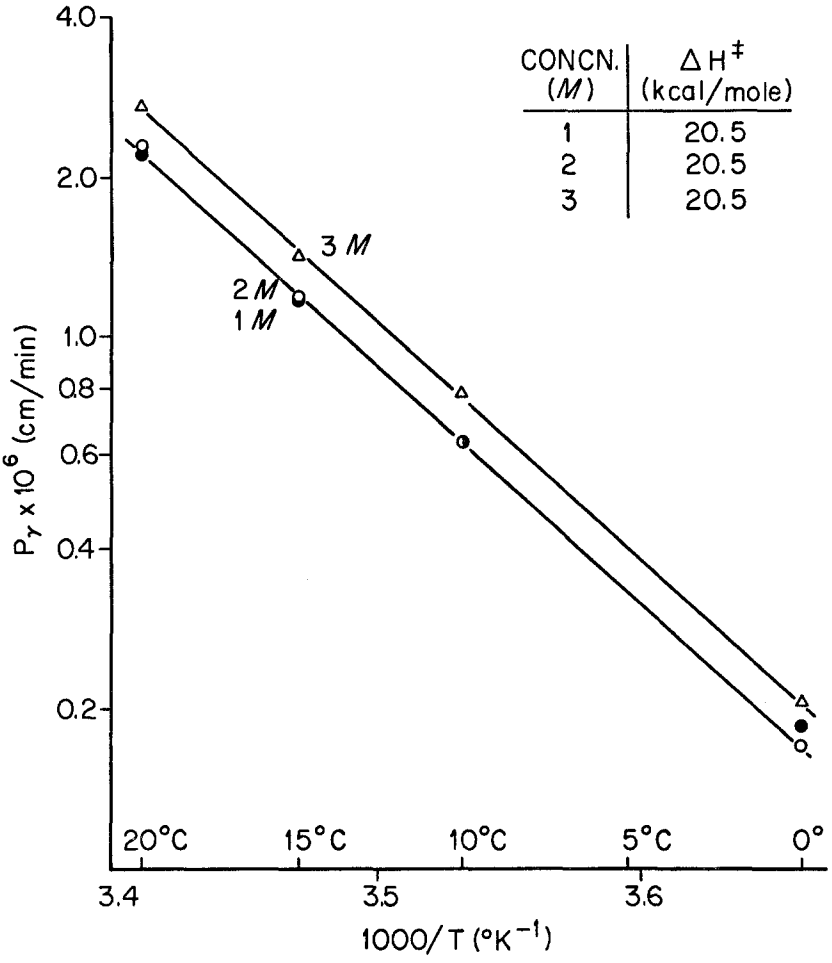

Fig. 7. Arrhenius plot of P_γ for bovine red cells in 1 M (•), 2 M (○), and 3 M (△) glycerol. The values of P_γ are those calculated by the osmotic shock method

times to hemolysis are less than 6% and usually less than 2%. The effect of these time differences on the estimates of P_γ is comparable; i.e. 6% or less. An Arrhenius plot of permeation and the calculated activation energies (ΔH^*) are shown in Fig. 7. The permeability constants for 1 and 2 M glycerol are nearly identical, but they are somewhat less than those for 3 M glycerol. The activation energies for permeation are the same in all three concentrations.

Comparison of Permeation Constants and Activation Energies Obtained by the Two Methods

Table 5 compares the values of P_γ and ΔH^* obtained by the two methods. The estimates of P_γ from osmotic shock are 0 to 55% higher than the estimates

Table 5. Comparison of P_γ and $\Delta H^\ddagger$ obtained from the two experimental methods

Nominal conc. of glycerol (M)	Temp. (°C)	P_γ			$\Delta H^\ddagger$		
		Osmotic shock (cm/min × 10^6)	Hypotonic hemolysis	Ratio [a]	Osmotic shock (kcal/mole)	Hypotonic hemolysis	Ratio [a]
1	20	2.21	1.58	1.40			
	15	1.18	0.83	1.42	20.5	21.9	0.94
	10	0.63	0.42	1.48			
	0	0.19	0.12	1.55			
2	20	2.29	2.24	1.02			
	15	1.19	1.15	1.04	20.5	21.1	0.97
	10	0.63	0.63	1.00			
3	20	2.71	2.31	1.17			
	15	1.42	1.17	1.21	20.5	22.8	0.90

[a] Value from osmotic shock/value from hypotonic hemolysis.

from hypotonic hemolysis, but the values for the activation energy are 3 to 10% lower. [The $\Delta H^\ddagger$ value of 22.8 kcal/mole for 3 M glycerol and hypotonic hemolysis was based on only two temperatures (*see* Fig. 4)].

