WATER AND CALCIUM IONS IN CELL FUSION INDUCED BY POLY(ETHYLENE GLYCOL)

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1. Introduction

There is currently considerable interest in the molecular modes of action of chemicals that induce cell fusion. This arises in part because of the continuing development of chemically-induced cell fusion as a laboratory tool. Thus poly(ethylene glycol), (PEG), has been used to fuse plant protoplasts [1,2], bacterial protoplasts [3,4], plant protoplasts with animal cells [5–7], and animal cells in culture [8–12]. The actions of chemical fusogens are also being investigated as a means of obtaining insights into the numerous instances of membrane fusion occurring in physiological and pathological systems in vivo [13,14]. To obtain information on the molecular mechanisms by which PEG induces the fusion of biological membranes we have studied:

(i) The effectiveness of differing concentrations of PEGs (mol. wt 200–20 000) in causing Lesch-Nyhan human skin fibroblasts to fuse;
(ii) The state of water in these solutions by means of differential scanning calorimetry;
(iii) The effect of PEG on the permeability of erythrocytes to $^{45}Ca^{2+}$.

Our observations indicate that PEG induces the fusion of fibroblasts by altering the physical state of bulk water adjacent to the cell surface and/or the water of hydration of the phospholipid polar groups in the cell membrane. Also, concentrated solutions of PEG act, in common with lipid-soluble fusogens, to allow Ca^{2+} to enter the cytoplasm of erythrocytes: this precedes the cell fusion reaction. It has been found, in addition, that PEG may be utilised in a controllable manner to induce fibroblasts to fuse by treating the cells with 35% and 40% (w/w) solutions for longer time periods than have been suggested.

2. Experimental

PEG-200, -400, -600, -1500 and -20 000 were from BDH, Poole and PEG-4000 and 6000 were from Koch-Light, Colnbrook.

Lesch-Nyhan human skin fibroblasts, between 15th and 25th passage, were grown at 37°C in Dulbecco's modification of Eagle's medium (DME) (Flow Laboratories) supplemented with 10% foetal calf serum (FCS), 2 mM glutamine, 100 IU/ml penicillin and streptomycin, buffered by bicarbonate–CO_2. Cells were routinely subcultured every 7 days. They were seeded at 0.7×10^5 cells in 3 replicate 30 mm diam. Petri dishes (each containing a cover slip) and grown for 24 h in DME–10% FCS. The cells were fused as in [15]. They were treated for the time shown with solutions of PEG in DME, washed, and then cultured for 6 h, before being fixed and stained with Leishman's stain. At least 300 cells on each cover slip were counted over randomly selected areas. The percentage fusion was calculated as [(number of nuclei counted) × (number of cells counted)$^{-1}$ − 1] × 100.

For differential scanning calorimetry, samples of polymer solutions were sealed in aluminium pans designed for volatile samples. Using a Perkin-Elmer model DSC-1B, in the low temperature mode with liquid nitrogen as coolant and an empty pan as reference, samples were cooled from 310–173 K and then heated to 310 K at 8 K/min.

The entry of $^{45}Ca^{2+}$ into hen erythrocytes was

investigated using centrifugation through a water-impermeable barrier of silicone oil to separate the cells from the radioactive solution [16,17].

3. Results and discussion

3.1. *Cell fusion in tissue culture*

High PEG concentrations are known to be necessary for the effective induction of cell fusion. As a result, the cells are normally treated with a concentrated solution of polymer for a very short time, usually 1 min [8,11] because a longer exposure leads to cell damage. Cell fusion is observed after removal of most of the PEG.

We have investigated the fusion of Lesch-Nyhan (LN) fibroblasts induced by treating these cells for up to 30 min with solutions of 25–50%, w/w, PEG-1500. Although the 50%, w/w, solution of polymer was most effective in producing cell fusion when used to treat cells for 1 min, maximum fusion with the 45% and 40% w/w solutions occurred when the cells were treated for 5 min and 15 min, respectively (table 1). It therefore seems that the use of more dilute solutions of PEG to treat animal cells for longer periods may be advantageous for the production of hybrid cells. The longer contact time can be more easily controlled, and cell damage thus minimised.

