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Osmotic forces in artificially induced cell fusion

Quet F. Ahkong and Jack A. Lucy *

Department of Biochemistry and Chemistry, Royal Free Hospital School of Medicine, Rowland Hill Street, NW3 2PF London (U.K.)

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The importance of cell swelling in the fusion of erythrocytes by three different chemical treatments has been investigated with cells that were cytoplasmically labelled with 6-carboxyfluorescein. Hen erythrocytes, which had been pre-incubated with ionophore A23187 and 5 mM Ca^{2+} to cause a proteolytic breakdown of the membrane skeleton, were induced to fuse by applying an osmotic shock. Human erythrocytes that had been incubated in an isotonic salt/buffer solution, which was progressively diluted and which contained 0.5 mM La^{3+} to minimise cell lysis, were also fused. In addition, the fusion of human erythrocytes by 40% poly(ethylene glycol) began only when the poly(ethylene glycol) was diluted, and it mostly occurred when the diluted polymer solution was subsequently replaced by isotonic buffer. In related experiments, the effect of an osmotic gradient on electrically induced cell fusion has been studied. Human erythrocytes in 150 mM erythritol fused more readily than less swollen cells in 200–400 mM erythritol when subjected to a 20 μs pulse of 3.5 kV · cm^{-1}, indicating that the extent of cell fusion induced by the breakdown pulse is governed by the combined electrical-compressive and osmotic forces. Since osmotic phenomena are already known to be important in exocytosis, we suggest that these observations on cell fusion indicate that osmotic forces may provide the driving force for many membrane fusion reactions in biological systems.

Introduction

The molecular mechanisms that govern membrane fusion reactions are currently of much interest because fusion is an essential feature of many biological phenomena e.g., fertilization, virus-cell fusion, myoblast fusion, and exocytosis. The production of monoclonal antibodies also depends on the fusion of myeloma cells with lymphocytes.

In early work on the chemically induced fusion of erythrocytes we concluded that cell swelling by colloid osmosis played an essential role in cell fusion [1]. Subsequently it has been demonstrated that solutions of high osmolarity inhibit exocytosis [2–4] and that phospholipid vesicles can be fused with a planar phospholipid bilayer by osmotic forces alone [5]. In the latter work, it has been shown that the vesicles first form a stable, tightly bound pre-fusion state with the planar membrane, and that osmotic swelling of the bound vesicles then provides the driving force for fusion of the two membranes. Since erythrocytes are, however, not induced to fuse by osmotic swelling alone, it has not previously been possible to decide whether fusion induced by chemical fusogens is causally related to the osmotic changes or whether it is merely accompanied by them. A possible reason for the failure of erythrocytes to fuse when they are subjected to osmotic swelling alone is that structural changes in the plasma membrane, which

* To whom correspondence should be addressed.

Abbreviations: PEG, poly(ethylene glycol); Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

are induced by chemical fusogens, do not occur. For example, it is known that endogenous proteinases that degrade membrane proteins play a rôle in the chemically-induced fusion of erythrocytes, since inhibitors of the enzymes have been shown to inhibit fusion [6–11]. Furthermore, the electrically induced fusion of these cells in low-ionic-strength media also seems to involve a perturbation of membrane proteins [12].

In this paper we report that osmotic swelling can drive the fusion of erythrocytes under conditions that are known to perturb the membrane skeleton. We also show that fusion induced by poly(ethylene glycol) occurs when water enters erythrocytes as they are rehydrated, rather than when the cells shrink as they are dehydrated, and that osmotic forces are involved in the fusion of erythrocytes by a high-voltage breakdown-pulse. It is therefore proposed that osmotic phenomena may be a general feature of fusion reactions in biological membranes. Aspects of this work have previously been reported in preliminary form [13].