In connection with the differences between the two sets of values of P_γ, it should be noted that the end point in the two procedures (i.e. 50% hemolysis) occurs at very different times during the time course of glycerol permeation. For example, in 2 M glycerol at 20 °C, 50% hemolysis from osmotic shock occurs after only 6.9 min (Table 4), whereas 50% hypotonic hemolysis requires 198 min (Table 3). Table 5 shows no comparisons for 2 M glycerol at 0 °C or for 3 M glycerol at 10 or 0 °C, because at these concentrations and temperatures the times to hypotonic hemolysis become prohibitively long ($\gg$29 hr).

Discussion

The two methods used to determine the permeability of bovine red cells to hyperosmotic concentrations of glycerol yielded similar results. The values of P_γ agree within 4, 20 and 50% for cells in 2, 3 and 1 M glycerol, respectively. The two methods give comparable temperature coefficients for permeation (the permeation rate is halved for every 5° drop in temperature), and they both yield values of P_γ that vary by less than 50% over concentrations of glycerol ranging from 1 to 3 M. However, there are differences both in the numerical values of P_γ and in their concentration dependence,

those estimated by the osmotic shock method being higher and less affected by concentration. As mentioned, estimates of P_γ from osmotic shock are derived from the first few minutes of glycerol permeation, when the cell is still shrunken prior to dilution, whereas those obtained by the hypotonic hemolysis method are derived from the terminal stages of permeation, when the cell has expanded to its hemolytic volume. One question, then, is whether the differences in P_γ result from true differences in the permeability of the shrunken and expanded cell or merely reflect inadequacies in our assumptions or in the flux equations.

Possible Flux Equations

In the past, most authors derived the rate of permeation by passive diffusion from Fick's law and expressed it as

$$ds/dt = PA(C_s^e - C_s^i), \tag{11}$$

where C_s was variously expressed in molar, molal, or osmolal units. With the recognition that this formulation neglects cross-coupling between solute and solvent flow, and with the development of techniques for handling these interactions by means of irreversible thermodynamics (Kedem & Katchalsky, 1958), the equation is now usually expressed as

$$ds/dt = PA(C_s^e - C_s^i) + (1-\sigma)\bar{C}_s dV/dt. \tag{12}$$

In this formulation, σ is the reflection coefficient, with values ranging from 0 to 1. The former value of 0 characterizes a membrane that cannot distinguish solute and solvent, and the value of 1 describes a membrane that is completely impermeable to solute. The parameter $\bar{C}_s$ is $(C_s^e - C_s^i)/(\ln C_s^e - \ln C_s^i)$ (Kedem & Katchalsky, 1958; Katchalsky & Curran, 1965).

To test whether the value of the permeability constant is sensitive to the choice of flux equations and to the choice of concentration units, we calculated P values for the two experimental methods using the following equations.

(a) First, expressing concentrations as molalities, the classical differential equation is

$$ds/dt = P_m A[(m_s^e/1000) - s(t)/V(t)], \tag{6a}$$

and its solution is

$$s(t+\Delta t) = \frac{V(t)\, m_s^e}{1000} - V(t)\left[\frac{m_s^e}{1000} - \frac{s(t)}{V(t)}\right] \exp[-P_m A \Delta t/V(t)]. \tag{7a}$$

The equation for $V(t)$ at osmotic equilibrium is Eq. (5).

(b) The irreversible thermodynamic equivalent of Eq. (6a) is

$$\frac{ds}{dt}=P_{m\sigma}A\left[\frac{m_s^e}{1000}-\frac{s(t)}{V(t)}\right]+(1-\sigma)\bar{C}_s\frac{V(t)-V(t-\Delta t)}{\Delta t} \tag{6b}$$

where

$$\bar{C}_s=\left[\frac{m_s^e}{1000}-\frac{s(t)}{V(t)}\right]\Big/\left[\ln\frac{m_s^e}{1000}-\ln\frac{s(t)}{V(t)}\right].$$

