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SOME ASPECTS OF THE OSMOTIC LYSIS OF ERYTHROCYTES

III. COMPARISON OF GLYCEROL PERMEABILITY AND LIPID COMPOSITION OF RED BLOOD CELL MEMBRANES FROM EIGHT MAMMALIAN SPECIES

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SUMMARY

1. The glycerol permeability coefficient at 37 °C and pH 7.5 was determined for erythrocytes from eight mammals and appeared to vary from $20 \cdot 10^{-6}$ cm·s^{-1} to $0.30 \cdot 10^{-6}$ cm·s^{-1} for the extremes, rat and sheep, respectively.

2. The variability of the permeability coefficient was relatively small (10–20 %) for all species, except for the rabbit (70 %).

3. Animals from the group with low glycerol permeability showed another sequence according to their permeability coefficients than according to their times of hemolysis.

4. No distinct correlation between the lipid composition, including phospholipid and fatty acid composition, and the glycerol permeability coefficient could be established. Only the animals with a low permeability demonstrated a sequence with an increase of the chain length and a decrease of the mean number of double bonds of the hydrocarbon chains of the lipids in combination with a decrease in the glycerol permeability of the red blood cell.

INTRODUCTION

In the preceding papers[1,2] a modified osmotic lysis method was introduced. Moreover, it was shown that the observed lysis delay was due to chloride–hydroxyl ion exchanges[2]. In this paper the glycerol permeability coefficients of red blood cell membranes from eight mammalian species will be compared with times of lysis. Although the time of hemolysis gives an impression of the red blood cell permeability the permeability coefficient determined with the osmotic lysis method proved to be more reliable.

De Gier and co-workers[3–5] demonstrated considerable differences in non-electrolyte permeability of liposomes with varying lipid composition. Earlier[6] they suggested a correlation between lipid composition and permeability of red blood cell membranes. Such a correlation seemed to be less probable later[7]. In the second part of this paper we compare our more accurate permeability data with our own,

and published data on the lipid composition of erythrocyte membranes to ascertain, if a correlation between these parameters exists. Differences in phospholipid composition did not correlate with the large variations in glycerol permeability. The fatty acid composition of the same erythrocytes was investigated to determine the characteristics of the hydrocarbon chains of the lipids.

MATERIALS AND METHODS

The permeability of untreated red blood cells to glycerol was determined at 37 °C in hypotonic and isotonic glycerol solutions, buffered with 1 mM sodium phosphate to pH 7.5. In the case of rat, man and rabbit the permeability was also studied in hypertonic glycerol solutions. Times of hemolysis were determined in 0.3 M glycerol, buffered with 1 mM sodium phosphate to pH 7.5. Technical details are described previously[1].

Mean cell volume in plasma or serum was determined with the [^{14}C]inulin dilution method[2]. The mean critical cell volume was determined by means of a modification of the same method[1].

Lipid extraction and analysis of fatty acids

Erythrocytes were treated three times with 2 vol. 0.9% NaCl solution after removal of the buffy coat. Lipids were extracted from 2.5 ml erythrocytes with methanol and chloroform, containing 50 mg butylated hydroxytoluene per l, according to Method I described by Broekhuyse[8]. The lipid extracts were dissolved in 1 ml benzene–methanol (4:1, v/v) and stored at −25 °C.

Aliquots of 0.4 ml lipid solution were used for the analysis of fatty acid composition. After evaporation at 25 °C *in vacuo*, 0.5 ml of hexane was added. Fatty acid methyl esters were prepared by methanolysis with 1 ml of BF_3–methanol (10%, w/v) at 100 °C for 15 min[9]. The fatty acid composition was assayed on a column of 15% diethyleneglycol succinate on 60–80 mesh Gas-Chrom P at 180 °C in a Packard Model 7821 gas chromatograph. Details of identification of the components were described previously[10].

RESULTS AND DISCUSSION

The ratio of the permeability coefficient (P) and the critical cell radius (R_h) were calculated from the lysis curves of untreated red blood cells in glycerol at 37 °C and pH 7.5 with Eqns 7 and 9 (ref. 1). We preferred to determine the permeability at 37 °C, since at low temperature the observed marked differences in times of lysis[6] might be due to pre-lytic loss of ions and to differences in hemoglobin release. The permeability coefficients at the 50% lysis level were calculated from the P/R_h ratios and the R_h values (Table I). These data are compared with the times of lysis, *i.e.* the time elapsing before 50% lysis has occurred in 300 mM glycerol. It is theoretically possible to determine the permeability coefficient at different levels of lysis, which represent different fractions of the red blood cell population. However, this appeared impossible in this study, since only the mean critical radius is known. The permeability coefficient at the 50% lysis level represents the average permeability of the red blood cell population. The hemolysis times suggest that

