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Formation and resealing of pores of controlled sizes in human erythrocyte membrane

APPLICATION of an electric pulse, at field intensities of a few kV cm^{-1} and of duration in the μs range, to an isotonic suspension of erythrocytes is known to cause haemolysis of the red cells[1–4]. Studies from different laboratories suggest that the haemolysis is due to the field-induced transmembrane potential[1,3,4]. Our recent experiments[5] indicate that once the transmembrane potential reaches a threshold of approximately 1 V, which corresponds to an applied field of 2.2 kV cm^{-1}, the erythrocyte membrane becomes leaky to normally impermeant ions or molecules. The permeation of solutes leads to the swelling and eventual lysis of the red cells. This type of haemolysis is known as colloid osmotic haemolysis[6,7]. The voltage-induced permeability change is consistent with the formation of pores in the membrane. We show here that the size of these pores can be varied in a controlled manner, and that the leaky membrane can be resealed while the haemolysis is prevented. Foreign molecules have successfully been incorporated into the resealed, but otherwise intact, erythrocytes.

In the experiment shown in Fig. 1, erythrocytes in an isotonic NaCl solution were treated with a 3.7 kV cm^{-1}, 20-μs pulse, and the subsequent change in cell volume V was monitored by light scattering measurement. In these conditions, intracellular K^+ leaks out, being replaced with Na^+, within a few minutes. Further entry of NaCl causes the swelling of the red cells[5]. When V reached 1.1 V_0 (where V_0 is the volume of the untreated cells) a small amount of isotonic solution of various substances was added to the suspension (time $t = 0$). Curve d in Fig. 1 is a control, where 1/10 volume of isotonic NaCl was added. The cells kept swelling because of the continuous influx of NaCl. However, when the same amount of sucrose solution was added, the swelling immediately stopped as shown in curve b. A separate tracer experiment showed that the cell membrane is practically impermeable to sucrose in these conditions. Therefore, the above result indicates that the reduction of the external NaCl concentration by 9% is just sufficient to abolish the electrochemical gradient, or the resultant uptake, of NaCl. As expected, the addition of a larger amount of sucrose solution reduced the external NaCl concentration further, and the cells began to shrink due to the efflux of NaCl (curve a).

When the added substance was xylitol (MW 152 whereas sucrose is 342), only partial blocking of the cell swelling was achieved as shown in curve c. The volume change in this situation is expected to satisfy the following relation

