Calcium Ions and Cell Fusion

EFFECTS OF CHEMICAL FUSOGENS ON THE PERMEABILITY OF ERYTHROCYTES TO CALCIUM AND OTHER IONS

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1. Fusogenic and non-fusogenic chemicals were tested for their ability to allow $^{45}\text{Ca}^{2+}$ and $^3\text{H}_2\text{O}$ to enter hen and human erythrocytes. 2. The ratio of $^{45}\text{Ca}^{2+}/^3\text{H}_2\text{O}$ in treated cells to that in untreated cells is referred to as the entry ratio. 3. Within 1 min at 37°C both water-soluble and lipid-soluble fusogens increased the value of the entry ratio, which reached maximum values in 5–10 min. 4. Values of the entry ratio in the range of 4–12 were found under conditions that led to cell fusion. 5. Closely related but non-fusogenic chemicals did not significantly alter the entry ratio. 6. The entry ratios for $^{86}\text{Rb}^+$, $^{22}\text{Na}^+$ and $^{35}\text{SO}_4^{2-}$ were also significantly increased by both lipid-soluble and water-soluble fusogens, though the increases were not as large as those for $^{45}\text{Ca}^{2+}$. 7. It is suggested that fusogenic compounds increase the permeability of biological membranes to ions, and that an increase in the concentration of intracellular Ca^{2+} initiates or facilitates events that lead to the chemically induced fusion of erythrocytes.

The fusion of hen erythrocytes by lipid-soluble and water-soluble molecules requires the presence of extracellular Ca^{2+} (Ahkong *et al.*, 1973, 1975a; Maggio *et al.*, 1976). With human erythrocytes treated with oleoylglycerol, increasing cell fusion occurs with increasing concentrations of Ca^{2+} , but some fusion has been observed without exogenous Ca^{2+} in the presence of 1 mM-EDTA (Quirk *et al.*, 1978). The bivalent cation ionophores X537A and A23187 are known to facilitate the movement of Ca^{2+} through membranes (Reed & Lardy, 1972; Pressman, 1973), and they can be used to fuse hen erythrocytes into multinucleated cells under appropriate conditions (Ahkong *et al.*, 1975c). It has therefore been proposed that the actions of lipid-soluble and water-soluble fusogens may be mediated by facilitating the entry of Ca^{2+} into the interior of treated erythrocytes (Ahkong *et al.*, 1975c; Lucy, 1977).

In the work reported in the present paper, the possibility that chemical fusogens allow Ca^{2+} to enter erythrocytes has been investigated by using techniques that are capable of measuring the entry of Ca^{2+} into cells within 1 min (Martin *et al.*, 1975; Freedman *et al.*, 1975). The observations made indicate that both water-soluble and lipid-soluble fusogens modify the permeability of erythrocyte membranes in a way that resembles the action of the bivalent cation

ionophore A23187. In addition, however, a non-specific entry of other ions (including Na^+) into the erythrocyte is facilitated by the fusogens although to a lesser extent than the entry of Ca^{2+} . A preliminary communication on this work has been published (Blow *et al.*, 1978b).

Materials and Methods

Oleoylglycerol, 1,2-dioleoylglycerol, 1,3-dioleoylglycerol, trioleoylglycerol, stearoylglycerol, oleic acid and dextran (average mol.wt. 60000–90000) were from Sigma (London) Chemical Co., London S.W.6, U.K. Crystalline retinol was from Roche Products, Welwyn Garden City, Herts., U.K. Poly(ethylene glycol) of mol.wt. 200, 400, 600, 1500 and 20000 was from BDH Chemicals, Poole, Dorset, U.K., and of mol.wt. 6000 was from Koch-Light Laboratories, Colnbrook, Bucks., U.K. Radiochemicals were from The Radiochemical Centre, Amersham, Bucks., U.K. Soluene-350 and Insta-gel were from Packard Instruments, Caversham, Berks. RG47AA, U.K. The bivalent cation ionophore A23187 was a gift from Eli Lilly, Windlesham, Surrey, U.K. A solution of the ionophore in ethanol (1 mg/ml; 10 μl) was added to 1.0 ml of the cell suspension, as described below for experiments with retinol, to give a final concentration of 20 μM -ionophore.

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Erythrocytes

Blood (approx. 2 ml) was removed either from the brachial vein of an adult hen or from a human donor (ORh⁺) and taken into a syringe containing citrate anti-coagulant solution (1 ml; De Gowin *et al.*, 1949). Blood thus obtained was centrifuged at 3000g for 5 min, and the erythrocytes were then washed by resuspension in the anti-coagulant solution (10 ml). Subsequently, the cells were washed twice in modified Eagle's basal salt solution, pH 7.4 or pH 5.6 (Ahkong *et al.*, 1973). This solution was 1.8 mM in Ca²⁺. The washed cells were pelleted by centrifugation at 3000g for 5 min.