The integrated version is

$$\begin{aligned}s(t+\Delta t)=&\frac{V(t)}{P_{m\sigma}A}\left(\frac{P_{m\sigma}Am_s^e}{1000}+h\right)\\&-\frac{V(t)}{P_{m\sigma}A}\left[\frac{P_{m\sigma}Am_s^e}{1000}+h-\frac{P_{m\sigma}A}{V(t)}s(t)\right]\cdot\exp\left[-P_{m\sigma}A\Delta t/V(t)\right]\end{aligned} \tag{7b}$$

where

$$h=(1-\sigma)\bar{C}_s[V(t)-V(t-\Delta t)]/\Delta t.$$

And the equation for $V(t)$ at osmotic equilibrium (when $V_{\text{iso}}=1\ \text{cm}^3$) is

$$V(t)=[M_{\text{iso}}^i+1000\,\sigma\varphi_s^i(t)s(t)]/(M_n^e+\sigma\varphi_s^e m_s^e). \tag{5a}$$

Note that $P_{m\sigma}$ is equivalent to ωRT, where ω is one of the phenomenological coefficients in the irreversible thermodynamic formulations. In the osmotic shock experiments, the volume of water after dilution becomes

$$V_d(t)=V(t)(M_n^e+\sigma\varphi_s^e m_s^e)/(M_{nd}^e+\sigma\varphi_{sd}^e m_{sd}^e), \tag{10a}$$

where the subscript d refers to the value of the parameter after dilution with isotonic saline-buffer.

(c) On the other hand, some authors express the diffusional driving force in terms of osmolalities (Macey & Tolberg, 1966; Sha'afi, Dakkuri & Tómey, 1971). In these terms, Eq. (6) becomes

$$d(\varphi s)/dt=P_\varphi A[\varphi_s^e m_s^e/1000-\varphi_s^i(t)s(t)/V(t)]. \tag{6c}$$

The solution is

$$N_s^i(t+\Delta t)=\frac{V(t)\varphi_s^e m_s^e}{1000}-V(t)\left[\frac{\varphi_s^e m_s^e}{1000}-\frac{\varphi_s^i(t)s(t)}{V(t)}\right]\cdot\exp\left[-P_\varphi A\Delta t/V(t)\right] \tag{7c}$$

where $N_s^i=\varphi_s^i s$. The equation for osmotic equilibrium is again Eq. (5).

(d) Finally, the concentrations can be expressed as activities. The irreversible thermodynamic counterpart for the activity equation [Eq. (6)] in the Theory section is

$$\frac{ds}{dt}=P_{\gamma\sigma}A\left[\frac{\gamma_s^e m_s^e}{1000}-\frac{\gamma_s^i(t)\,s(t)}{V(t)}\right]+(1-\sigma)\,\bar{C}_s\frac{V(t)-V(t-\Delta t)}{\Delta t} \tag{6d}$$

where

$$\bar{C}_s=\left[\frac{\gamma_s^e m_s^e}{1000}-\frac{\gamma_s^i(t)\,s(t)}{V(t)}\right]\Big/\left[\ln\frac{\gamma_s^e m_s^e}{1000}-\ln\frac{\gamma_s^i(t)\,s(t)}{V(t)}\right].$$

The integrated version is

$$\begin{aligned}s(t+\Delta t)=&\frac{V(t)}{P_{\gamma\sigma}A\gamma_s^i(t)}\left(\frac{P_{\gamma\sigma}A\gamma_s^e m_s^e}{1000}+h\right)\\&-\frac{V(t)}{P_{\gamma\sigma}A\gamma_s^i(t)}\left[\frac{P_{\gamma\sigma}A\gamma_s^e m_s^e}{1000}+h-\frac{P_{\gamma\sigma}A\gamma_s^i(t)}{V(t)}s(t)\right]\\&\cdot\exp\left[-P_{\gamma\sigma}A\gamma_s^i(t)\,\Delta t/V(t)\right]\end{aligned} \tag{7d}$$

in which h is the last term of Eq. (6d). The volume of cell water, $V(t)$, is again given by Eq. (5a).