TABLE I

COMPARISON OF CALCULATED PERMEABILITY COEFFICIENTS AND LYSIS TIMES FOR GLYCEROL

The mean cell volume in plasma or serum (V_i in μm^3), the critical cell volume (V_h in μm^3) and the critical radius (R_h in 10^{-4}cm) were determined in duplicate for 3 subjects. P/R_h ($\times 10^3$) ratios were determined for the number of animals given between parentheses. Permeability coefficients (P in 10^{-6} cm·s^{-1}) and times of hemolysis (t_h in s)with their standard deviations were determined at the 50% lysis level for the number of animals given between parentheses.

Species	V_i	V_h	R_h	P/R_h	P	t_h
Rat (6)	62.7 ± 3.7	100.2 ± 3.3	2.89 ± 0.07	69.1 ± 11.4	19.96 ± 3.28	5 ± 1
Man (7)	87.7 ± 5.7	137.6 ± 3.6	3.20 ± 0.01	57.1 ± 13.2	18.27 ± 4.23	13 ± 2
Rabbit (10)	64.3 ± 2.3	104.3 ± 1.1	2.92 ± 0.01	12.4 ± 9.0	3.65 ± 2.62	57 ± 30
Dog (3)	65.1 ± 1.3	79.8 ± 2.1	2.67 ± 0.05	5.1 ± 0.8	1.37 ± 0.20	164 ± 8
Horse (8)	49.7 ± 2.5	58.6 ± 2.5	2.40 ± 0.06	4.3 ± 0.5	1.02 ± 0.12	114 ± 12
Pig (9)	61.5 ± 2.3	93.5 ± 1.5	2.85 ± 0.05	2.4 ± 0.2	0.70 ± 0.05	177 ± 11
Ox (7)	51.1 ± 3.5	99.8 ± 0.9	2.88 ± 0.02	1.3 ± 0.2	0.43 ± 0.09	562 ± 50
Sheep (8)	35.3 ± 2.5	55.4 ± 0.4	2.34 ± 0.01	1.4 ± 0.1	0.32 ± 0.01	258 ± 21

dog erythrocyte membranes are less permeable to glycerol than those of horse and the same can be said for ox and sheep red blood cell membranes. The glycerol permeability coefficients, however, demonstrate the reverse.

The variability in the permeability coefficient and the time of hemolysis do not seem to be due to variations in the P/R_h ratios and to inaccuracies of the determination of R_h, since the reproducibility of the determinations appeared to be sufficiently precise (Table I). Since few values of the critical cell volume are given in the literature, the mean cell volumes (V_i) found in plasma or serum were compared with data from the literature[11-13] in order to check the determination of cell volume with [^{14}C]inulin. Our data appeared to be quite consistent. The determination of the critical volume appears to be reliable, since the figure obtained for human erythrocytes comes close to the values given by Canham[14] and Seeman *et al.*[15] (147 and 152 μm^3, respectively).

Obviously the discrepancies in sequence of the glycerol permeability coefficients and the times of hemolysis are real. Probably the discrepancies are due to variations in osmotic resistance and lysis delay. Coldman *et al.*[16] observed considerable variations in osmotic resistance for erythrocytes from some mammalian species. Whereas in general within each species the standard deviations for the P/R_h ratio, as well as the permeability coefficient ranged from 10 to 20%, much higher standard deviations (up to 72%)were observed for rabbit erythrocytes. This led us to study the permeability behaviour of these erythrocytes to glycerol in more detail. The results will be presented in an other paper. It is impossible to compare our glycerol permeability coefficients and times of hemolysis with data from the literature[17-19], since the published experiments were performed at undefined room temperatures. Though de Gier *et al.*[7] carried out some experiments at different temperatures, no comparable times of hemolysis can be obtained from their publication.

Comparison of lipid composition and glycerol permeability of mammalian red blood cells

In Table II the permeability coefficients for glycerol are compared with the relative amounts of phospholipid, cholesterol and glycolipid in red blood cell mem-

TABLE II

RELATIVE AMOUNTS OF PHOSPHOLIPID, CHOLESTEROL AND GLYCOLIPID IN RED BLOOD CELL MEMBRANES COMPARED WITH THEIR GLYCEROL PERMEABILITY COEFFICIENTS

Lipid components are given in percentages of total lipid. Moreover, the molar ratio of cholesterol and phospholipid is presented. The glycerol permeability coefficient, P (glycerol), at pH 7.5 and 37 °C is given in $cm \cdot s^{-1} \times 10^6$. Data on lipid composition were taken from Rouser *et al.*[20].