Incubation of erythrocytes with retinol

Packed erythrocytes (0.4 ml) were mixed with 1.6 ml of the modified Eagle's basal salt solution, pH 5.6 (see above), which additionally contained dextran (80 g/litre) as described by Ahkong *et al.* (1973). Retinol was stored and handled as described by Dingle & Lucy (1962); stock and working solutions were prepared as described previously (Ahkong *et al.*, 1973). Retinol (1040 µg) was injected, with a Hamilton syringe (Micromedex N.U., The Hague, The Netherlands), as a solution in ethanol (100 µl) into the modified Eagle's basal salt solution, pH 5.6, containing dextran (3.12 ml). The dispersion of retinol (0.25 ml) prepared in this way was added without delay to the suspension of erythrocytes (0.4 ml). Between 10 and 40 µl of a mixture of ⁴⁵CaCl₂ and ³H₂O was then added (this was sufficient to give 0.4 µCi of ⁴⁵Ca and 2.0 µCi of ³H), and the volume made up to 1.0 ml with the modified Eagle's basal salt solution, pH 5.6, containing dextran. The cells were incubated at 37°C, and samples removed at intervals for observation by optical microscopy with phase-contrast or Nomarski differential-interference-contrast optics (Standard WL Research Microscope; Carl Zeiss, Degenhardt and Co., London W1P 8AP, U.K.), at a magnification of ×400. After 15 min, 3 samples of 250 µl were taken for centrifugation in a Beckman 'microfuge' (model B; Beckman-RIIC, Glenrothes, Fife KY7 4NG, Scotland, U.K.) in 400 µl polyethylene tubes containing approx. 100 µl of silicone fluid DC550 (Hopkin and Williams, P.O. Box 1, Romford, RM1 1HA, Essex, U.K.) overlaid with 25 µl of 5.5 mM-LaCl₃. Centrifugation for 45 s was sufficient to separate cells from medium, and the tubes were cut between the interposed oil layer with a scalpel. The tips of the tubes containing the cells were put into glass scintillation vials containing 5 ml of Soluene 350/isopropyl alcohol (1:5.67, v/v) and vortex-mixed rapidly to disperse cells. When the cells had completely dissolved, the solution was bleached by the addition of 0.25 ml of 100-volume H₂O₂ (30%, w/v) and left to stand for 2 h at room temperature (18°C). Acidified Insta-gel solution (5 ml; 0.5 M-HCl/Insta-gel, 1:9 v/v) was then added and the vials

placed in a model 3385 Packard Tri-Carb liquid-scintillation counter for temperature equilibration for at least 2 h. ³H radioactivity was recorded on the ³H-channel, which discriminates against ¹⁴C radioactivity; ⁴⁵Ca radioactivity was recorded on the ¹⁴C-channel, which discriminates against ³H radioactivity.

Oleoyleglycerol, dioleoyleglycerol, trileoyleglycerol, stearoyleglycerol and oleic acid

Dispersions of these lipids were prepared by sonication in the modified Eagle's basal salt solution (5 ml), at pH 5.6 or 7.4, by the procedure described by Ahkong *et al.* (1973). Erythrocytes were treated with suspensions of these lipids as described above for experiments with retinol.

Poly(ethylene glycol)

Packed erythrocytes (0.4 ml) were mixed with 1.6 ml of the modified Eagle's basal salt solution, normally at pH 7.4 (see above), not containing dextran. This cell suspension (0.4 ml) was centrifuged at 3000g for 5 min. The packed erythrocytes were then resuspended in 0.4 ml of poly(ethylene glycol) at various concentrations in the range 10–50% (w/w). Solid poly(ethylene glycol) was dissolved in modified Eagle's basal salt mixture not containing dextran, and the concentration of CaCl₂ was adjusted to give a final concentration of 1.8 mM Ca²⁺. After 1 or 15 min of treatment, the erythrocyte suspension was diluted by the addition of modified Eagle's basal salt solution not containing dextran (3.5 ml) and centrifuged at 3000g for 5 min. The cells were washed with a further 3.5 ml of the same solution, centrifuged at 3000g for 5 min, and finally resuspended in the same salt solution (1.0 ml) containing ⁴⁵CaCl₂ and ³H₂O with the same radioactivity as described above for retinol. To minimize cell lysis, all solutions were kept at 37°C and the duration of exposure of the cells to the concentrated solution of poly(ethylene glycol) was carefully controlled. After addition of the radioactive isotopes, the cells were incubated for a further 15 min; samples were then taken at intervals for observation by optical microscopy, and for measurements of ⁴⁵Ca²⁺ as described above for retinol.