With preparations of PEG having mol. wt 200–20 000, the optimum concentration for cell fusion varied with the molecular weight of the polymer when the cells were treated with PEG for 1 min. As the molecular weight was increased, the concentration at which the polymer was most effective decreased, from 60% for PEG-200, to 45% for PEG-20 000. Cytological studies showed that extensive cell damage was caused by solutions of PEG that were more concentrated than the optimum for cell fusion.

3.2. *Differential scanning calorimetry on solutions of PEG*

Thermal transitions from 173–310 K were observed on heating the solutions of PEG that depended on the concentrations and molecular weights of PEG. Most solutions of PEG exhibited an endothermic peak between 255 K and 279 K, the enthalpy (per g sample) and temperature of which decreased with increasing polymer concentrations (fig.1). We have ascribed this transition to water that is able to freeze and to melt, i.e., the 'free water' of the solution. The quantity of free water was always less than the total water, presumably because the polymer interacts strongly with some water molecules [18], which are then unable to freeze or melt. No free water was present at high concentrations of low molecular weight preparations of PEG, e.g., 60% PEG-200, 55% PEG-400 and 55% PEG-600 (fig.1).

The calorimetry curves for solutions of higher molecular weight preparations of PEG (-1500, -4000, -6000 and -20 000) were more complex. Solutions of these polymers showed an additional endothermic transition at 255 K (fig.1). We tentatively consider that this represents the melting of a polymer hydrate. As with the smaller polymers, the higher melting exotherm (255–270 K) is thought to represent the

Table 1
Effect of contact time with PEG-1500 on fusion of LN fibroblasts

PEG 1500	Percentage fusion ± SD				
(% w/w)	1 min	2.5 min	5 min	15 min	30 min
0	1.40 ± 0.29 (3)	1.21 ± 0.11 (3)	1.77 ± 0.92 (3)	1.50 ± 1.24 (3)	1.49 ± 1.22 (3)
25	1.40 ± 0.33 (3)	1.28 ± 0.22 (3)	1.28 ± 0.40 (3)	1.39 ± 0.56 (3)	2.13 ± 1.34 (3)
30	1.36 ± 0.33 (3)	1.62 ± 1.08 (3)	1.60 ± 1.14 (3)	1.95 ± 0.70 (3)	4.52 ± 4.00 (3)
35	2.28 ± 0.40 (3)	2.67 ± 0.33 (3)	3.57 ± 0.64 (3)	7.72 ± 2.17 (3)	9.68 ± 2.32 (3)
40	6.84 ± 2.57 (3)	9.89 ± 2.26 (3)	14.57 ± 5.52 (3)	24.40 ± 3.04 (3)	18.33 ± 10.31 (3)
45	13.33 (2)	25.41 ± 2.69 (3)	26.65 ± 7.56 (3)	1.74 (2)	
50	17.53 ± 4.00 (3)	14.93 ± 7.74 (3)	2.80 (2)		

The values shown are the means ± SD where appropriate, of the number of independent experiments shown in brackets

