

Glycerol permeability of human fetal and adult erythrocytes and of a model membrane

THOMAS J. MOORE

St. Luke's Hospital Center, New York 10025, and Columbia University College of Physicians and Surgeons, New York 10032

ABSTRACT When erythrocytes from different mammalian species are compared, the hemolysis rate in 0.3 M glycerol is seen to be directly related to the percentage of lecithin in the erythrocyte phospholipid. Since this percentage is higher in erythrocytes from human adults than in those from infants, the hemolysis times in 0.3 M glycerol were compared. As expected, hemolysis was more rapid in the adult cell, which is therefore more permeable to glycerol under these conditions.

The permeability to glycerol of a film of erythrocyte lipids in vitro was next examined in a model system containing the two phases water and butanol. Lipid introduced into the bulk butanol appears as a film at the interface. When equal amounts of total lipid extracted from adult and fetal erythrocytes were introduced into the butanol phase of two such chambers, the initial flux of glycerol-¹⁴C across the lipid boundary was greater in the cell containing lipid from adult erythrocytes than in the cell containing fetal erythrocyte lipid. This difference corresponds qualitatively to the difference in hemolysis time measured in the intact erythrocytes.

KEY WORDS adult · fetal · erythrocyte · phospholipid · composition · interfacial transfer · glycerol · permeability coefficient · hemolysis

DURING HUMAN development the phospholipid composition of erythrocyte membranes changes. Crowley, Ways, and Jones, studying fetal and adult erythrocytes (1), reported small but significant differences in the contents of lecithin, phosphatidyl ethanolamine, and pooled phosphatidyl inositol, and sphingomyelin. The alteration in the phospholipid composition raises the interesting question whether the permeability of the cell membrane is concomitantly altered.

Abbreviation: TLC, thin-layer chromatography.

Van Deenen and de Gier (2) have summarized data from a number of laboratories and have related the 75% hemolysis time in 0.3 M glycerol to the amount of sphingomyelin and lecithin present in the erythrocyte membrane for a variety of mammalian species. As the content of sphingomyelin increases relative to that of lecithin, the hemolysis time is prolonged.

The purpose of the work reported here was to repeat the studies of Crowley et al. (1) on erythrocytes from adults and term infants with respect to composition of the membrane lipid, to measure differences in glycerol permeability of the erythrocytes, and to measure glycerol transport in vitro across films of lipid extracted either from adult erythrocytes or from fetal erythrocytes.

MATERIALS AND METHODS

Analysis of Lipid Composition

Venous blood for adult samples was aspirated from the antecubital fossa of 20 donors; for fetal (term infant) samples blood was aspirated from an umbilical vessel in the Delivery Suite from an equal number of donors.

Total lipids were extracted by procedure III of Ways and Hanahan (3). The extracted lipid was a clear, amber oil. Gravimetric estimation of lipid content gave 4.79 mg of lipid per ml of packed cells (obtained at 1,000 g) on nine determinations from normal adult blood. These values agreed closely with a mean of 4.88 mg of lipid per ml of packed cells previously reported (3).

Lipid classes were isolated by the method of Gluck (4). The eluates yielded three fractions: nonphospholipid, "nonacidic phospholipid" (lecithin, sphingomyelin, phosphatidyl ethanolamine), and "acidic phospholipid" (phosphatidyl serine and phosphatidyl inositol).

Thin-Layer Chromatography

250 g of Silica Gel H was washed with 2 liters of methanol-chloroform-formic acid 2:1:1 followed by 1 liter of doubly distilled water. The washed silica gel was dried at 100°C for 48 hr. A slurry of the gel (30 g/70 ml of water) was applied to thin-layer plates in a layer 250 μ thick. The plates were dried at 110°C for 30 min and lipid, at a concentration of 2–5 mg/ml, was applied by means of a 50 μ l Hamilton microsyringe.

Brinkmann TLC tanks lined with Whatman No. 1 filter paper were filled with the appropriate solvent mixture and allowed to settle for 30 min before the thin-layer plate was introduced. The solvent mixtures used were petroleum ether–diethyl ether–glacial acetic acid 130:30:2 for nonphospholipid, and chloroform–methanol–water 130:50:8 for “nonacidic phospholipid” and “acidic phospholipid.” The solvent front was allowed to run 10 cm. The plate was then air-dried and transferred to a tank containing iodine crystals until the spots just became visible. The spots were outlined with a needle, the residual iodine was allowed to evaporate in air, and the spots were scraped off the plate and transferred to 20-ml Pyrex test tubes (5). 2 ml of concentrated sulfuric acid was added to each tube. The tubes were heated to 200°C for 15 min in an aluminum block inside a Blue M Stabil-Therm oven, and then immediately transferred to an ice bath. 3 ml of water was added and the contents were mixed and centrifuged. Absorbance was measured in a Zeiss model PMQ II spectrophotometer at 375 $m\mu$.