Dimethyl sulphoxide

Packed erythrocytes (0.4 ml) were mixed with 1.6 ml of the modified Eagle's basal salt solution, normally at pH 7.4, not containing dextran. This cell suspension (0.4 ml) was centrifuged at 3000g for 5 min. The packed erythrocytes were then resuspended in 1.0 ml of dimethyl sulphoxide dissolved at various concentrations in the modified Eagle's basal salt solution not containing dextran (with a final CaCl₂ concentration of 1.8 mM) containing ⁴⁵CaCl₂ and ³H₂O of the same activity as described above for retinol.

The high concentration of this reagent that was necessary to cause cell fusion altered the density of

the erythrocyte suspension such that both pellet and supernatant centrifuged through the oil layer. Hence the silicone oil layer was not used in these experiments. Instead the supernatant was removed by aspiration with a Pasteur pipette, and the sides of the tube were carefully swabbed, before the bottom section of the tube was cut off and placed in Soluene-350/isopropyl alcohol as detailed above for experiments with retinol.

Entry of ⁴⁵Ca²⁺ into erythrocytes

Since equilibration of water across phospholipid and biological membranes at 37°C is very rapid (Paganelli & Solomon, 1974), the inclusion of ³H₂O in the incubation medium used in these experiments served as an indicator of cell volume, or cell number assuming a constant cell volume. Data expressed as a ratio of ⁴⁵Ca c.p.m. to ³H c.p.m. thus correct for variations in cell size that occur as a result of cell-rounding or swelling (Freedman *et al.*, 1975).

The entry ratio was calculated (see the Results section) from radioactivity (c.p.m.) in treated compared with untreated cells. Both sets of cells were treated so that the degree of quenching was the same and hence the entry ratio obtained was independent of the degree of quenching. Loss of cell volume owing to cell lysis was not a complicating factor in these experiments, because lysed cells do not penetrate through the oil layer.

By adding [³H]sucrose to the cell suspension to act as a marker of entrained extracellular water space, it was demonstrated that 0.6% of the volume of the loading solution was transferred through the silicone oil layer with 90 × 10⁶ cells (approx. 20 μl of packed cells). When a chilled solution containing ⁴⁵Ca²⁺ was added to chilled cells in modified Eagle's basal salt solution at pH 5.6, containing dextran (80 g/litre), and the cells then immediately centrifuged, a small 'apparent entry' of Ca²⁺ into the cells was observed, amounting to about 0.67% of the total ⁴⁵Ca²⁺ radioactivity. This value was not significantly

increased by incubation of the cells at 37°C for 15 min (see Table 1). Thus the apparent entry of Ca²⁺ into cells in control incubations was, to a large extent, attributable to entrained medium, with only a negligible contribution from cations that were adsorbed on the cell surface or had been transported into the cell interior.

Each measurement of the entry of Ca²⁺ and water was based on triplicate sampling for the experimental and control incubations. The ratio of ⁴⁵Ca²⁺/³H₂O was calculated as a mean value (±s.d.) for the experimental and the control incubations. The experimental value was then divided by the control value to give the 'entry ratio' and the standard deviation of this was calculated (Finney, 1964). When the entry of Ca²⁺ under a given condition was studied in three or more independent incubations, the entry ratio was calculated as a mean value (±s.d.) for the independent incubations.

For any given preparation of erythrocytes the entry ratio was very reproducible (see Table 1). Different preparations of cells, however, sometimes gave entry ratios that varied rather widely (see Table 2). This was probably a corollary of the observation that the degree of cell fusion observed also varied from one preparation of cells to another. Comparisons of the ability of different chemical agents to cause the entry of Ca²⁺ into erythrocytes were normally therefore made within the same experiment, with a single preparation of erythrocytes.

Results

Measurement of the entry of ⁴⁵Ca²⁺ into erythrocytes

Hen erythrocytes were treated with oleoylglycerol (final concentration 0.28 mM), or with the bivalent cation ionophore A23187 (final concentration 20 μM) as described in the Materials and Methods section. In control experiments, these cells were treated with ⁴⁵Ca²⁺ and with ³H₂O in the absence of oleoylglycerol or ionophore A23187, either at 0°C (chilled in ice) and

Table 1. Measurement and calculation of ⁴⁵Ca²⁺ entry

Erythrocytes (3.5 × 10⁸ cells/ml final concentration) were incubated with ⁴⁵Ca²⁺ and ³H₂O at 37°C for 15 min in the presence of the agents listed, except for the unincubated control (which was kept on ice and centrifuged immediately after addition of the radioactive isotopes). The incubated control results were used for the entry-ratio calculations, as described in the Materials and Methods section. Means (±s.d.) are given for the radioactivity determined in triplicate samples of cell pellets.