$$dV/dt = (j_x + j_{NaCl})V/C \quad (1)$$

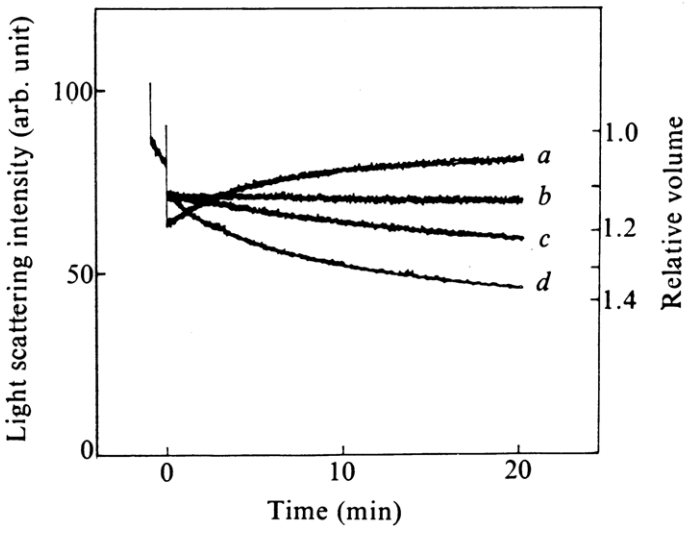

Fig. 1 Changes in cell volume after the electric pulsation. Washed human erythrocytes were suspended in an isotonic NaCl solution (150 mM NaCl, 7 mM phosphate buffer, pH 7.0) at a volume concentration of 1%, and treated with a single square-wave electric pulse of intensity 3.7 kV cm^{-1}, duration 20 μs, between a pair of stainless steel electrodes. After 10–20 s, an aliquot was taken and mixed with 50 volumes of the isotonic NaCl solution, and the change in light scattering intensity at 600 nm was recorded with an Aminco–Bowman spectrofluorometer equipped with a magnetic stirrer. The scattering intensity was calibrated against the cell volume V by a separate haematocrit measurement, as shown in the right-hand scale which applies to the $t > 0$ portion of curves b–d. When V reached 1.1 times that of the untreated V_0, the following additions were made ($t = 0$). Curve a, 2/10 volume of isotonic (272 mM) sucrose solution to 1 volume of the suspension; curve b, 1/10 volume of the isotonic sucrose solution; curve c, 1/10 volume of isotonic (285 mM) xylitol solution; curve d, 1/10 volume of the isotonic NaCl solution. Temperature was 25 °C. In these conditions, the extent of haemolysis is negligible for the 20-min period shown; however, all the cells haemolyse by 15 h unless the carbohydrates are added.

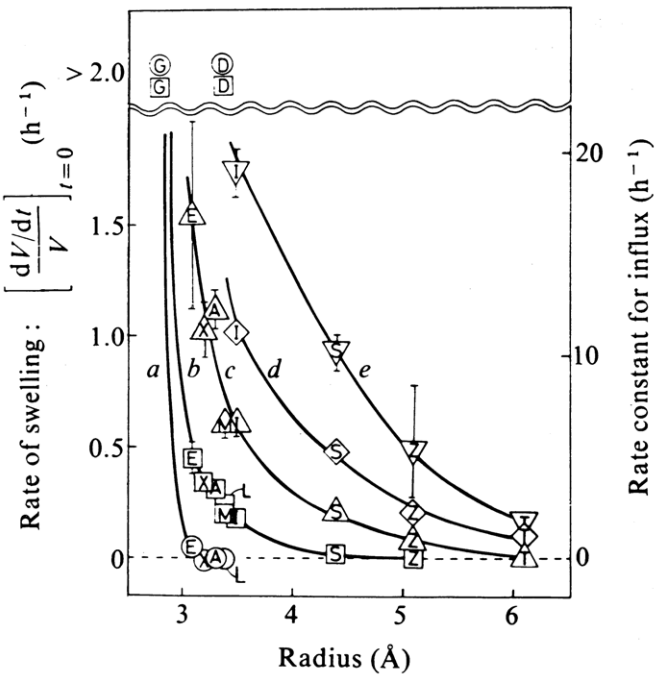

Fig. 2 Permeability of the pulse-treated erythrocyte membrane to various carbohydrate molecules. Curve a, untreated cells; curve b, cells treated with a 3.7 kV cm^{-1}, 20-μs pulse in isotonic NaCl solution; curve c, 5.3 kV cm^{-1}, 20-μs, in NaCl; curve d, 3.7 kV cm^{-1}, 80 μs, in NaCl; curve e, 3.7 kV cm^{-1}, 20 μs, in a 3:7 mixture of isotonic NaCl and isotonic sucrose solutions. After the pulsation, swelling of the cells due to the influx of various carbohydrate molecules was recorded as in curve c of Fig. 1. The rate of swelling was determined from the initial slope of the swelling curve; the rate of permeation was calculated from equation (2). The average radius in the abscissa denotes $(r_1r_2r_3)^{1/3}$ where the r_i values are three orthogonal radii measured in space-filling molecular models. G, glycerol; E, meso-erythritol; X, xylitol; A, D-arabitol; D, D-glucose; L, L-glucose; M, D-mannitol; I, myo-inositol; S, sucrose; Z, melezitose; T, stachyose.