Estimates of P Using Different Flux Equations

Values of P were estimated from the above equations, using parameters derived from the two procedures described earlier. The results are tabulated in Table 6 along with values for $\Delta H^{\ddagger}$. From Table 6 and Fig. 8*a* and *b*, we can conclude the following:

(1) For a given procedure, concentration, and temperature, all the equations give values of P that agree within 15%. However, the estimates of P from the hypotonic hemolysis method are about 50% more sensitive to the choice of the flux equation than are the estimates from the osmotic shock method (*cf.* Fig. 8*a* and *b*), probably because the end point for the osmotic shock measurements occurs much earlier in the time course of glycerol permeation. Since less glycerol has entered the cell at these early times, the values of γ_s^i and φ_s^i depart less from 1 than they would at later times, and as a result it makes less difference whether the internal concentration is expressed as m_s^i, $\gamma_s^i m_s^i$, or $\varphi_s^i m_s^i$.

(2) The choice of flux equation has little or no effect on the estimate of the activation energy for permeation.

Table 6. Estimates of P and $\Delta H^{\ddagger}$ using various flux equations

Nominal conc. glycerol (M)	Temp. (°C)	Estimates of P^a (cm/min × 10^6)								
		Hypotonic hemolysis method					Osmotic shock method			
		P_γ	$P_{\gamma\sigma}$	P_m	$P_{m\sigma}$	P_φ	P_γ	P_m	$P_{m\sigma}$	P_φ
0.27	20	1.12	0.99	1.13	1.00	1.13	—	—	—	—
	15	0.61	0.54	0.62	0.55	0.62	—	—	—	—
	10	0.30	0.26	0.30	0.26	0.30	—	—	—	—
	0	—	—	—	—	—	—	—	—	—
	$\Delta H^{\ddagger}$ (kcal/mole)	21.9	22.0	22.0	22.0	21.9	—	—	—	—
1	20	1.58	1.47	1.65	1.53	1.63	2.21	2.29	2.34	2.27
	15	0.83	0.76	0.87	0.79	0.86	1.18	1.23	1.26	1.22
	10	0.42	0.38	0.44	0.40	0.44	0.63	0.65	0.66	0.64
	0	~0.12	~0.11	~0.12	~0.12	~0.12	0.19	0.19	0.20	0.19
	$\Delta H^{\ddagger}$ (kcal/mole)	21.9	22.2	21.8	22.2	21.7	20.5	20.7	20.5	20.7
2	20	2.24	2.07	2.46	2.28	2.38	2.29	2.47	2.48	2.42
	15	1.15	1.04	1.26	1.14	1.22	1.19	1.29	1.29	1.26
	10	0.63	0.60	0.70	0.66	0.67	0.63	0.68	0.68	0.67
	0	—	—	—	—	—	0.17	0.19	0.19	0.18
	$\Delta H^{\ddagger}$ (kcal/mole)	21.1	20.6	21.0	20.4	20.9	20.5	20.6	20.6	20.7
3	20	2.31	2.09	2.69	2.43	2.65	2.71	3.07	3.06	3.00
	15	1.17	1.11	1.36	1.29	1.34	1.42	1.61	1.60	1.57
	10	—	—	—	—	—	0.78	0.89	0.88	0.87
	0	—	—	—	—	—	0.21	0.23	0.23	0.23
	$\Delta H^{\ddagger}$ (kcal/mole)	22.8	21.2	22.9	21.3	22.8	20.5	20.4	20.6	20.4

[a] The subscripts for P refer to the various flux equations used (*see text*).

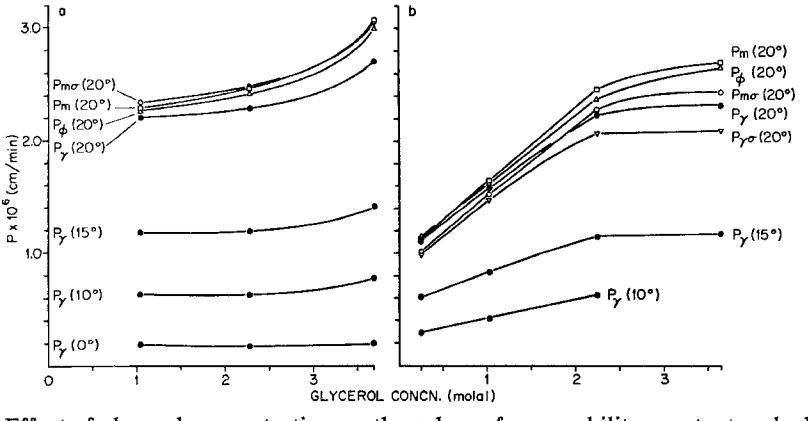

Fig. 8. Effect of glycerol concentration on the values of permeability constants calculated using various flux equations. (*a*) Values of P calculated by the osmotic shock method. (*b*) Values of P calculated by the hypotonic hemolysis method

(3) The use of irreversible thermodynamic expressions for the flux affects the values of P by less than 10% (*cf.* $P_{m\sigma}$ *vs.* P_m, and $P_{\gamma\sigma}$ *vs.* P_γ). This is not surprising in view of the fact that σ is 0.95 for glycerol permeation in the bovine red cell at 25 °C (Farmer & Macey, 1972).