Addition	Time of incubation (min)	³ H ₂ O radioactivity (c.p.m.)	⁴⁵ Ca ²⁺ radioactivity (c.p.m.)	⁴⁵ Ca ²⁺ / ³ H ₂ O	⁴⁵ Ca ²⁺ entry ratio
None	0	6493 ± 98	342 ± 8	0.0528 ± 0.001	—
None	15	6687 ± 315	378 ± 46	0.0619 ± 0.003	1.00 ± 0.04
Oleoylglycerol (0.28 mM)	15	13552 ± 1257	3842 ± 435	0.2847 ± 0.04	4.60 ± 0.36
Ionophore A23187 (20 μM)	15	39293 ± 2148	12958 ± 1154	0.3297 ± 0.02	5.33 ± 0.24
None	15	5162 ± 182	138 ± 11	0.0268 ± 0.001	1.00 ± 0.04
Poly(ethylene glycol) (mol.wt. 1500; 40% w/w; 1 min treatment)	15	4618 ± 1724	798 ± 288	0.1738 ± 0.004	6.49 ± 0.21

centrifuged immediately, or at 37°C and centrifuged after 15 min. The entry of $^{45}\text{Ca}^{2+}$ and $^3\text{H}_2\text{O}$ into the unincubated and incubated control cells were similar (Table 1). In this and in subsequent experiments the incubated control was used for the calculation of the entry ratio, as described in the Materials and Methods section. Both ionophore A23187 and oleoylglycerol caused increases in the ratio of $^{45}\text{Ca}^{2+}/^3\text{H}_2\text{O}$, compared with the control experiment. This is clearly shown by the values of the entry ratio for the treated cells (Table 1). Oleoylglycerol gave a similar value for the entry ratio to that of the ionophore at the concentrations used in this experiment, though the former substance caused cell fusion, whereas the latter did not. It may therefore be concluded that the entry of Ca^{2+} by itself is not sufficient to cause cell fusion. This is consistent with the results of previous experiments with hen erythrocytes in which it was found that for cell fusion to occur rapidly with the ionophore it was necessary to pretreat the cells with neuraminidase and to incubate them for a short time at 47°C (Ahkong *et al.*, 1975c). (In a related experiment, neuraminidase treatment did not alter the entry ratio with either the control or ionophore-treated hen erythrocytes.)

Kinetics of the entry of Ca^{2+}

Hen erythrocytes were treated with oleoylglycerol, ionophore A23187, or poly(ethylene glycol) of mol.wt. 1500, as described in the experiments of Table 1 and the cells brought to 37°C or

Fig. 1. Entry of $^{45}\text{Ca}^{2+}$ into erythrocytes. Erythrocytes were treated as described in the text; $^{45}\text{Ca}^{2+}$ and $^3\text{H}_2\text{O}$ were added at zero time. Symbols: ●, with oleoylglycerol at 0°C; ○, with oleoylglycerol at 37°C; ▲, with ionophore A23187 at 37°C; △, with ionophore A23187 at 0°C; ■, with poly(ethylene glycol) (mol.wt. 1500) at 37°C. Mean values ($\pm$ s.d.) for the entry ratio are given for the triplicate samples referred to in the text (see also the Materials and Methods section).

Table 2. The effect of lipid-soluble fusogens and related chemicals on the entry of Ca^{2+} into hen and human erythrocytes. For single experiments the entry ratio was determined in triplicate (cf. Table 1), and from this data the mean value and standard deviation were calculated. For experiments done more than twice, the individual mean values were averaged. The time to fusion was the number of minutes of incubation before fusion was observed in high incidence by light microscopy (Ahkong *et al.*, 1973). All incubations were for 15 minutes at 37°C in the presence of the agents listed and the isotope mixture, as described in the Materials and Methods section.

Addition	pH	Species	Concn. (mM)	Number of experiments	Ca^{2+} entry ratio (mean $\pm$ s.d.)	Time to fusion (min)
Oleic acid	5.6	Hen	0.35	8	9.71 ± 3.10	15
	7.4	Hen	0.35	1	1.36 ± 0.01	>90
Oleoylglycerol	5.6	Hen	0.28	12*	6.35 ± 0.86	15
	5.6	Hen	0.28	4†	4.29 ± 1.00	15
	7.4	Hen	0.28	1	1.31 ± 0.08	>90
1,3-Dioleoylglycerol	5.6	Hen	0.28	5	1.60 ± 0.39	>90
1,2-Dioleoylglycerol	5.6	Hen	0.28	5	1.30 ± 0.20	>90
Trioleoylglycerol	5.6	Hen	0.28	1	1.26 ± 0.03	>90
Stearoylglycerol	5.6	Hen	0.28	1	1.19 ± 0.12	>90
Retinol	5.6	Hen	0.35	1	1.71 ± 0.05	30
Benzyl alcohol	5.6	Hen	100.00	1	5.40 ± 1.22	30
Oleoylglycerol	5.6	Human	0.28	1	1.79 ± 0.23	30
	7.4	Human	0.28	1	1.26 ± 0.17	>90

* Data from experiments on one hen, gathered over a period of 5 weeks.

