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Lack of influence of membrane cholesterol depletion on anion and nonelectrolyte permeability of pig erythrocytes

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SUMMARY

Anion and nonelectrolyte permeabilities of normal and cholesterol-depleted pig erythrocytes were studied by net and tracer flux measurements. In contrast to findings reported for other membrane systems, removal of 35% of pig erythrocyte membrane cholesterol did not induce notable changes of the normal permeability. Enhancements of membrane permeability, however, induced by amphotericin B were partly abolished by the cholesterol depletion. The results are discussed in terms of a model involving two pools of membrane cholesterol.

As is well established, the permeability of artificial phospholipid membranes diminishes considerably after incorporation of cholesterol. This effect has been demonstrated for the permeabilities to water[1], nonelectrolytes[2-6], anions[6,7] and cations[8]. The concomitant physical alterations of the phospholipid membranes have been discussed in detail[2,5,9].

In analogy, the cholesterol present in plasma membranes is also thought to influence the permeability of these structures. This concept mainly relies on results obtained with *Mycoplasma laidlawii* B. The nonelectrolyte permeability of this microorganism diminishes considerably when cholesterol is incorporated into the membrane during growth[5]. Studies using erythrocytes have provided ambiguous results: Glycerol permeability of human erythrocytes was reported to increase after partial removal of cholesterol[10]. A decrease of nonelectrolyte and Na^+ transfer was demonstrated in guinea pig erythrocytes after dietary elevation of membrane cholesterol[11]. On the other hand, the hydraulic water permeability of human erythrocytes was found to be independent of membrane cholesterol content[12].

In a further attempt to elucidate the influence of cholesterol on erythrocyte membrane permeability, passive non-mediated transfer of anions and nonelectrolytes was

analyzed in cholesterol-depleted pig erythrocytes. In addition it was examined whether cholesterol depletion affects the action of amphotericin B on the membrane permeability of erythrocytes.

The membrane cholesterol content of fresh pig erythrocytes was reduced by incubation of the cells (24 h, 37 °C, pH 7.35, Hct. 10–15%) in cholesterol-depleted pig serum[13]. This procedure diminished membrane cholesterol, determined[14] in isopropanol–chloroform extracts[15] of the washed cells, from a mean value (nmoles/μmole hemoglobin ± S.E.) of 754 ± 15 in fresh cells to 448 ± 13 in depleted cells. In control cells, incubated in serum (heated to 56 °C for 1 h[13]) with normal cholesterol level, the cholesterol content decreased only slightly to a value of 712 ± 13 nmoles/μmole hemoglobin. Membrane lipid phosphorus was also determined[16] in a number of experiments. No significant changes of this fraction were observed, values amounting to 850 ± 38, 839 ± 27 and 804 ± 37 nmoles P/μmole hemoglobin (± S.E., $n = 6$) in fresh, control and cholesterol-depleted cells. Transfer processes in control and cholesterol-depleted cells were analyzed by measuring either the net exchange of extracellular anions with cellular chloride[17], or tracer fluxes of anions and of nonelectrolytes under equilibrium conditions[18]. In both cases the solute movements followed first-order kinetics and could be characterized by a rate constant. Phosphate influx was measured as described previously[19].