Table 1 Incorporation of sucrose in resealed erythrocytes

Treatment	Procedure Medium	Time	Assay % survival	Relative cell volume	Sucrose content	Na^+ content (mmol l^{-1} cells)	K^+ content
a Pulsation 3.7 kV cm^{-1}, 80 μs, 25 °C	NaCl 135 mM, NaP* 6 mM, sucrose 27 mM, *p*H 7.0	before *a*	100	1.00	0.0	(9)	(10)
b Resealing 1 37 °C, 1 h	NaCl 119 mM, NaP 7 mM, sucrose 27 mM, stachyose 21 mM, $MgSO_4$ 2 mM, *p*H 7.2	after *b*	99	0.99	3.2	92	11
c Resealing 2 37 °C, 6 h	NaCl 107 mM, KCl 5 mM, sucrose 27 mM, stachyose 21 mM, adenosine 3 mM, inosine 1 mM, supplement†, *p*H 7.5	after *c*	97	0.94	3.5	98	17
d Resealing 3 37 °C, 12 h	NaCl 107 mM, KCl 5 mM, $CaCl_2$ 2 mM, stachyose 40 mM, adenosine 0.3 mM, inosine 0.1 mM, supplement, *p*H 7.5	after *d*	95	1.01	3.2	81	27
e Test incubation 37 °C, 72 h	NaCl 133 mM, KCl 5 mM, $CaCl_2$ 2 mM, adenosine 0.15 mM, inosine 0.05 mM, supplement, *p*H 7.5	24 h in *e*	74	1.00	3.3	63	42
		48 h in *e*	65	0.99	3.3	55	46
		72 h in *e*	58	0.95	3.2	—	—

* NaP, Sodium phosphate buffer.

† Supplement: NaP 7 mM, $MgSO_4$ 2 mM, D-glucose 11 mM, chloramphenicol 0.1 mg ml^{-1}, penicillin G 500 units ml^{-1}, bovine serum albumin 30 mg ml^{-1}. Sucrose contained trace amount of [^{14}C] sucrose.

Volume concentration of the cells was ~ 20% at stage *a*, 10% at *b*, 3–5% at *c* through *e*. The suspension was gently rocked during the incubations. In *e*, medium was changed at every 12 h, during which *p*H dropped by 0.2–0.3 unit. Percentage survival was calculated from the amount of haemoglobin released in the supernatant. Relative cell volume was assumed to be inversely proportional to the amount of haemoglobin contained in a unit volume of packed cells (haemoglobin content of the untreated cells was 315±6 g l^{-1} cells). Sucrose content was determined from the radioactivity of the cells packed in microhaematocrit tubes. Na^+ and K^+ were assayed by flame photometry. Assay values except % survival refer to survived (unlysed) cells. All values are averages of several determinations on two independent samples; variations were within ±1% for % survival, ±0.02 for cell volume, ±0.2 mmol l^{-1} cells for sucrose, ±5 mEq l^{-1} cells for Na^+ and K^+.

where j_x and j_{NaCl} are the net influxes of xylitol and NaCl respectively, in mOsmol l^{-1} cells h^{-1} and C the total osmotic concentration of the medium in mOsmol l^{-1}. At $t \approx 0$ or $V \approx V_0$, the net influx j_{NaCl} is negligible, as shown in curve *b*, and the swelling is almost entirely due to the xylitol influx j_x. Thus the rate of permeation of xylitol molecule k_x can be roughly estimated as