Evaluation of the Method

Standard curves for each fraction isolated were prepared by applying to the TLC plate a commercial standard (Applied Science Laboratories Inc., State College, Pa.) serially from 20 to 80 μ g. A plot of absorbance against amount of lipid applied to the thin-layer plate indicated linearity throughout the range studied for each lipid class.

Completeness of recovery was assessed as follows. Triplicate microcolumns were prepared as described by Gluck (4). 5–7 mg samples of total erythrocyte lipid were added to columns 1 and 2; 1 mg samples of various commercially available lipid species (Applied Science Labs.) were added to columns 2 and 3. Elution, TLC, and charring spectrophotometry were performed on each of the three original samples. The procedure was repeated for each of the isolated groups of lipids. Percentage recoveries for a single trial were as follows: cholesterol 93%; cholesteryl esters 96.1%; lecithin 94.3%; sphingomyelin 98.8%; phosphatidyl ethanolamine 106.7%; phosphatidyl serine 110%. When the samples were ultimately determined, duplicate samples,

duplicate standards for each lipid class, and a blank were run for the TLC and charring spectrophotometry.

Permeability Studies

0.5 ml of sedimented erythrocytes was brought to 50 ml volume in phosphate-buffered (pH 7.4) isotonic saline. 1 ml of these resuspended cells was transferred to 15 ml of phosphate-buffered saline; another 1 ml was transferred to an equal volume of phosphate-buffered 0.3 M glycerol and allowed to hemolyze completely. Serial dilutions of hemolyzed cells were prepared by mixing at 1 ml increments 1–10 ml of the unhemolyzed solution with 9–0 ml of the completely hemolyzed solution. Absorbance was immediately read at 675 $m\mu$. A linear plot of absorbance against percentage of hemolysis was obtained for every sample in the study.

The number of seconds after initial mixing at which ten predetermined absorbance values were reached was measured. The absorbance values were translated into percentage of hemolysis by means of the standard curve. In this fashion a hemolysis curve was obtained.

Model Studies

Diffusion of solute across a butanol–water interface differs from the diffusion through a homogeneous medium. When lipid is introduced into the butanol phase an oily film appears at the interface, offering a greater resistance to the movement of solute compared to its movement across a clean interface. Variation of the chemical composition of the lipid introduced into the butanol phase might have an effect upon the resistance offered to the movement of solute.

The model used was the Schulman chamber (6, 7). The apparatus consists of battery jars with glass covers and double-bladed stirrers on each stirring shaft. The stirring blades were rotated at a constant speed of 20 rpm. A six-unit Phipps and Bird electric stirrer allowed four simultaneous experiments to be performed. For all experiments 10 ml each of 1 M sodium and potassium propionate and 250 ml of distilled water and 200 ml of *n*-butanol, mutually saturated, were placed in the jars and allowed to equilibrate. 2 mg of total lipid from the erythrocyte sample under investigation was introduced into each cell. Then 10 ml of 8.4 M glycerol containing 40 μ l of glycerol- U - ^{14}C , 0.1 mc/ml, was injected into the aqueous phase so that the glycerol concentration of the aqueous phase at time zero was 0.3 M. The butanol and aqueous phases were independently stirred by the double-bladed stirrers, and six 0.6 ml samples were taken from the butanol phase at 10-min intervals. After samples had been collected the whole system was vigorously mixed and stirred and then allowed to clear for several hours before samples were drawn from both phases for the determination of the equilibrium distribution coeffi-

cient (P_{ow}). Samples were transferred to scintillation vials containing 15 ml of Bray's solution and counted in a Packard model 4000 spectrometer. Cpm were converted to dpm.

The coefficient for rate of transfer of solute across the interface from butanol to water, K_{ow} , is given by the following expression (8).

$$K_{ow}t = -\ln(1 - C_o/C_e) / \{A/V_o(aP_{ow} + 1)\}$$

where C_o = concentration of glycerol in the alcohol phase at time t , C_e = concentration of glycerol in the alcohol phase at equilibrium, A = area of interface, V_o = volume of alcohol phase, a = ratio of V_o to volume of water phase, and P_{ow} = distribution coefficient of glycerol, oil/water. The rate of transfer from water to butanol is given by the simple relationship $K_{wo} = P_{ow}K_{ow}$. Results are expressed in this paper in terms of K_{ow} , which is the slope of the straight line that results when the right-hand side of the equation is plotted against t .