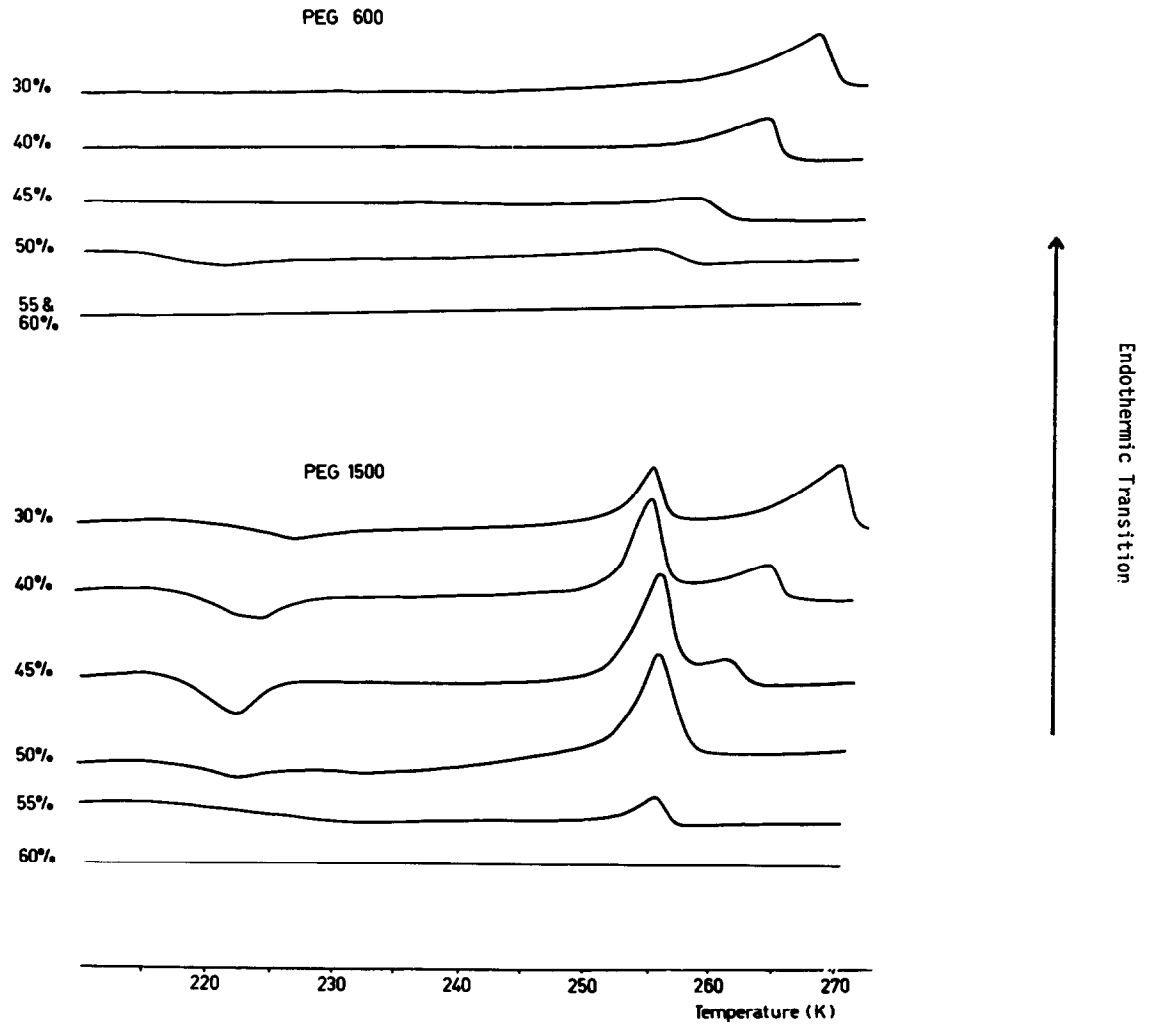

Fig.1. Differential scanning calorimetry traces for solutions of PEG (% w/w) for PEG-600 and PEG-1500. The sample weights were 5–10 mg, and the traces shown are not directly comparable for the 2 polymers in terms of peak area.

melting of frozen free water. This peak was not detected in solutions of 50%, w/w, PEG-1500, and 45% w/w PEG-4000, -6000 and -20 000.

Table 2 relates the maximum percentage of cell fusion observed with LN fibroblasts, induced by solutions of PEG, to the concentration of free water in the PEG solutions used. The free water (% w/w) in the solutions that induced maximum cell fusion was zero or, with PEG-20 000, extremely low. It therefore seems that the ability of PEG to bind water and to decrease the free water content of its aqueous solutions is important in causing cell fusion. This may explain why cell fusion was observed at progressively lower concentrations of polymer when the molecular weight was increased, as shown in table 2, and why PEG caused cell damage at high concentrations. It is interesting that the more dilute solutions of PEG, which induced fibroblasts to fuse only when used to treat the cells for longer time periods, contained increasing quantities of free water (fig.2). For example a 35% w/w solution of PEG 1500 (giving 10% fusion in 30 min) contained ~20% w/w free water.