$$k_x \equiv j_x/C_x = (V^{-1}dV/dt)_{t=0}(C/C_x) \qquad (2)$$

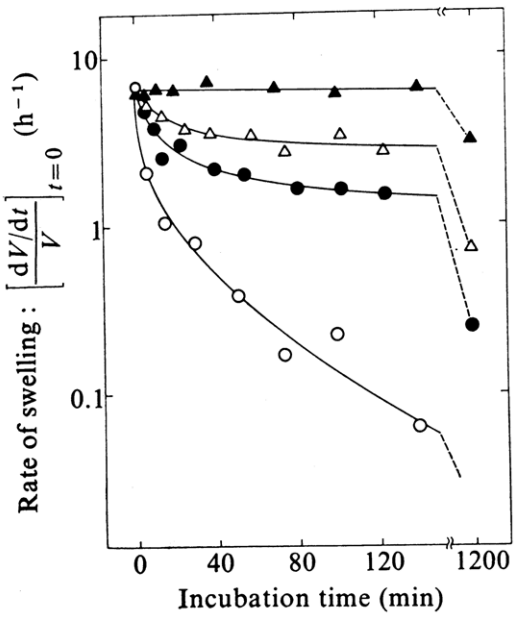

Fig. 3 Time courses of the resealing of pulse-treated erythrocytes at different temperatures. ▲, 3 °C; △, 17 °C; ●, 25 °C; ○, 37 °C. The washed erythrocytes in isotonic NaCl were treated with a 3.7 kV cm^{-1}, 80-μs pulse at 25 °C, and immediately mixed with a large volume of 85:15 mixture of isotonic NaCl and isotonic stachyose solutions kept at the specified temperatures. At intervals, an aliquot was taken and added to an isotonic NaCl solution of the same temperature, and the initial rate of swelling was measured as described in Fig. 1. Haemolysis in the incubation media (NaCl–stachyose) was negligible except at 3 °C, where a small portion of the cells haemolysed by 20 h presumably because of the very slow penetration of stachyose.

where C_x is the external osmotic concentration of xylitol ($C/C_x = 11$).

In this way, the permeability of the pulse-treated membrane to 11 different carbohydrates was measured in various conditions. (Tracer influx measurements on a few selected carbohydrates gave values consistent with those obtained by the above method.) The results are plotted in Fig. 2 against the average radius of the tested molecule. Curve *a* gives data for the untreated cells; molecules larger than erythritol do not enter the cells to any appreciable amount, except D-glucose which is known to be carried by a specific transport system[7]. This agrees with the idea that pores of a radius of about 3.5–4.2Å exist in human erythrocyte membrane[8]. Curve *b* shows that the treatment with a 3.7 kV cm^{-1}, 20, μs pulse increases the critical size for permeation: molecules smaller than sucrose can penetrate the membrane, and the rate decreases with the size of the molecule. Again, an exception to this curve is D-glucose; the specific transport system seems to be intact even after the pulse treatment. As can be seen from curves *c*, *d* and *e*, larger pores are obtained either by using a higher field intensity, by increasing the pulse duration, or by reducing the ionic strength of the pulsation medium.

As suggested by curve *b* in Fig. 1, addition of a sufficient amount of impermeant substance to the suspension of pulse-treated erythrocytes retards the haemolysis indefinitely. While the cells are prevented from lysis, the membrane spontaneously reseals as in the case of ghosts obtained by hypotonic lysis[9]. The resealing process is strongly temperature-dependent, as shown in Fig. 3. The ordinate is the rate of swelling measured in isotonic NaCl; as before, this rate is proportional to the net influx of NaCl. At 37 °C the treated membrane rapidly regains its impermeability to cations, whereas at 3 °C the cells remain highly permeable even after 20 h.

The electric pulsation followed by an appropriate resealing procedure makes it possible to prepare erythrocytes (not ghosts) with altered intracellular compositions. For example, the ionic composition of the resealed cells reflects that of the resealing medium: incubation in NaCl or KCl media yields cells loaded predominantly with Na^+ or K^+ respectively. Although the alteration of cellular cations can also be achieved by lactose treatment[10] or chemical modification of the cell membrane[11], the present method allows the incorporation of larger molecules such as sucrose by introducing pores of adequate size. Results of a typical experiment are shown in

Table 1. The intactness of the loaded cells was tested by incubation for an additional 72 h in a simulated physiological medium at 37 °C. Although some haemolysis occurred, especially during the first 24 h of the test incubation, about 60% of the original cells survived the 3-d period. The cells in the medium assumed either normal biconcave or slightly cup-shaped disk forms and maintained approximately normal cell volume. The resealed erythrocytes accumulated K^+ and extruded Na^+ against a concentration gradient, indicating that the pores were almost completely annealed and the Na–K pump was intact.

At least two applications of the present technique are conceivable: (1) the alteration of intracellular compositions will simplify experimental designs, especially in transport studies. (2) Erythrocytes may be used as intravenous drug reservoirs; gradual release from loaded erythrocytes could help maintain the drug level in a patient's circulation.

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