Four chambers were used simultaneously, two containing lipid from fetal erythrocytes and two containing lipid from adult cells. The difference between the slopes of the plots of $K_{ow}t$ against t was tested for significance by an analysis of covariance. To exclude the possibility that we were measuring differences between chambers or stirrers, we marked the chambers and reversed the sequence for each experiment.

RESULTS

Lipid Composition

Lipid composition of adult and term fetal erythrocytes (Table 1) show results for cholesterol and cholesteryl ester in reasonable agreement with those published elsewhere (1,2). Adult and fetal erythrocytes showed no significant difference. The percentage of lecithin in the phospholipids was higher in adult than in fetal erythrocytes, apparently at the expense of the other phospho-

TABLE 1 LIPID COMPOSITION OF ADULT AND TERM FETAL ERYTHROCYTES

	Adult	Fetal	Significance
<i>mg/ml packed cells</i>			
Cholesterol	1210 ± 242	1340 ± 174	NS
Cholesteryl esters	123 ± 30.2	107 ± 43.5	NS
<i>% of total phospholipids</i>			
Lecithin	30.1 ± 4.1	22.6 ± 2.4	$P < 0.05$
Sphingomyelin	28.7 ± 6.5	29.6 ± 7.7	NS
PE + PS + PI	41.0 ± 8.5	46.4 ± 9.5	NS

Values are means ± SD ($n = 9$). PE, PS, PI are phosphatidyl ethanolamine, serine, and inositol, respectively. Values for phospholipids expressed as mg/ml of packed cells showed greater variability.

glycerides, although the difference for the latter between adult and fetal was not significant because of the high degree of variability after the several chromatographic steps (9). The results of Crowley et al. (1) indicated that the increase in lecithin in the adult was at the expense of sphingomyelin and inositol glycerophosphatides.

Hemolysis Time in 0.3 M Glycerol

Fig. 1 illustrates a hemolysis curve in 0.3 M glycerol for adult and fetal blood samples. The hemolysis time, for any percentage of hemolysis measured, is longer in the fetal cells. Table 2 shows that the mean 50% hemolysis time in 0.3 M glycerol is significantly shorter in the adult cells.

Glycerol Transfer Across Interface

Fig. 2 is a plot of $K_{ow}t$ against t for experiment 2 of Table 3. Table 3 indicates consistently and significantly greater K_{ow} values for the chambers containing lipid from the adult erythrocytes. The considerable variability in K_{ow} values among the various trials may reflect uncontrollable fluctuations in stirring speed during each experiment. The intercept of the lines in Fig. 2 on the abscissa can be attributed to a finite time of mixing and diffusion of the injected glycerol in the water layer before it penetrates the interfacial film.

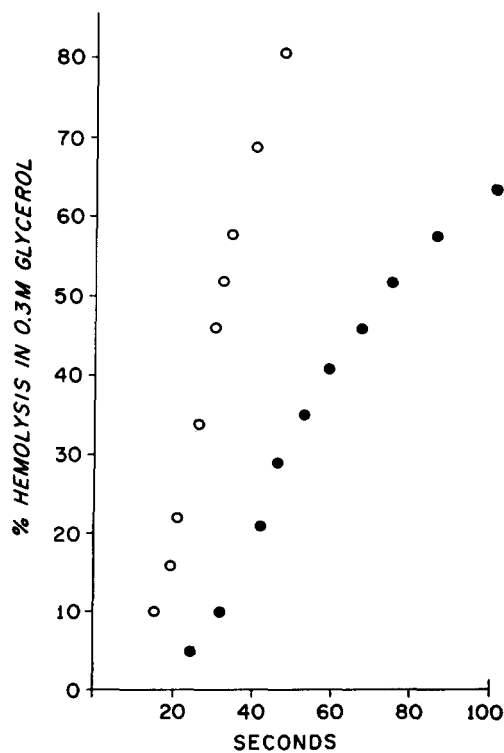


FIG. 1. Hemolysis curve of human adult (O) and term fetal (●) erythrocytes in 0.3 M glycerol at 21°C.