Table 2
Fusion of LN fibroblasts and the binding of water by PEG

PEG mol. wt	Maximum cell fusion[a] observed (%)	Concentration of PEG[b] (% w/w) for maximum cell fusion	Concentration of free water[c] (% w/w) for maximum cell fusion
200	17.8 (2)	60	0.0
400	73.0 ± 4.6 (4)	55	0.0
600	37.2 ± 4.2 (4)	55	0.0
1500	27.1 (2)	50	0.0
4000	25.2 (2)	50	0.0
6000	13.1 ± 2.4 (4)	50	0.0
20 000	5.2 ± 1.2 (4)	45	0.6

[a] Cell fusion was measured after a 30–60 s treatment of the cells with PEG. The cells were seeded at 2×10^5 cells onto 50 mm diam. Petri dishes, each containing one 2 × 2 mm glass cover slip, and the values given are means of the fusion observed in replicate dishes, ± SEM where appropriate, with the number of dishes shown in brackets

[b] Cells were treated with PEG solutions of 0%, 30%, 40%, 45%, 50%, 55% and 60%, w/w

[c] The free water content (% sample weight) was obtained from the differential scanning calorimetry trace by measuring the area of the peak due to the melting of ice and relating this to a standard curve of peak area against weight of water. The area of each melting peak was measured in triplicate with a planimeter. Samples were cooled and heated 3 times and the mean values obtained

3.3. *Ca^{2+} and cell fusion*

Ca^{2+} is usually essential for cell fusion and LN fibroblasts treated with PEG-6000 in the presence of 8 mM EGTA gave no fusion greater than that observed in the control cultures in 3 independent experiments. On the basis of observations on hen erythrocytes treated with the ionophore A23187, it was suggested that Ca^{2+} may mediate cell fusion by interacting with the cytoplasmic side of the plasma membrane following an initial increase in membrane permeability to Ca^{2+} [19].

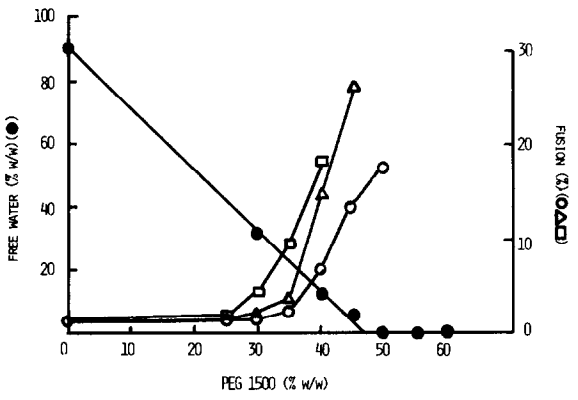

Fig.2. Fusion of LN fibroblasts following treatment with PEG-1500 for 1 min (○), 5 min (△) and 30 min (□). The free-water content of the solutions of PEG (○) was obtained from differential scanning calorimetry traces.

The entry of $^{45}Ca^{2+}$ into hen erythrocytes treated with PEG has now been measured. Inclusion of ^3H_2O in the medium, which rapidly penetrates cells, gives a measure of intracellular volume, and thus the $^{45}Ca^{2+}$: ^3H_2O cpm ratio provides an index of the concentration of intracellular Ca^{2+}. The ratio of this index for treated cells, relative to control cells, is referred to here as the entry-ratio. The data of fig.3 were obtained with hen erythrocytes that were incubated for 1 min with 3 different preparations of PEG: the cells were washed and then resuspended for 15 min in medium, free from PEG, containing $^{45}Ca^{2+}$. The cells exhibited an abnormal permeability to Ca^{2+} only after treatment with concentrations of PEG that were >30% w/w, i.e., only after treatment with fusogenic concentrations of PEG. Above this concentration PEG-4000 and -6000 induced cell fusion, but PEG-1500 which is less fusogenic with erythrocytes [9] did not. Cell fusion was always preceded by the entry of Ca^{2+} into the erythrocytes. The change in membrane permeability induced by PEG is probably extremely rapid since in [9] addition of EDTA, when

the erythrocytes were washed to remove most of the PEG, was unable to inhibit fusion.