Estimates of the Intracellular Concentration of Glycerol Based on the Various Estimates of P

Effect of Choice of Flux Equations. Fig. 9 compares the kinetics of the permeation of glycerol in isotonic saline-buffer at 20 °C using P_γ, $P_{\gamma\sigma}$, P_m, $P_{m\sigma}$ and P_φ. The solid curves give the results using the P values obtained by the hypotonic hemolysis method; the dashed curves give the results using the values from the osmotic shock method. The osmolal concentrations of glycerol in the cell at time t are simply $1000\,\varphi_s^i(t)\,s(t)/V(t)$.

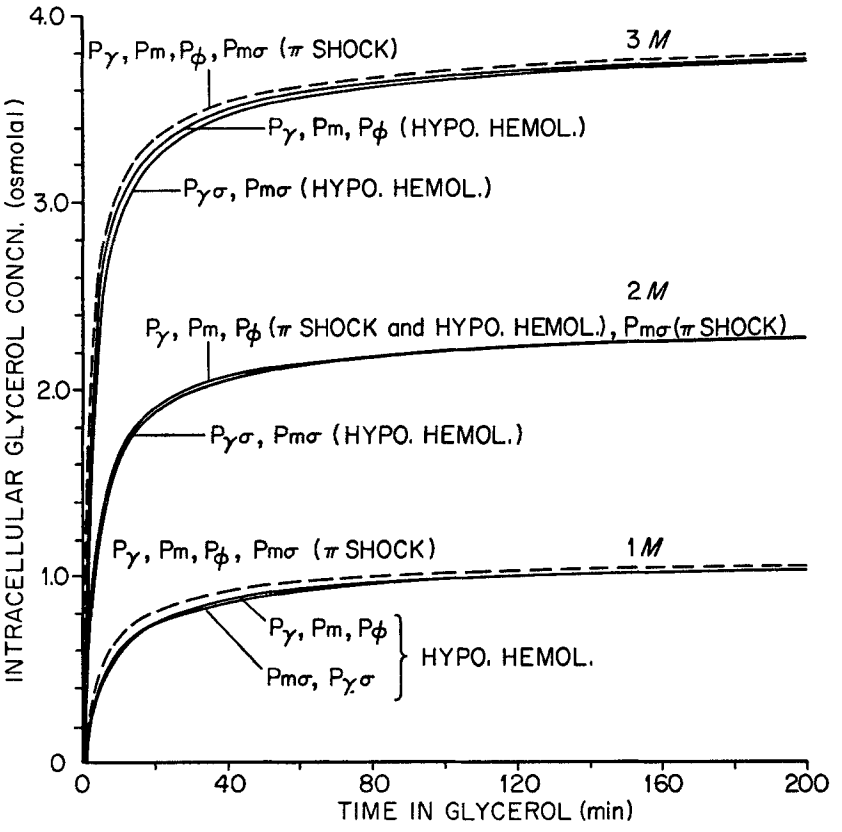

Fig. 9. Effect of the choice of flux equation and associated P on the calculated kinetics of permeation of glycerol into the bovine red cell at 20 °C, using values of P calculated by the hypotonic hemolysis method (——) and by the osmotic shock method (— — —) (*see* Table 6). The dashed and upper solid curves overlap in the case of 2 M glycerol

Even though the values of the various P's for a given technique and concentration of glycerol differ by as much as 15%, the concentrations of glycerol in the cell calculated by the several flux equations are practically indistinguishable at the scale drawn in Fig. 9. The reason that the calculated concentration of glycerol and the value of P differ in sensitivity to the choice of flux equations is that our approach puts constraints on the former but treats P as a completely adjustable parameter, which is adjusted to force the calculated volume of water in the cell to be equal to the critical volume at the observed time of hemolysis.