Materials and Methods

Fluorescent labelling of erythrocytes. Erythrocytes were washed and freed from leucocytes as previously described [6]. The cells were then suspended in salt solution (110 mM NaCl/40 mM Hepes buffer at pH 7.4). 6-Carboxyfluorescein diacetate (from Molecular Probes, Eugene, OR, U.S.A.) was used to label erythrocytes as follows. The cells (0.2 ml packed cell volume) were incubated at 37°C in 1 ml of the above NaCl/Hepes buffer at pH 7.4 for 30 min with 200 μg 6-carboxyfluorescein diacetate (prepared from a 10 mg · ml^{-1} stock solution in acetone). The cells were washed twice in the NaCl/Hepes buffer, 0.1 ml of packed, labelled cells was added to 0.9 ml of packed, non-labelled cells, and the mixed cell population was suspended in a total of 5 ml of NaCl/Hepes buffer. 1 ml of this cell suspension was placed in an Alcian blue-coated [14] plastic dish (35 mm). After 30 min, non-attached erythrocytes were washed off with NaCl/Hepes buffer, and the attached cells were maintained in 1 ml of NaCl/Hepes buffer at 37°C.

Chemically induced cell fusion. PEG 6000 was obtained from BDH (Poole, Dorset, U.K.), and was dissolved in a buffered salt solution (pH 7.4) that contained 110 mM NaCl and 40 mM Hepes buffer. Dextran (M_r 60000–90000) was from Sigma Chemical Co., Poole.

Electrically induced cell fusion. The chemicals used were obtained from Sigma (erythritol), BDH (histidine), and Serva, Heidelberg, F.R.G. (pronase from *Streptomyces griseus*). Erythrocytes in a small chamber were aligned by dielectrophoresis in an a.c. field of 400 $V \cdot cm^{-1}$ (from a function generator type TE7702, Toellner Electronic, M. Werner, Frankfurt, F.R.G.) applied between two electrodes that were 200 μm apart. The subsequent electrical breakdown pulse was applied to the cells from a pulse generator (type 214B, Hewlett-Packard). Electrically induced cell fusion was undertaken at room temperature.

Results

Fusion of hen erythrocytes by an osmotic shock

The effect of osmotic swelling on erythrocytes is conveniently investigated with monolayers of cells that are attached to an Alcian blue-coated surface [14]. When such cells are exposed to a hypotonic media they merely lyse without fusing, whether or not Ca^{2+} is present. Thus unlike the fusion of phospholipid vesicles with a phospholipid bilayer [5], erythrocytes are not fused by simple osmotic swelling. However, since the cells swell much less than a perfect osmometer in mildly hypotonic solutions because of their spectrin-actin network [15], it seemed possible that osmotic swelling might drive cell fusion if the restraining membrane skeleton is previously damaged or destroyed.

Hen erythrocytes were therefore pre-incubated with ionophore A23187 and Ca^{2+}. This induces a proteolytic breakdown of spectrin and goblin [8], and release intramembranous particles from the restraining effects of the membrane skeleton [16]. Prolonged incubation of cells treated in this way or incubation at 47°C results in cell fusion [17], which is probably facilitated by a Ca^{2+}-stimulated phosphodiesterase that yields a diacylglycerol [18] which may be fusogenic [19]. However, under the conditions of our experiments, little fusion was apparent after 2 h at 37°C in the pre-treated cells (Fig. 1a) but, only 5 min after adding water to