Cell fusion induced by PEG, in common with that caused by lipid-soluble fusogens [20], thus seems to depend on a change in membrane permeability to Ca^{2+}. The changed permeability may be related to the capacity of PEG to decrease the surface potential of monolayers of phospholipid [9] but it is apparent from the inability of PEG-1500 to fuse erythrocytes that Ca^{2+} entry is not itself sufficient to induce the fusion process. An increase in the cytoplasmic concentration of Ca^{2+} could give rise to several membrane changes [19,21,22] that may lead to cell fusion. Recent work has raised the additional possibility that a Ca^{2+}-dependent proteolytic degradation of certain membrane proteins may also be an important feature of cell fusion [23].

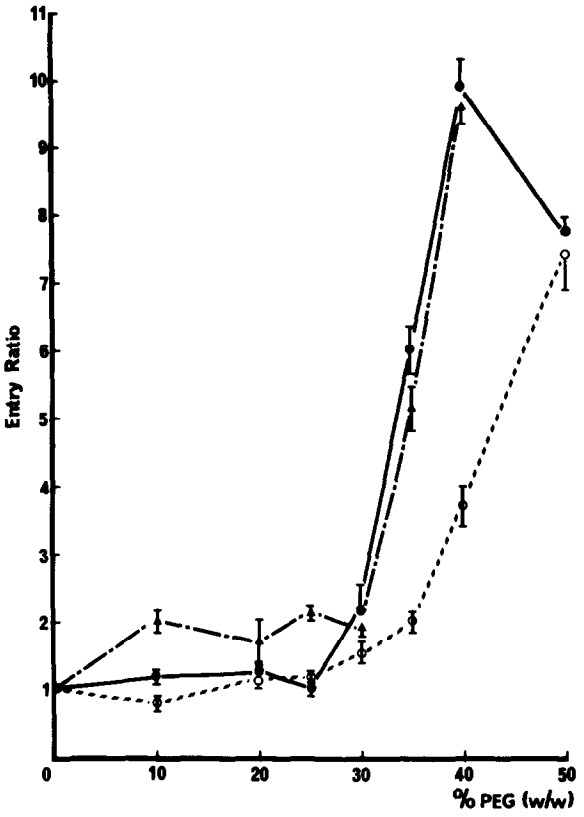

Fig.3. Dose response of PEG-induced $^{45}Ca^{2+}$ entry into hen erythrocytes. Erythrocytes (3.5×10^8 cells/ml final conc.) were resuspended at 37°C in 1 ml buffered solution A: NaCl 116 mM; $CaCl_2$ 1.8 mM; Me_2AsO_2Na 10 mM; glucose 1 g/l; penicillin G 200 000 units/l; streptomycin sulphate 100 mg/l (pH 7.4), containing PEG as indicated. After 1 min, 3.5 ml solution A at 37°C was added, the suspension centrifuged at 3000 × *g* for 5 min, and the cells washed with a further 3.5 ml solution A at 37°C, recentrifuged and resuspended in 1 ml solution A containing 0.4 μCi $^{45}Ca^{2+}$ and 2.0 μCi $^3H^+$, at 37°C. After 15 min, triplicate 250 μl samples of cells were separated from the radioactive loading solution by centrifugation at 12 000 × *g* for 1 min through silicone oil, overlaid with 25 μl 5.5 mM $LaCl_3$ to reduce surface bound Ca^{2+} from the erythrocytes. The points shown are means ± SD of triplicate assays. PEG-1500 (•); PEG-4000 (○); PEG-6000 (▲).

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