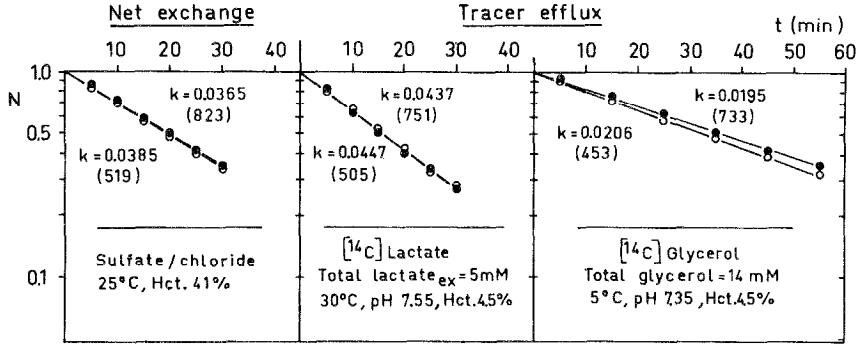

Fig.1. Time course of membrane transfer processes in control and cholesterol-depleted pig erythrocytes. Net exchange was initiated by suspending the erythrocytes in 100 mM sodium sulfate. The increase of chloride in the medium (= Cl_{ex} (mM)) served as a measure for the anion exchange. Tracer efflux was studied by following the release of isotope from preloaded cells into the medium (cpm/ml = cpm_{ex}). Incubation media (mM): NaCl (70); sodium phosphate (13); glucose (5); glycylglycine (55); sucrose (75); permeant (see figure). Rate constants k (min^{-1}), corresponding to the slope of the regression line were calculated from t and ln N. $N = (Cl_{ex\,\infty} - Cl_{ex\,t}) / (Cl_{ex\,\infty} - Cl_{ex\,o})$ for net exchange; $N = (cpm_{ex\,\infty} - cpm_{ex\,t}) / cpm_{ex\,\infty} - cpm_{ex\,o})$ for tracer efflux. Subscripts o and ∞ refer to values obtained initially and after attainment of equilibrium. Numbers in parentheses: cholesterol content (nmoles/μmole hemoglobin). ●, control cells; ○, cholesterol-depleted cells.

Typical time courses of the transfer of sulfate, lactate and glycerol across membranes of control and cholesterol-depleted erythrocytes are shown in Fig.1. In none of these experiments the removal of about 35% of the total membrane cholesterol induced relevant changes of the rate constants of transfer, indicating that cholesterol depletion of this extent does not alter erythrocyte membrane permeability to the three

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TABLE I

CHOLESTEROL CONTENT AND RATES OF SOLUTE TRANSFER IN CONTROL AND CHOLESTEROL-DEPLETED PIG ERYTHROCYTES

Experimental conditions as described in legend to Fig.1.

Penetrant and experimental conditions	*Control cells (preincubated in 56 °C serum)*		*Cholesterol-depleted cells*		*Significance* §
	*Cholesterol**	*Rate constant** or *phosphate influx****	*Cholesterol**	*Rate constant** or *phosphate influx****	
Anions					
Net exchange					
Sulfate (c_o = 100 mM) 25 °C, Hct. 41%	720 ± 26	3.69 ± 0.19	451 ± 18	3.94 ± 0.16	$0.1 > P > 0.05$ (n=5)
Isethionate (c_o = 155 mM), 35 °C, Hct. 38%	663 ± 28	6.99 ± 0.42	378 ± 26	7.65 ± 0.39	$0.05 > P > 0.025$ (n=6)
Tracer influx (^{32}P)					
Phosphate (10 mM), 37 °C, pH 7.35, Hct. 10%	681 ± 34	86.4 ± 3.6	435 ± 25	88.6 ± 4.3	$0.25 > P > 0.20$ (n=12)
Tracer efflux (^{14}C)					
Lactate (5 mM), 30° C, pH 7.55, Hct. 4.5%	718 ± 26	4.50 ± 0.06	477 ± 16	4.59 ± 0.11	$0.1 > P > 0.05$ (n=3)
Glycolate (5 mM), 5 °C, pH 8.0, Hct. 4.5%	756 ± 23	0.98 ± 0.05	487 ± 36	1.03 ± 0.03	$0.15 > P > 0.10$ (n=5)
Nonelectrolytes					
Tracer efflux (^{14}C)					
Glycerol (14 mM), 5 °C, pH 7.35, Hct. 4.5%	736 ± 26	1.93 ± 0.08	469 ± 13	2.04 ± 0.08	$0.25 > P > 0.20$ (n=4)
Erythritol (31 mM), 30 °C, pH 7.55, Hct. 4.5%	762 ± 21	1.43 ± 0.09	479 ± 16	1.52 ± 0.06	$0.10 > P > 0.05$ (n=6)

* nmoles/μmole hemoglobin ± S.E.

** $min^{-1} \times 10^2$ ± S.E.

*** nmoles $P \cdot g^{-1} \cdot min^{-1}$ ± S.E.

§ Significance of difference between transfer rates in control and cholesterol-depleted cells, calculated by the paired Student's t test.

solutes. This finding was confirmed and generalized in further experiments compiled in Table I. The data support the view that neither the net movements nor the tracer fluxes of anions and polyols become enhanced to any notable extent in cholesterol-depleted pig erythrocytes. Even the minimal changes observed are only of dubious significance.