† Data from experiments with four hens.

Fig. 2. Influence of dextran on the entry of ⁴⁵Ca²⁺ in hen erythrocytes treated with oleoylglycerol

Dextran was added to the modified Eagle's basal salt solution used for preparing the suspension of oleoylglycerol and for the cell suspension. The values shown are mean values ($\pm$ S.D.) for the entry ratio (see the Materials and Methods section).

left at 0°C as appropriate. ⁴⁵Ca²⁺ and ³H₂O were then immediately added (at zero-time), and the contents of the tubes were mixed. Triplicate samples were transferred to centrifuge tubes and the cells were pelleted within approx. 45–60 s of adding the radioactive isotopes. The entries of ⁴⁵Ca²⁺ and ³H₂O into the cells were measured for the three substances studied at intervals from 1 to 16 min (Fig. 1). With at 37°C, more than 50% of the labelled Ca²⁺ entered the cells in 1 min and after 10 min no further entry occurred. At 0°C the entry of ⁴⁵Ca²⁺ was much slower.

Lipid-soluble fusogens

In previous work it was shown that a number of carboxylic acids, their esters, and retinol, induced the fusion of hen erythrocytes *in vitro* (Ahkong *et al.*, 1973). We have therefore investigated the ability of some of these agents to affect the permeability of hen erythrocytes to Ca²⁺. Oleoylglycerol (referred to previously as glycerol mono-oleate; Ahkong *et al.*, 1973) was previously found to be an effective fusogen at pH 5.6 in the presence of dextran and 1.8 mM-Ca²⁺, whereas di- and tri-oleoylglycerol were found to be only weakly fusogenic and non-fusogenic respectively. In the present studies, a similar relationship was found with regard to their ability to allow exogenous Ca²⁺ to enter hen erythrocytes (Table 2). Stearoylglycerol, a non-fusogenic lipid, did not make the cells permeable to Ca²⁺. Oleic acid, a rather more haemolytic fusogen than oleoylglycerol (Ahkong *et al.*, 1973), gave values for the entry ratio that were greater than those found with oleoylglycerol at pH 5.6. It is noteworthy that the relatively weak fusogenic properties of these agents at pH 7.4 (Ahkong *et al.*,

Table 3. The effects of poly(ethylene glycol)s of different molecular weights and of dimethyl sulphoxide on the entry of Ca²⁺ into hen erythrocytes

Unless otherwise stated, the cells were incubated at pH 7.4 for 1 min in the solution of poly(ethylene glycol) or for 15 min with dimethyl sulphoxide. In experiments with the polymers the cells were washed and resuspended in polymer-free modified Eagle's medium (as described in the Materials and Methods section) before the entry of ⁴⁵Ca²⁺ was measured.

Agent	Concn. (% w/w)	No. of expts.	Ca ²⁺ entry ratio (mean $\pm$ S.D.)	Time to fusion (min)
Poly(ethylene glycol) (mol.wt. 200)	30	1	21.81 $\pm$ 1.85	Lysis*
Poly(ethylene glycol) (mol.wt. 400)	40	1	3.28 $\pm$ 0.26	> 90
Poly(ethylene glycol) (mol.wt. 600)	40	1	12.44 $\pm$ 1.10	Lysis*
Poly(ethylene glycol) (mol.wt. 1500)	40	3	9.67 $\pm$ 1.30	60
Poly(ethylene glycol) (mol.wt. 4000)	40	1	3.71 $\pm$ 0.22	15
Poly(ethylene glycol) (mol.wt. 6000)	40	5	3.96 $\pm$ 3.30	10
Poly(ethylene glycol) (mol.wt. 6000)	40	10†	11.13 $\pm$ 6.31	5
Poly(ethylene glycol) (mol.wt. 20000)	40	1	15.83 $\pm$ 0.94	5
Dimethyl sulphoxide	4.5 M	1	11.01 $\pm$ 1.21	45

* In these experiments, the cells were lysed before fusion was observed.

† Cells were treated with poly(ethylene glycol) for 15 min, instead of 1 min.

1973) were paralleled by smaller values of the entry ratio for $^{45}\text{Ca}^{2+}$. With retinol, an effective fusogen, the entry ratio was relatively small; its value was, however, significantly greater than unity (Table 2).