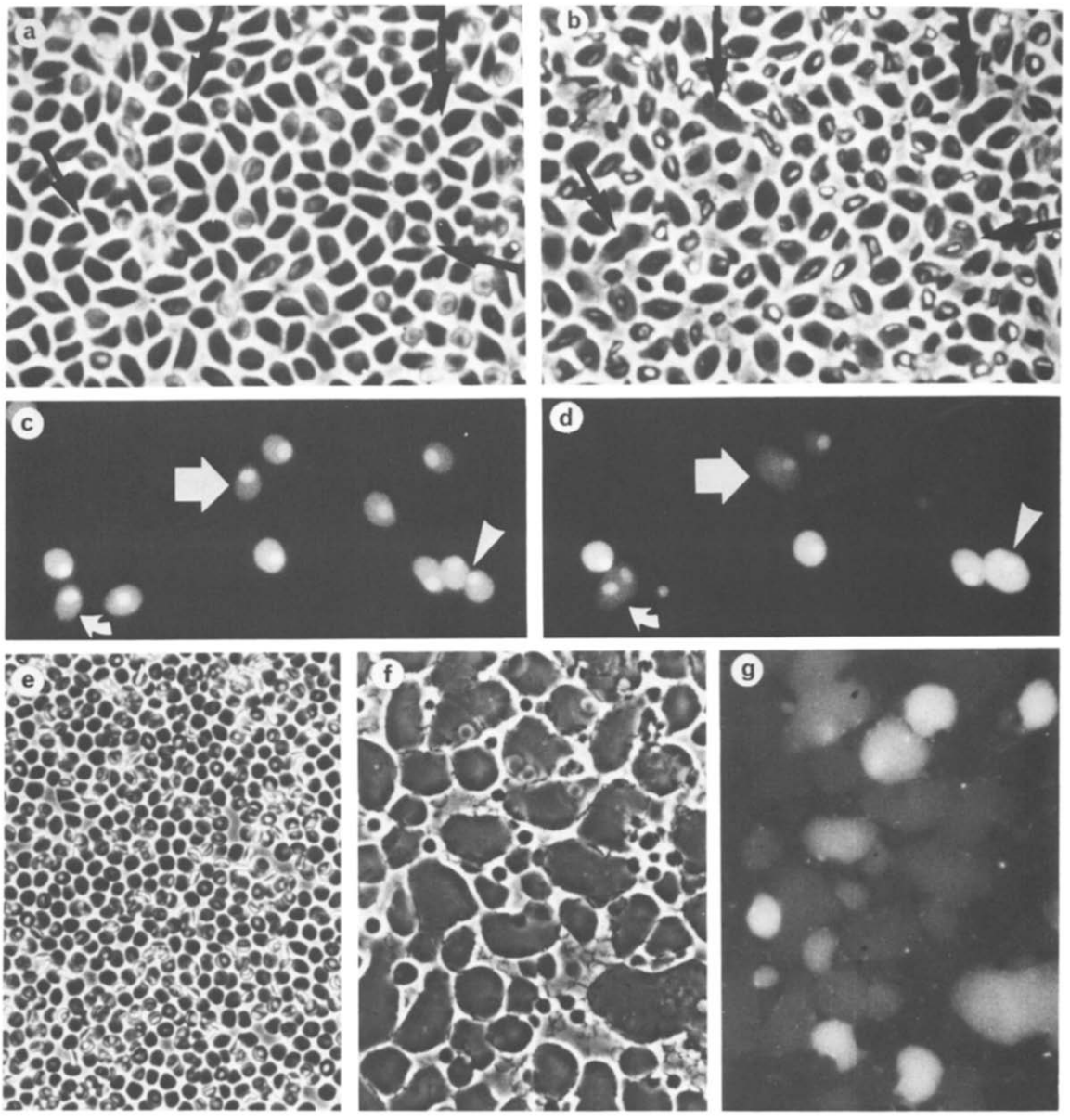


Fig. 1. (a) A monolayer of hen erythrocytes was attached to the surface of an Alcian blue-coated plastic dish. The NaCl/Hepes buffer was removed and replaced by 1 ml of NaCl/Hepes buffer that contained 10 $\mu g \cdot ml^{-1}$ ionophore A23187, 5 mM Ca^{2+} and 80 $mg \cdot ml^{-1}$ dextran (M_r 60000–90000), and the cells were then incubated at 37°C for 2 h. (b) A micrograph taken 5 min after the addition of 1.5 ml of a solution of 80 $mg \cdot ml^{-1}$ dextran to the cells shown in (a) to dilute the buffered-salt solution by 2.5-fold. Four binucleate cells (arrowed) are seen after the osmotic shock, which were not present in (a). (c) Hen erythrocytes were labelled with 6-carboxyfluorescein as described in Materials and Methods, treated as in (a) and visualised by fluorescence microscopy. (d) A micrograph taken, like that in (b), 5 min after dilution of the buffered-salt solution by 2.5-fold. Following the osmotic shock, two fluorescent cells have fused with previously non-fluorescent cells (arrows), and a pair of fluorescent cells have fused with each other (arrowhead). (e) Human erythrocytes were labelled with 6-carboxyfluorescein and attached as a monolayer to a plastic dish as described in Materials and Methods. (f) For osmotically induced cell fusion, the medium was removed from the cells in (e) and replaced by 1 ml NaCl/Hepes buffer containing 0.5 mM La^{3+} and 80 $mg \cdot ml^{-1}$ dextran. After 5 min at 37°C, 0.5 ml of a solution containing 0.5 mM La^{3+} and 80 $mg \cdot ml^{-1}$ dextran was added. After a further 5 min at 37°C, a further 1 ml of La^{3+}/dextran solution was added. After 10 min at 37°C, the whole volume of solution (2.5 ml) was withdrawn and replaced by 1 ml of a solution containing 0.5 mM La^{3+} only. The micrograph (f) shows that, after this exposure to increasingly dilute media, very large polycells are present which have retained their haemoglobin. The outlines of lysed, single cells are also discernible between the polycells. (g) When the cells of (f) were visualised by fluorescence microscopy, very large cells of varying fluorescent intensity were seen to have been formed by the swelling procedure. Magnifications: (a)–(d), ×540; (e)–(g), ×315.