TABLE 2 HEMOLYSIS TIME OF HUMAN ADULT AND FETAL ERYTHROCYTES IN 0.3 M GLYCEROL

Erythrocytes	Mean Temperature °C	Mean 50% Hemolysis Time sec	n	P
Adult	20.8	32.0 ± 2.9	6	<0.001
Term fetal	19.3	65.5 ± 8.0	6	

Values are means ± SD. Each of the 12 samples was obtained from a different donor.

TABLE 3 INTERFACIAL TRANSFER COEFFICIENT FOR GLYCEROL ACROSS FILMS OF LIPID FROM ADULT AND FROM TERM FETAL ERYTHROCYTES

Expt.	No. of Samples Taken for Counting	K_{ow}		F Value of Analysis of Covariance, K_{ow}	P
		Adult	Fetal		
		mmoles/hr			
1	24	56.6	35.7	17.7	<0.01
2	23	28.0	23.4	8.18	<0.01
3	24	60.1	51.2	5.22	<0.05
4	40	64.1	54.8	28.9	<0.01

DISCUSSION

Analysis of Lipid Composition

Implicit in the analysis of lipid composition is the assumption that the measured differences in lecithin percentage reflect differences that occur at the surface of the cell. Conditional acceptance of this assumption requires evidence that the lipids occupy sites on the surface of the cell and that differences in cell age in otherwise normal individuals do not significantly influence the reported values.

Van Deenen and de Gier (2) report that the total lipid extracted from ghosts and from intact cells is virtually the same. Alterations in lipid content during cell aging have been studied in the normal adult. Westerman, Pierce, and Jensen (10) found no difference in total lipid or phospholipid phosphorus between old and young cells from the same subject when results were expressed per surface area.

The reticulocyte count of the term infant averages 2% (11), and was presumably lower in the samples analyzed since buffy coat was removed three times during the erythrocyte washing procedure. For these reasons the measured difference in lecithin content is not attributed to the presence of this cell type in the fetal blood.

Hemolysis

The difference in hemolysis time might be accounted for in three ways: (a) the two cell types differ in the permeability to glycerol; (b) the differences measured represent enrichment of the fetal sample with immature

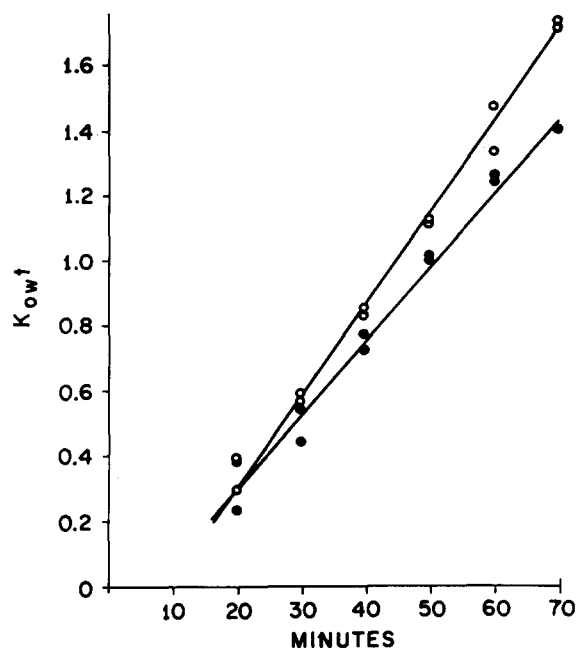


FIG. 2. Plot of $K_{ow}t$ against t for 0.3 M glycerol diffusing from water to butanol across a lipid film. Two chambers contained lipid from adult erythrocytes (○), two contained lipid from term fetal erythrocytes (●). The slope is K_{ow} , the interfacial transfer coefficient.

cells; (c) adult and fetal erythrocytes differ in mean cell diameter.

If the difference in hemolysis time were due to an enrichment of the fetal cells with immature cell types, the mature cell types in the fetal blood, representing the majority of the sample, should hemolyze at the same rate as the adult cell. The expected effect of the immature cell type on the hemolysis curve would be a departure from the sigmoid hemolysis curve after the majority of cells had hemolyzed. Inspection of Fig. 2 indicates that already at 10% hemolysis there exists a measurable difference in hemolysis time. This difference is seen to increase in a regular fashion as more cells become hemolyzed. The notion that immature cell types are in fact determining the difference in hemolysis curves can therefore be rejected.