An unexplained aspect of the fusion of erythrocytes induced by lipid-soluble agents in the extent to which fusion is facilitated by dextran of mol.wt. approx. 80000. Without oleoylglycerol or oleic acid, dextran (80mg/ml) causes neither agglutination nor fusion of hen erythrocytes, but in their presence cell fusion is positively increased by dextran (Ahkong *et al.*, 1973). Fig. 2 shows that the dextran also markedly increased the entry ratio for $^{45}\text{Ca}^{2+}$. It therefore appears that oleoylglycerol and dextran have a synergistic effect on the entry of Ca^{2+} into hen erythrocytes, as well as on cell fusion. This may explain why the low-molecular-weight dextran is itself unable to fuse cells, and why oleoylglycerol is much less effective in inducing cell fusion without the polymer. [Long incubation (30–48 h) of hen erythrocytes with 80–100 mg of a high-molecular-weight dextran (mol.wt. 2×10^6)/ml has been shown by Ahkong *et al.* (1975a) to induce cell fusion.]

The entry of $^{45}\text{Ca}^{2+}$ into human erythrocytes treated with oleoylglycerol was less at pH 7.4 than at pH 5.6 (Table 2). Also, entry of $^{45}\text{Ca}^{2+}$ into the human cells was lower than for hen erythrocytes; this is consistent with the fact that a higher concentration of oleoylglycerol is required to fuse human erythrocytes by comparison with the hen cells.

Water-soluble fusogens

The ability of high concentrations of dimethyl sulphoxide and of poly(ethylene glycol) to fuse hen erythrocytes has been reported previously (Ahkong *et al.*, 1975b,c; Maggio *et al.*, 1976). Like fusion caused by the lipid fusogens, cell fusion induced by these

water-soluble agents is dependent on Ca^{2+} (1.8 mM) and it is inhibited by EDTA (5 mM). Table 3 shows that the water-soluble fusogens all allow Ca^{2+} to enter hen erythrocytes in 15 min at 37°C. In contrast with the behaviour of cells treated with lipids, the optimum pH for cell fusion with water-soluble reagents is usually 7.4 and at this pH a marked entry of Ca^{2+} occurred with the water-soluble fusogens.

Hen erythrocytes that agglutinate and shrink on treatment with poly(ethylene glycol) (mol.wt. 6000) do not fuse *in situ*, possibly because polymer molecules between these cells act as a barrier. Extensive fusion occurs after removal of most of the poly(ethylene glycol) between swollen less-aggregated cells (Maggio *et al.*, 1976). Hence in contrast with experiments with all the other fusogens studied in the present investigations, measurements of the entry of Ca^{2+} into hen erythrocytes treated with poly(ethylene glycol) were made in the absence of poly(ethylene glycol). The cells were thus treated for a short period (usually 1 min), the polymer was then washed away from the cells, which were resuspended in fresh medium to which radioactive isotopes were added. Cells treated in this way retained their permeability to Ca^{2+} for at least 1 h; the entry ratios for 'zero-time', 30 and 60 min of incubation at 37°C [after treatment for 1 min with 40% (w/w) poly(ethylene glycol) (mol.wt. 6000)] were 6.6, 7.3 and 6.9 respectively.

Studies with other cations and anions

The entry of $^{86}\text{Rb}^+$, $^{22}\text{Na}^+$ and $^{35}\text{SO}_4^{2-}$ into hen erythrocytes on treatment with oleoylglycerol and poly(ethylene glycol) (mol.wt. 6000) was studied in a similar manner to that used for $^{45}\text{Ca}^{2+}$. Conditions were chosen that led to extensive fusion in each experiment (except in the absence of unlabelled

Table 4. The effects of poly(ethylene glycol) (mol.wt. 6000) and of oleoylglycerol on the entry of $^{86}\text{Rb}^+$, $^{22}\text{Na}^+$ and $^{35}\text{SO}_4^{2-}$ into hen erythrocytes

Entry of the ions listed was measured as for $^{45}\text{Ca}^{2+}$ after incubation at 37°C for 15 min in the presence of the radioactive ions. In experiments with poly(ethylene glycol), the cells were treated with the polymer at the concentration stated for 15 min before the entry-ratio measurement. All incubations were done in the presence of unlabelled 1.8 mM- Ca^{2+} , at pH 5.6 (with oleoylglycerol as effector) or pH 7.4 [poly(ethylene glycol)]. Extensive cell fusion occurred in all incubations. $^3\text{H}_2\text{O}$ was not included in experiments with $^{35}\text{SO}_4^{2-}$, owing to the difficulties of discriminating between the two radioactive isotopes. The entry of $^{45}\text{Ca}^{2+}$ was measured in a parallel experiment with each of the other ions, and the values given for $^{45}\text{Ca}^{2+}$ are means ($\pm$ S.D.) obtained from the three measurements. Values for the other ions are means ($\pm$ S.D.) obtained from triplicate samples of the cell pellets.