In spite of the fact that the choice of flux equations has little effect on the calculated concentrations of internal glycerol, we favor the view that the P of choice is P_γ and that the equations of choice are (5), (6) and (7). Eqs. (6) and (7) are the most satisfying conceptually, in that they express the driving force for permeation as the difference in *activities* of glycerol rather than as the difference in concentrations. Furthermore, P_γ shows the least dependence on the concentration of glycerol, a finding that is more consistent with those of Macey and Tolberg (1966) and Farmer and Macey (1972), i.e., that P for glycerol (estimated by photometric methods involving small volume changes) is constant over osmolalities ranging from 0.24 to 1.

Eqs. (5), (6) and (7) are "classical" equations that neglect σ and cross-coupling. Our reasons for emphasizing them rather than their irreversible counterparts [Eqs. (5a), (6d), and (7d)] are the following:

(1) From the pragmatic point of view, the value of σ in these cells is so nearly 1 (0.95 at 25 °C) that the introduction of σ produces little effect on the calculated value of P (*cf.* P_m *vs.* $P_{m\sigma}$, and P_γ *vs.* $P_{\gamma\sigma}$ in Fig. 8*a* and *b* and in Table 6) and only a barely detectable effect on the kinetics of permeation (Fig. 9).

(2) The value of σ will be even higher than 0.95 at lower temperatures, since lower temperatures increase the ratio of water permeability to glycerol permeability. The ratio increases because the activation energy for water permeation is only 4 kcal/mole (Farmer & Macey, 1970), whereas that for glycerol permeation is 21 kcal/mole.

(3) The derivation of Eq. (12) requires that rigorous equations describing the diffusional force in terms of differences in the chemical potential be transformed to an equation describing the force as terms of differences in the concentration of solute (Kedem & Katchalsky, 1958). This transformation involves a number of simplifying assumptions that are justified when the concentration of permeating additive is low and when the difference between the external and internal concentration is small. Unfortunately, in

the present studies neither of these restrictions applies, and because of this it is not clear that expressing fluxes in terms of Eq. (12) would in fact represent an improvement in either numerical accuracy or conceptual precision.

P_γ from the Hypotonic Hemolysis Method vs. P_γ from the Osmotic Shock Method. We noted in Table 5 that values of P_γ estimated by the osmotic shock method were 0 to 55% higher than those estimated by hypotonic hemolysis. (It can be seen from Table 6 that higher values from osmotic shock were also found in the case of P_m, $P_{m\sigma}$ and P_φ.) The dashed and solid curves in Figs. 9 and 10 show the extent to which these differences affect the estimated concentration of glycerol in the cell as a function of time. The differences in internal concentration are smaller than the differences between the two sets of P_γ values. Thus, in 1 M glycerol at 20 °C (Fig. 10), the estimated concentration of glycerol after 20 min is 0.82 osmolal using P_γ from osmotic shock, whereas it is 0.75 osmolal using P_γ from hypotonic hemolysis – a difference of some 9%. On the other hand, the two P values differ by 40% (Table 5).

One possible explanation for the discrepancy between estimates of P_γ made by the two methods lies in the way we determined V_c – namely, by abrupt dilution of the isotonic saline-buffer. This procedure produces a rate of dilution that is similar to that used to determine P from osmotic shock, both procedures causing the cell volume to increase rapidly. The

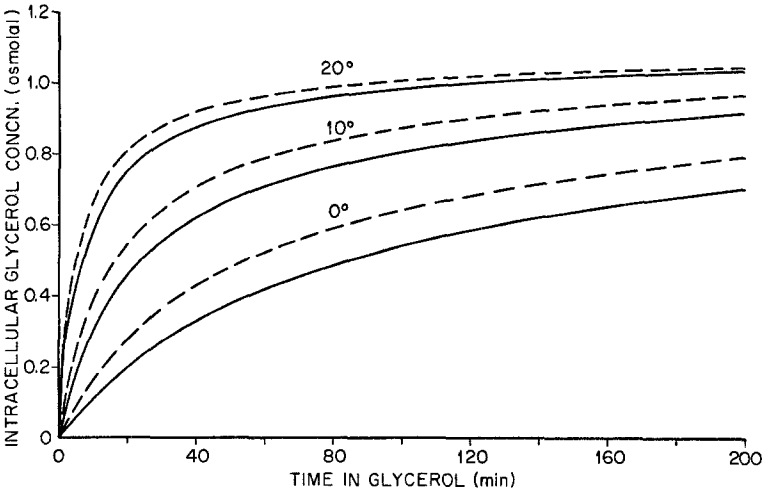

Fig. 10. Effect of experimental method of estimating P_γ on the calculated kinetics of permeation of glycerol into bovine red cells suspended in a solution of 1 M glycerol in isotonic saline-buffer at 0, 10 and 20 °C. P_γ values were calculated by the hypotonic hemolysis method (——) or by the osmotic shock method (– – –)