The adult erythrocyte possesses a mean cell diameter of 7.5 μ , while the fetal erythrocyte at term possesses a mean cell diameter of 8.0 μ . That these differences might account for differences in hemolysis times is refuted by the following discussion. Schiödt (12), studying penetration rates of solute entering the human erythrocyte, has developed the following expression for relating the rate of entrance of permeant to changes in cell volume:

$$Kt = \frac{1}{2} [(V - X)^2 - (V_0 - X)^2] / (V_0 - X)$$

where K = permeability coefficient, V = cell volume at time t , expressed as percent of initial volume; V_0 = ini-

tial cell volume, expressed as 100 in isosmotic solutions; and X = the volume of the dispersion phase of the erythrocyte that takes no part in swelling or shrinking. Schiödt assigned to X an empirical value of 50.

The above equation relates changes in the volume of a cell to the permeability coefficient. V_0 under the conditions of the present experiment is the mean cell volume, and V the maximum cell volume or the cell volume at which hemolysis occurs.

Guest and Wing (13) found the maximum cell volume to be 171–185% of the mean cell volume in the adult and 153–165% in fetal blood. Introducing these values and the time values reported in Table 2 into the original expression should yield K values in reasonable agreement with each other if the difference in hemolysis time were, in fact, due only to a difference in cell size and change in cell volume.

$$\text{Adult } K_A = \frac{1}{2}[(178-50)^2 - (100-50)^2]/(100-50)$$
$$K_A = 4.34$$

$$\text{Fetal } K_F = \frac{1}{2}[(159-50)^2 - (100-50)^2]/(100-50)$$
$$K_F = 1.45$$

The two K values disagree by a factor of three. It seems reasonable, therefore, to conclude that the differences in hemolysis time reflects not differences in cell size but differences in the rate of entry of glycerol into the two cell types.

Model Studies

The permeation of glycerol was further studied in a two-phase system of water and butanol that contained erythrocyte lipids at the interface. Minutes after introducing the lipid into the butanol a continuous film appears at the interface. No attempt was made to measure the composition of lipid in the bulk butanol phase as opposed to the interface. The rate of glycerol transfer across

this interface depended on whether adult or fetal lipid was used. It qualitatively corresponded to the differences in hemolysis time in the intact cells; that is to say, glycerol moved across the "adult lipid" boundary faster, just as adult erythrocytes were hemolyzed in a shorter time. The mechanism for this difference is unknown at present.

The author acknowledges the generosity and encouragement of the late Professor Jack Schulman, of Columbia University; the use of the Stanley Thompson Laboratories, Henry Krumb School of Mines, Columbia University; and the technical assistance of Mrs. Vera David and Miss Barbara Schlowsky.

This investigation was supported by U. S. Public Health Service General Research Support Grant FE-05501 from the Division of Research Facilities and Resources.

Manuscript received 27 November 1967; accepted 25 June 1968.

REFERENCES

1. Crowley, J., P. Ways, and J. W. Jones. 1965. *J. Clin. Invest.* **44**: 989.
2. Van Deenen, L. L. M., and J. de Gier. 1964. In *The Red Blood Cell*. C. Bishop and D. M. Surgenor, editors. Academic Press, Inc., New York, Chapter 7.
3. Ways, P., and D. Hanahan. 1964. *J. Lipid Res.* **5**: 318.
4. Gluck, L., M. V. Kulovich, and S. J. Brody. 1966. *J. Lipid Res.* **7**: 570.
5. Marsh, J. B., and D. B. Weinstein. 1966. *J. Lipid Res.* **7**: 574.
6. Rosano, H. L., P. Duby, and J. H. Schulman. 1961. *J. Phys. Chem.* **65**: 1704.
7. Schulman, J. H. 1966. *Ann. N.Y. Acad. Sci.* **137**: 860.
8. Ting, H. P., G. L. Bertrand, and D. F. Sears. 1966. *Biophys. J.* **6**: 813.
9. Dodge, J. T., and G. B. Phillips. 1966. *J. Lipid Res.* **7**: 387.
10. Westerman, M. P., L. E. Pierce, and W. N. Jensen. 1959. *J. Clin. Invest.* **38**: 1054.
11. Smith, N. J., and V. C. Vaughan. 1964. In *Textbook of Pediatrics*. W. E. Nelson, editor. W. B. Saunders Co., Philadelphia, 8th edition. 1007.
12. Schiödt, E. 1933. *J. Gen. Physiol.* **16**: 977.
13. Guest, G. M., and M. Wing. 1942. *J. Clin. Invest.* **21**: 257.