dilute the buffered salt solution 2.5-fold, binucleate cells were clearly seen to have been formed by the fusion of previously separate cells (Fig. 1b). The fused cells were unable to round up because they were firmly attached to the Alcian blue-coated surface. (This point is considered further below in relation to cells fused by poly(ethylene glycol).)

In a similar experiment, a proportion (10%) of the erythrocytes were previously treated with 6-carboxyfluorescein diacetate, which enters cells and is enzymatically hydrolysed to 6-carboxyfluorescein [20]. 5 min after the osmotic shock, fluorescent cells had fused with non-fluorescent cells to produce weakly fluorescent cells: they had also fused with each other (Figs. 1c and 1d).

Fusion of human erythrocytes in progressively diluted media

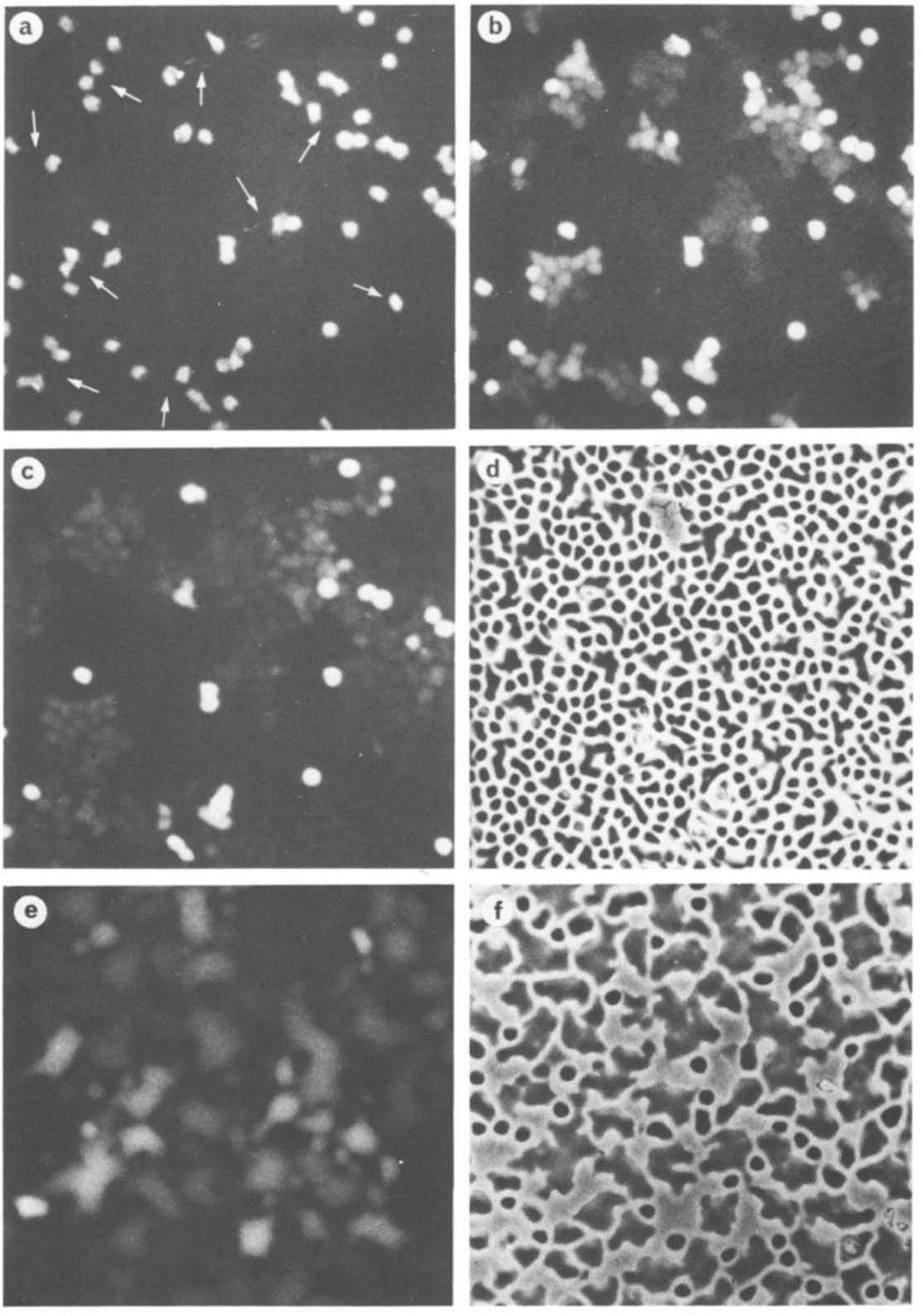
Human erythrocytes show greatly restricted volume changes at osmolarities between 200–700 mosM. This behaviour has been attributed to a mechanical restriction caused by the membrane skeleton, since the restricted volume change is abolished by pretreating the cells with diamide or by heating them for 60 min at 50°C [15]: conditions that are known to disrupt the spectrin-actin network. An appropriate disruption of the membrane skeleton that could facilitate the fusion of human erythrocytes might thus be caused by cell swelling in very dilute media, as human erythrocytes swell like a simple osmometer in solutions that are more dilute than 150 mM [15].