The rate constants determined in our experiments are not a direct measure of permeabilities. For converting one into the other, the diffusional area has to be considered. Since cholesterol depletion reduces erythrocyte surface area[13,20], identical rate constants might be observed in spite of different permeabilities. The uncertainty arising from this complication, however, does not invalidate our conclusion, since a 35% reduction of membrane cholesterol diminishes erythrocyte surface area only by about 8%[20].

The lack of a response of the pig erythrocyte membrane permeability to a decrease of the molar cholesterol/phospholipid ratio from 0.84 to 0.53, *i.e.* approximately from 1:1 to 1:2, stands in marked contrast to the response of artificial lipid membranes, in which similar changes may enhance permeability more than 3-fold[3]. Moreover, our findings concerning nonelectrolyte permeabilities do not agree with results of other investigators[10] reporting a 6-fold increase of glycerol permeability in cholesterol-depleted human erythrocytes. This discrepancy may be due to the fact that glycerol transfer proceeds by different mechanism in porcine and human erythrocytes[21], although other explanations cannot be excluded. The reduced permeability of cholesterol-loaded guinea pig erythrocytes[11] seems compatible with our results on the basis of the following hypothesis:

Cholesterol in the pig erythrocyte membrane might be localized in two pools. An "external" pool, which can be depleted *in vitro*, is supposed to contain about 40% of the

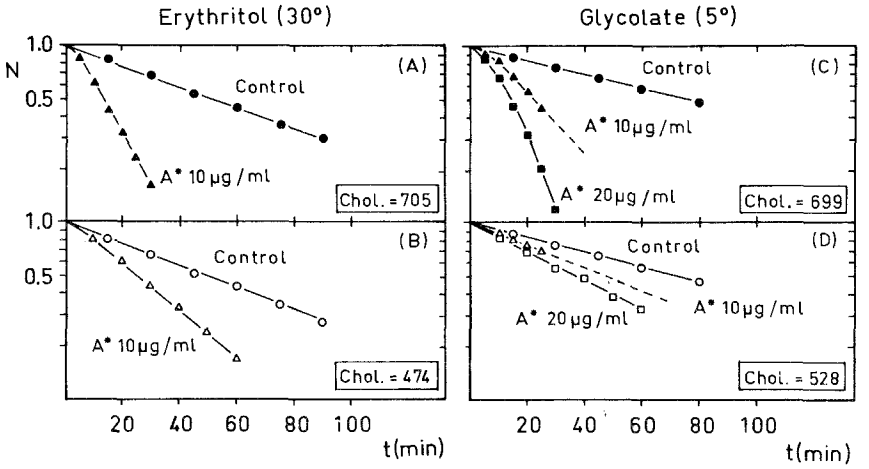

Fig.2. Influence of amphotericin B (A*) on the efflux of [^{14}C]erythritol and [^{14}C]glycerol from normal and cholesterol-depleted pig erythrocytes. Amphotericin B (Squibb) was added at the beginning of the efflux period. Further experimental conditions as in Fig.1 and Table I. For amphotericin-treated cells, k values refer to the linear part of the kinetics. Chol. = Cholesterol content (nmoles/µmole hemoglobin).

total cholesterol. This assumption is supported by the finding that only about 40% of the pig erythrocyte membrane cholesterol can exchange with lipoprotein-bound serum cholesterol[22] or with other sterols[10]. The other, "internal" pool, in contrast, cannot be depleted without disruption of the membrane. Additional cholesterol incorporated into the membrane[11] is taken up by both pools. The "internal" pool is supposed to be part of the barrier limiting the passive transfer of anions and nonelectrolytes. Additional cholesterol increases the resistance of this barrier[11] which thus behaves like an artificial lipid bilayer or a *Mycoplasma* membrane. The "external" pool of cholesterol, on the other hand, has no influence on normal membrane permeability.

This "external" pool, however, may become involved in membrane permeability under special experimental conditions. The action of the polyene antibiotic, amphotericin B, which enhances the permeability of artificial and natural membranes[23–27] requires the presence of cholesterol or other sterols as receptors[24,25,27,28]. As is evident from Figs 2A and 2C, amphotericin B, in addition to its effects on cation permeability[29], considerably increases the permeability of erythrocytes to small anions and nonelectrolytes (for details *cf.* ref.18). In agreement with the findings on other membranes, this enhancement of the erythrocyte permeability depends on the cholesterol content of the membrane, being considerably less pronounced after cholesterol depletion (Figs 2B and 2D). In terms of the model outlined above and of present concepts concerning the molecular basis of amphotericin B-induced membrane transformations[24,28], this finding may indicate, that the "external" pool of cholesterol, although not part of the normal pathway of anion and nonelectrolyte transfer, may nevertheless contribute to the formation of new "channels" of solute transfer[24,26] in the presence of amphotericin B.