Ion	Effector	Concentration	Entry ratio
$^{45}\text{Ca}^{2+}$	Oleoylglycerol	0.42 mM	5.51 ± 1.73
	Poly(ethylene glycol) (mol.wt. 6000)	40%	9.31 ± 5.96
$^{86}\text{Rb}^+$	Oleoylglycerol	0.42 mM	3.48 ± 0.12
	Poly(ethylene glycol) (mol.wt. 6000)	40%	3.42 ± 0.23
$^{22}\text{Na}^+$	Oleoylglycerol	0.42 mM	2.52 ± 0.06
	Poly(ethylene glycol) (mol.wt. 6000)	40%	2.33 ± 0.08
$^{35}\text{SO}_4^{2-}$	Oleoylglycerol	0.42 mM	2.52 ± 0.06
	Poly(ethylene glycol) (mol.wt. 6000)	35%	1.21 ± 0.08

Ca²⁺). All of the ions studied exhibited a significant increase in entry ratio with erythrocytes treated with oleoylglycerol at concentrations of 0.42 mM (Table 4) and at 0.28 mM (A. M. J. Blow, G. M. Botham & J. A. Lucy, unpublished work), both in the presence and absence of unlabelled 1.8 mM-Ca²⁺. (The results shown in Table 4 were obtained in the presence of unlabelled Ca²⁺.) Entry of ⁸⁶Rb⁺ and of ²²Na⁺ (but not significantly of ³⁵SO₄²⁻) was also observed for cells treated with 40% poly(ethylene glycol) (mol.wt. 6000). The values of the entry ratios for labelled Rb⁺, Na⁺ and SO₄²⁻ were lower than that for ⁴⁵Ca²⁺.

Discussion

The increase in the entry ratio found on treating hen erythrocytes with the ionophore A23187 (Table 1) is consistent with its well known ability to facilitate the movement of Ca²⁺ through biological membranes; Volsky & Loyter (1977) have also reported that Ca²⁺ enters hen erythrocytes in the presence of the ionophore. The similar increase in the entry ratio observed in the present paper on treating hen and human erythrocytes with lipid-soluble (Table 2) and water-soluble fusogens (Table 3) is thought to indicate that these agents also allow Ca²⁺ to enter the cells.

It might nevertheless be suggested that treatment of erythrocytes with fusogenic molecules leads not to entry of Ca²⁺ into the cells, but to binding of Ca²⁺ on the cell surface. If this were the case, it would be expected that omission of LaCl₃ from the procedure used to separate the cells from the incubation medium would increase the observed value of the entry ratio. In fact, when LaCl₃ was omitted the entry ratios were unchanged (A. M. J. Blow, G. M. Botham & J. A. Lucy, unpublished work). The absence of any effect of neuraminidase on the entry ratio also indicated that binding of Ca²⁺ to externally located sialic acid residues is unlikely.

A further argument in favour of fusogenic lipids allowing Ca²⁺ and other ions to enter cells, rather than enhance their surface binding, relates to previous work on the interactions of fusogenic lipids with monolayers of phospholipids at the air/water interface (Maggio & Lucy, 1975). On the basis of this work it was suggested by Maggio & Lucy (1976) that the presence of low-melting molecules of fusogenic lipid might co-operatively increase the number of rotational isomers in clusters of hydrocarbon in a membrane interior, and that these changes might increase the permeability of the lipid bilayer. Hence it seems reasonable to interpret the present behaviour of oleic acid and oleoylglycerol, compared with stearyl glycerol and trioleoylglycerol, in terms of an effect on the lipid bilayer in the erythrocyte membrane that allows Ca²⁺ to enter the cell.

Increases in cell volume, which have been regarded

previously as indicating the occurrence of alterations in membrane permeability, are a marked feature of cell fusion induced by chemicals such as retinol and oleic acid that (in the longer term or higher concentrations) also cause cell lysis (Akhong *et al.*, 1973, 1975c). Poste & Pasternak (1978) have commented, in view of the highly lytic properties of most of these agents, that it is unclear how far permeability changes reflect changes related specifically to fusion. It should be emphasized in this respect, however, that, apart from the experiments with dimethyl sulphoxide, all of the treated cells analysed in the present work had been centrifuged through a layer of silicone oil that excludes lysed cells. It is also noteworthy that with oleoylglycerol more than 50% of the labelled Ca²⁺ entered these cells in 1 min. Furthermore, preparations of poly(ethylene glycol) with mol.wts. of 1500–6000 are of low toxicity to cells and are now widely used for the preparation of viable hybrid cells. Thus, although it is not possible to say conclusively that the observed permeability changes are specifically associated with membrane fusion, it is clear that the changes occur very rapidly in unhaemolysed cells after treatment not only with potentially lytic substances, e.g. oleic acid, but also with the much less damaging preparations of poly(ethylene glycol).