hypotonic hemolysis technique, on the other hand, produces a slow increase in cell volume (Fig. 1). It is well known that as the swelling rate is lowered, so too is the osmotic fragility of the red cell (Seeman *et al.*, 1969; Livne & Raz, 1971). That is to say, hemolysis in slowly diluted cell suspensions occurs at lower concentrations of NaCl than in rapidly diluted suspensions. Seeman *et al.* (1969) have shown by hematocrit measurements that although rates of dilution do not affect the hemolytic volumes of the human red cell, they do affect the prelytic leak of K^+ out of the cell, a conclusion supported by Livne and Raz (1971). Cells in slowly diluted suspensions lose more K^+ than do cells in rapidly diluted suspensions, and it is this leakiness that allows them to withstand somewhat lower external NaCl concentrations. If a leak occurs, the time required for 50% of the cells to swell to their hemolytic volume will be longer than it would be in the absence of a leak, and as a result our estimate of P will be too low. Whether this explanation can account quantitatively for the differences in the estimates of P by the two techniques remains to be seen.

Comparison with Published Data

Where comparisons are possible, our estimates for P and for the activation energy of P agree reasonably well with published values. For cells in isosmotic glycerol, Davson and Danielli (1952) and Jacobs (1952) have reported values of P at 20 °C of 1.02×10^{-6} and 1.17×10^{-6} cm/min. Our values (Table 6) range from 0.99 to 1.13×10^{-6} cm/min, depending on the flux equation used. The data of Jacobs *et al.* (1935) on cells in isosmotic glycerol correspond to an activation energy of 22.3 kcal/mole between 10 and 20 °C. Our value is 22.0 kcal/mole (Table 6).

Macey and Tolberg (1966) and Farmer and Macey (1972) report somewhat higher values for P in isosmotic glycerol: 2.9×10^{-6} and 6.5×10^{-6} cm/min, respectively (corrected to 20 °C). Since they found no concentration dependence of P, these values also apply to their data for 1 M glycerol. The value of 2.9×10^{-6} corresponds fairly closely to our values of 2.2 to 2.3×10^{-6} cm/min estimated by the osmotic shock technique for cells in 1 M glycerol at 20 °C (Table 6). (Their P in our terminology would be a $P_{\varphi\sigma}$.)

Appendix

Discussion of Assumptions

Variables. The integration of Eq. (6) assumes that s and t are the only variables. Although $\gamma_s^i(t)$ and $V(t)$ are not constants, they are held constant

Table 7. Effect of the value of N_n^i (osmoles of nonpermeating solute in the cell) on the calculated values of $V(t)$, $s(t)$, and $M_s^i(t)$ (osmolal concentration of intracellular glycerol)[a]

Time (min)	$V(t)$ when N_n^i is		$s(t)$ when N_n^i is		$M_s^i(t)$ when N_n^i is		Ratio[b]
	0.0003	0.0006	0.0003 (moles × 10^5)	0.0006	0.0003 (osmolal)	0.0006	
0	0.115	0.229	0	0	0	0	–
0.2	0.124	0.239	2.35	2.40	0.189	0.101	1.89
0.4	0.133	0.247	4.52	4.69	0.342	0.190	1.80
0.6	0.140	0.256	6.54	6.90	0.469	0.271	1.72
0.8	0.148	0.264	8.45	9.03	0.576	0.344	1.67
1	0.155	0.272	10.02	11.09	0.668	0.410	1.64
2	0.185	0.308	18.09	20.48	0.988	0.670	1.47

[a] Calculated for cells in 2 M glycerol plus isotonic saline-buffer at 20 °C, using $P_\gamma = 2.29 \times 10^{-6}$ cm/min.

[b] $M_s^i(t)$ when N_n^i is 0.0003/$M_s^i(t)$ when N_n^i is 0.0006.

during a given integration from t to $t + \Delta t$, and their values are recomputed for each successive iteration. The surface area of the cell, A, has been shown to remain constant in a given mammalian red cell regardless of its volume (Ponder, 1955).