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REFERENCES

1 A. Finkelstein and A. Cass, *Nature*, 216 (1967) 717.
2 J. de Gier, J.G. Mandersloot and L.L.M. van Deenen, *Biochim. Biophys. Acta*, 173 (1969) 143.
3 J. de Gier, J.G. Mandersloot and L.L.M. van Deenen, *Biochim. Biophys. Acta*, 150 (1968) 666.
4 R.A. Demel, K.R. Bruckdorfer and L.L.M. van Deenen, *Biochim. Biophys. Acta*, 255 (1972) 321.
5 B. de Kruyff, R.A. Demel and L.L.M. van Deenen, *Biochim. Biophys. Acta*, 255 (1972) 331.
6 S.C. Kinsky, S.A. Luse, D. Zopf, L.L.M. van Deenen and J. Haxby, *Biochim. Biophys. Acta*, 135 (1967) 844.
7 D. Papahadjopoulos and J.C. Watkins, *Biochim. Biophys. Acta*, 135 (1967) 639.
8 J.M. Vanderkooi and A. Martonosi, *Arch. Biochem. Biophys.*, 147 (1971) 632.
9 D. Chapman and D.F.H. Wallach, in D. Chapman, *Biological Membranes*, Academic Press, New York, pp.125–202.
10 K.R. Bruckdorfer, R.A. Demel, J. de Gier and L.L.M. van Deenen, *Biochim. Biophys. Acta*, 183 (1969) 334.
11 J. Kroes and R. Ostwald, *Biochim. Biophys. Acta*, 249 (1971) 647.
12 R.J. Sha'afi, C. Gary-Bobo and A.K. Solomon, *Biochim. Biophys. Acta*, 173 (1969) 141.
13 J.R. Murphy, *J. Lab. Clin. Med.*, 60 (1962) 86.
14 R. Richterich and K. Lauber, *Klin. Wochenschr.*, 40 (1962) 1552.

15 H.G. Rose and M. Oklander, *J. Lipid Res.*, 6 (1965) 428.
16 E. Gerlach and B. Deuticke, *Biochem. Z.*, 337 (1963) 447.
17 B. Deuticke, in P. Astrup, *Oxygen Affinity of Hemoglobin and Red Cell Acid–Base Status*, Alfred Benzon Symp. IV, Munksgaard, Kopenhagen, in the press, p.549.
18 B. Deuticke, M. Kim and Chr. Zöllner, to be published.
19 B. Deuticke, *Pflügers Arch. Ges. Physiol.*, 296 (1967) 21.
20 R.A. Cooper and J.H. Jandl, *J. Clin. Invest.*, 47 (1968) 809.
21 M.H. Jacobs, H.N. Glassman and A.K. Parpart, *J. Cell. Comp. Physiol.*, 7 (1935) 197.
22 F.P. Bell and C.J. Schwartz, *Biochim. Biophys. Acta*, 231 (1971) 553.
23 T.E. Andreoli, V.W. Dennis and A.M. Weigl, *J. Gen. Physiol.*, 53 (1969) 133.
24 V.W. Dennis, N.W. Stead and T.E. Andreoli, *J. Gen. Physiol.*, 55 (1970) 375.
25 A. Cass, A. Finkelstein and V. Krespi, *J. Gen. Physiol.*, 56 (1970) 100.
26 R. Holz and A. Finkelstein, *J. Gen. Physiol.*, 56 (1970) 125.
27 H. van Zutphen, R.A. Demel, A.W. Norman and L.L.M. van Deenen, *Biochim. Biophys. Acta*, 241 (1971) 310.
28 S.C. Kinsky, *Annu. Rev. Pharmacol.*, 10 (1970) 119.
29 S.F. Blum, S.B. Shohet, D.G. Nathan and F.H. Gardner, *J. Lab. Clin. Med.*, 73 (1969) 980.