Species	*Glycolipid*	*Phospholipid*	*Cholesterol*	*Cholesterol/ Phospholipid*	*P(glycerol)*
Rat	8.3	67.0	24.7	0.74	20.0
Man	—	58.0	23.0	0.79	18.3
Rabbit	5.3	65.8	28.9	0.88	3.6
Dog	22.7	52.6	24.7	0.94	1.4
Horse	23.5	52.0	24.5	0.94	1.0
Pig	13.4	59.8	26.8	0.90	0.7
Ox	7.7	64.8	27.5	0.85	0.4
Sheep	10.3	63.2	26.5	0.84	0.3

TABLE III

PHOSPHOLIPID COMPOSITION AND GLYCEROL PERMEABILITY OF MAMMALIAN RED BLOOD CELLS

Data from Nelson[21] for all animal species and from Rouser *et al.*[20]. for human red blood cells were compared with the glycerol permeability coefficients, P(glycerol), in $cm \cdot s^{-1} \times 10^6$. Abbreviations: LPC: lysophosphatidylcholine; S: sphingomyelin, PC: phosphatidylcholine, PI: phosphatidylinositol, PS: phosphatidylserine, PE: phosphatidylethanolamine, PA: phosphatidic acid, U: unidentified phospholipid.

Species	*LPC*	*S*	*PC*	*PI*	*PS*	*PE*	*PA*	*U*	*P(glycerol)*
Rat	3.8	12.8	47.8	3.5	10.8	21.5	0.3	—	20.0
Man	—	26.9	28.9	1.3	13.0	27.2	2.2	—	18.3
Rabbit	0.3	19.0	33.9	1.6	12.2	31.9	1.6	—	3.6
Dog	1.8	10.8	46.9	2.2	15.4	22.4	0.5	—	1.4
Horse	1.7	13.5	42.4	0.3	18.0	24.3	0.3	—	1.0
Pig	0.9	26.5	23.3	1.8	17.8	29.7	0.3	—	0.7
Ox	—	46.2	—	3.7	19.3	29.1	0.3	1.7	0.4
Sheep	—	51.0	—	2.9	14.1	26.2	0.3	4.8	0.3

branes. Data on lipid composition were taken from Rouser *et al.*[20]. Obviously neither a correlation of the permeability coefficient with the lipid composition, nor with the cholesterol/phospholipid ratio exists.

The phospholipid composition of mammalian red blood membranes is presented in Table III. Data were taken from Nelson[21] except those for human red blood cells are from Rouser *et al.*[20]. No clear correlation between phospholipid composition and red blood cell permeability to glycerol seems to exist. The data used for phospholipid composition can be considered reliable, since for human red blood cell membranes essentially the same results were obtained by Dodge and Phillips[22] and by Broekhuyse[8] and for rabbit red blood cells by Gercken and Brockmann[23] and ourselves.

The fatty acid composition of red blood cell lipids determined by different authors shows considerably variations (*cf.* Rouser *et al* [20]). Many investigators[24-27] reported that changes in fatty acid composition of red blood cell lipids can be

caused by differences in dietary composition, whereas the phospholipid composition remains unchanged[24]. They[7,26,27] showed that considerable variations in fatty acid composition hardly affected the lysis time of rat red blood cells in glycerol solutions. The results of our analyses on the fatty acid composition of erythrocytes from 8 mammalian species are given in Table IV. The erythrocytes were except for the ox and man obtained from the same animals, on which the permeability coefficients were determined. The relative standard deviation for the major fatty acids (> 5 %) was less than 5 % within one species. The values for the human erythrocytes agree quite well with those of Dodge and Phillips[22]. The values for six animals are in the same range as reported by de Gier *et al.*[7], who did not determine the long-chain fatty acids. Comparable values were reported for sheep and rabbit erythrocytes by Nelson[28] and Gercken and Brockmann[23], respectively. The first author could not detect arachidonic acid in sheep erythrocytes and found also a large amount of nervonic acid. The fatty acid composition of horse erythrocytes was not reported earlier.

TABLE IV

FATTY ACID COMPOSITION OF ERYTHROCYTES FROM EIGHT MAMMALIAN SPECIES

The fatty composition is given in g/100 g as the means of duplicate analyses of lipid extracts of the number of animals given between parentheses. The fatty acid methyl esters are designated by the number of carbon atoms, followed by the number of double bonds. tr denotes presence in an amount less than 0.5%.