It is relevant that previous work has shown that, although excess EDTA inhibits the fusion of hen erythrocytes by poly(ethylene glycol), EDTA is unable to inhibit fusion when added after the cells have been incubated with the polymer in the presence of Ca²⁺. As pointed out previously (Maggio *et al.*, 1976), it appears that once events leading to cell fusion have been initiated by the treatment with poly(ethylene glycol) exogenous Ca²⁺ is not required for fusion to be completed in this system. In the light of the present observations it thus seems that the entry of Ca²⁺ into erythrocytes, which occurs on treatment with poly(ethylene glycol), acts as a trigger that initiates the fusion process.

It is nevertheless important to assess the role of the entry of Ca²⁺ into cells in relation to other factors in the fusion process. First, it is pertinent that the entry ratio is a measurement of the flux of Ca²⁺ and not of the concentration of intracellular Ca²⁺. Also, the magnitudes of the values observed for the entry ratio can only usefully be compared for a given mixture of radioactive isotopes used with a given batch of cells. In addition it is noteworthy that, under the conditions of the experimental work in the present paper, hen erythrocytes treated with the ionophore A23187 did not fuse. The entry of Ca²⁺ into cells is therefore not itself sufficient to induce fusion. It seems likely that at least the lipid-soluble fusogens have additional effects on the cell membrane that facilitate the fusion process, e.g. the exposure of a normally latent Ca²⁺-dependent proteinase; cf. Quirk *et al.* (1978). In this connection it is interesting

that retinol, an effective fusogen that behaves like other fusogenic lipids in its actions with choline-containing phospholipids in monolayers, was only weakly effective in allowing Ca^{2+} to enter the erythrocytes. Finally, the fusogenic properties of preparations of poly(ethylene glycol) of different molecular weight are not directly related to the entry ratio for Ca^{2+} (Table 3). However, this may possibly be ascribed to impurities in the polymer, which is often prepared from ethylene oxide with sodium ethoxide or strontium carbonate as catalysts. Traces of these compounds may remain in the commercial preparation of poly(ethylene glycol) used.

We have, nevertheless, not observed erythrocytes to fuse in the absence of an entry of Ca^{2+} . Furthermore, a number of other findings combine to indicate that the entry of Ca^{2+} into the cytoplasm of erythrocytes is of major importance in the fusion process even though other parameters are without doubt also involved. At 0°C the entry of Ca^{2+} into cells treated with oleoylglycerol was very slow. Cell fusion in the cold is also extremely slow (Fisher, 1975). Similarly, the relatively weak fusogenic properties of the lipid fusogens at pH 7.4 were paralleled by smaller values of the entry ratio for Ca^{2+} at this pH. By contrast, the optimum pH for fusion with water-soluble reagents is usually 7.4, and at this pH a marked entry of Ca^{2+} occurred with water-soluble fusogens. Additionally, the hitherto unexplained action of dextran of low molecular weight in facilitating the fusion of erythrocytes induced by lipid-soluble agents has been shown here to be related to an increase in the entry ratio for Ca^{2+} . Finally it has been briefly reported elsewhere (Blow *et al.*, 1978a) that hen erythrocytes treated with poly(ethylene glycol) exhibit an abnormal permeability to Ca^{2+} only after treatment with concentrations of polymer that are greater than 30% (w/w), i.e. only after treatment with fusogenic concentrations of poly(ethylene glycol).

In conclusion we consider that the observations reported in the present paper, taken together with those that have been made previously, indicate that both lipid-soluble and water-soluble fusogens increase the permeability of erythrocyte membranes to cations, including Ca^{2+} , and that the entry of Ca^{2+} into the cells initiates the process of cell fusion. Lipid-soluble fusogens may alter membrane permeability by influencing the structure of the hydrocarbon chains and/or the polar groups of membrane phospholipids (Maggio & Lucy, 1975, 1976). The effects of water-soluble fusogens may be mediated either through surface potential effects (Maggio & Lucy, 1978), and/or actions on the water of hydration of the phospholipid polar groups in the cell membrane (Blow *et al.*, 1978a). How an increased concentration of intracellular Ca^{2+} initiates cell fusion is by no means clear at present, and it seems possible that more than one pathway may be involved. The

movement of integral membrane proteins, which appears to be important in the fusion process, has been suggested to involve Ca^{2+} -mediated movements in the cytoskeletal elements of cells, and/or Ca^{2+} -mediated phase separations of membrane phospholipids (Ahkong *et al.*, 1975c; Lucy, 1978; Papahadjopoulos, 1978). In addition, an important feature of membrane fusion may be the proteolytic degradation of certain membrane proteins that results in an increased freedom of movement of membrane proteins (Ahkong *et al.*, 1978; Quirk *et al.*, 1978).

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