Osmoles of Intracellular Solute. In the iteration we assume that the number of osmoles of nonpermeating solutes in the cell (N_n^i) [Eq. (3)] or its equivalent M_{iso}^i [Eq. (4)] remains constant ($N_n^i = 0.0003$, $M_{iso}^i = 0.301$). This is not strictly true. Gary-Bobo and Solomon (1968) have shown that the charge on hemoglobin in the shrunken human red cell is reduced, and as a consequence there is a net inflow of Cl^- to neutralize the released cations. The net result is an increase in the number of moles of solute in the shrunken cell at time zero. Furthermore, the osmotic coefficients of electrolytes and of hemoglobin rise considerably above 1 at high concentrations (Robinson & Stokes, 1959; Gary-Bobo & Solomon, 1968). The result of both effects is to make our calculated values of $V(t)$ smaller at the early stages of permeation than they would be in actuality (Table 7). If we underestimate $V(t)$, the effect in Eq. (7) is to underestimate $s(t)$ slightly; i.e., slightly more glycerol enters the cell in the early stages than we are calculating. However, the error is small. As shown by the example in Table 7, underestimating N_n^i by as much as a factor of 2 results in an underestimate of $s(t)$ by about 10%.

Although our calculations slightly underestimate the moles of glycerol that enter the cell, they *overestimate* the *concentration* of intracellular glycerol, since the concentration is proportional to $s(t)/V(t)$. As shown in

the last column of Table 7, the factor by which the concentration of glycerol, $M_s^i(t)$, is overestimated is somewhat less than the factor by which N_n^i is underestimated; i.e., if our value of $N_n^i = 0.0003$ were only half of what it should be early in permeation, then the concentration of intracellular glycerol that we are calculating would be 90% too high initially and 50% too high after 2 min. Of course as the cell swells, the value of N_n^i will decrease towards 0.0003, and this source of error will diminish and finally disappear. The magnitude of the error is difficult to estimate, since we know neither the correct value of N_n^i in highly shrunken bovine cells nor by how much it changes as the cell swells.

Impermeability of Other Solutes. The assumption is made on p. 112 that all solutes other than glycerol are nonpermeating. We have discussed on p. 132 the consequences of departures from this assumption which occur in the form of a K^+ leak in cells approaching their hemolytic volume. Cation leaks also occur in shrunken human red cells in hyperosmotic solutions of nonpermeating additives (Meryman, 1971*b*; Farrant & Woolgar, 1972*a*, *b*) but apparently do not occur in red cells in hyperosmotic solution of the permeating solute DMSO (Farrant, 1972). If they occur in bovine cells shrunken in hyperosmotic glycerol, and if as a result there were a net outward flow of ions, the result would be a reduction in the number of moles of solute in the shrunken cell, i.e., the opposite of the effects discussed in the preceding paragraph.

Calculation of Osmolalities and Activities. The osmolalities of the various components in the solutions were calculated by multiplying the molality of each by its osmotic coefficient (φ), using published values of φ for the single solutes in water. The osmolality of the whole solution was taken to be $(\varphi \nu m)_{\text{glycerol}} + (\varphi \nu m)_{\text{NaCl}} + (\varphi \nu m)_{\text{buffer}}$, where ν is 1 and 2 for glycerol and NaCl, respectively, and $\varphi \nu$ is 2.43 for the buffer.

The approximate error involved in such calculations can be estimated from freezing point depression measurements of solutions of glycerol in NaCl plus water. A. MacKenzie and D. Rasmussen (*personal communication*) have made such measurements. If one calculates the osmolalities by the usual relation, $(\varphi \nu m)_{\text{total}} = \Delta T_f / 1.86$ (Lewis & Randall, 1961), the resulting values exceed our estimates of total osmolalities by about 6%. (Similar calculations for solutions of dimethyl sulfoxide, NaCl, and water give agreement between calculated and measured osmolalities of better than 1%.)

Although we could have computed the total osmolality of the suspending media from freezing-point measurements, we would still not have known

the osmotic coefficients of the individual solutes required in Eqs. (5), (5a), (6c) and (10a).

This research was sponsored by the U.S. Atomic Energy Commission under contract with the Union Carbide Corporation. We thank Drs. J. S. Cook and W. D. Fisher for detailed review of the manuscript.

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