Fatty acid	*Rat* (4)	*Man* (6)	*Rabbit* (10)	*Dog* (4)	*Horse* (6)	*Pig* (4)	*Ox* (7)	*Sheep* (4)
16:0	25.1	21.4	20.6	15.2	16.5	19.5	10.7	9.0
16:1	1.5	1.9	1.2	0.6	1.0	0.6	1.0	0.8
18:0	15.1	15.2	18.7	26.7	15.0	16.0	18.6	13.6
18:1	11.5	12.2	11.3	11.3	25.0	30.9	35.7	53.6
18:2	11.0	10.2	27.5	10.9	35.6	21.8	17.8	7.8
18:3	0.5	0.5	2.4	tr	1.9	1.6	1.7	1.8
20:3	tr	1.3	0.8	1.1	tr	tr	tr	tr
20:4	22.7	15.0	5.2	28.8	1.1	4.0	4.5	1.5
20:5	1.9	0.5	0.5	0.7	0.9	0.6	0.5	0.6
22:4	tr	3.7	1.3	tr	—	—	—	—
24:0	0.8	5.3	0.5	0.8	0.5	1.4	5.5	1.3
24:1	tr	4.0	2.7	1.5	1.7	1.8	1.0	8.7
22:5	3.4	3.2	1.3	tr	—	—	—	—
22:6	4.4	4.1	4.4	1.1	tr	—	—	—

We could calculate from the fatty acid composition in g/100 g the composition in moles/100 moles by means of the detector response factor[22]. For calculation of the composition of the hydrocarbon chains of the lipids one has to take into account the plasmalogen and sphingosine content. The plasmalogen content was neglected in our calculations, because it amounted to 5 % (human erythrocytes) or lower. The value for the sphingosine base was calculated from the sphingomyelin content, reported by Nelson[21] and Rouser *et al.*[20]. We could calculate from the molar composition the mean effective chain length and mean number of double bonds per molecule, taking into account the sphingosine base to be composed of only 4-sphingenine. The results are given in Table V. Comparison with the glycerol

TABLE V

COMPOSITION OF THE HYDROCARBON CHAINS OF THE LIPIDS AND GLYCEROL PERMEABILITY OF ERYTHROCYTES

The amount of the fractions are given in mole % of the sum of fatty acids and sphingosine. The value for the sphingosine was calculated from the sphingomyelin content, given by Nelson[21] and Rouser *et al.*[20]. The mean effective length and the mean number of double bonds of the hydrocarbon chains were calculated from the molar composition with the assumption that the sphingosine base was only composed of 4-sphingenine. The glycerol permeability coefficient is expressed in $cm \cdot s^{-1} \times 10^6$. tr denotes an amount less than 0.5%.

Fraction	*Rat*	*Man*	*Rabbit*	*Dog*	*Horse*	*Pig*	*Ox*	*Sheep*
Unsaturated fatty acids	51.0	45.6	53.9	51.2	62.4	52.4	49.4	55.6
20:5 + 22:6	5.1	3.5	3.8	1.5	0.8	0.5	0.4	0.4
18:3 + 20:4 + 22:5	22.5	15.3	7.4	25.4	2.8	4.5	4.6	2.5
16:1 + 18:2 + 20:3 + 22:4	12.3	15.1	29.5	12.6	33.7	19.4	15.1	6.6
16:0 + 18:1	38.6	33.2	31.6	27.9	40.9	45.7	37.9	48.7
18:0	14.7	13.8	17.1	26.1	13.8	14.6	14.8	10.5
24:1 + 22:0	tr	4.2	0.8	1.0	1.2	1.1	0.6	5.1
24:0	0.5	3.6	tr	0.5	tr	0.9	3.2	0.7
Sphingosine	6.4	13.4	9.5	5.4	6.7	13.2	23.1	25.5
Mean chain length	14.83	15.89	15.23	14.98	15.39	15.82	16.03	16.32
Mean number double bonds	1.63	1.44	1.32	1.52	1.12	0.98	1.01	0.95
P(glycerol)	20.0	18.3	3.65	1.37	1.02	0.70	0.43	0.32

permeability coefficients does not show any correlation with the percentage of unsaturated fatty acids. Rat, man and rabbit do not demonstrate any relation in the characteristics of the hydrocarbon residues of the erythrocyte lipids with the glycerol permeability. For these species a facilitated diffusion system for glycerol has been suggested[29–33]. Comparison of the erythrocytes of the other five animals shows a decrease of the glycerol permeability together with an increase of the mean effective chain length and a decrease of the mean number of double bonds. A clear correlation does not seem to exist, however. A correlation between the fatty acid composition and glycerol permeability of erythrocytes was obviously absent in the case of a group of 10 individual rabbits, where a very variable glycerol permeability existed together with a uniform lipid composition.

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