Monolayers of human erythrocytes (Fig. 1e) were therefore incubated in an isotonic salt/buffer solution that was progressively diluted, first by 1.5-fold, then by a further 1.7-fold, and finally replaced by water alone. Dextran was present until the final stage to reduce the rate of swelling, and La^{3+} (0.5 mM) was present throughout to inhibit lysis. This procedure resulted in the formation of very large polycells of irregular shape that retained their haemoglobin (Fig. 1f), as well as giving rise to lysed single cells and polyghosts. Some of the erythrocytes were prelabelled with carboxyfluorescein, and corresponding, very large cells of varying fluorescent intensity were formed by the swelling procedure (Fig. 1g). Incubation of erythrocytes with 0.5 mM La^{3+} for 30 min at 37°C in an isotonic medium containing dextran had no effect on the cells. When La^{3+} was omitted or replaced by 2 mM Ca^{2+}, swelling procedure caused lysis without fusion. Cations (including Ca^{2+}) are known to decrease the size of holes produced in human erythrocyte membranes by osmotic lysis [21] but La^{3+} (possibly because of its high charge) is clearly particularly effective in this respect. These experiments confirm that, under appropriate conditions, osmotic swelling can induce the fusion of erythrocytes. It should be noted, however, that, as well as minimising lysis, 0.5 mM La^{3+} may additionally facilitate osmotically driven cell fusion by perturbing the polar groups [22] of erythrocyte phospholipids. Thus, 0.1–0.3 mM La^{3+} disrupts phospholipid vesicles [23] and, at much higher concentrations (10 mM), La^{3+} causes human erythrocytes to fuse in 325 mosM saline [24].

Fusion induced by poly(ethylene glycol)

Is cell fusion induced by poly(ethylene glycol) driven by osmotic forces? In concentrations that are sufficiently high (30–50%) to induce cell fusion, the polymer has such an avidity for water that no unbound water is present [25], and this withdrawal of water is considered to force membranes together. However, despite much work on the perturbation of phospholipid systems by poly(ethylene glycol), the way in which its dehydrating action causes cell fusion rather than just the close apposition of membranes has not been elucidated. It is significant in this connection that, although fusion and rearrangement of phospholipid vesicles occurs in the presence of concentrated poly(ethylene glycol) [26,27], cytoplasmic bridges between cells are not seen until the polymer solution has been diluted with isotonic buffer, and the cells subsequently resuspended in an isotonic buffer [28,29]. This indicates that, while the dehydration of poly(ethylene glycol)-treated cells brings them into close contact, it is their rehydration that causes cell fusion, and this possibility has been substantiated in our experiments.

In the present work we found that entrapped carboxyfluorescein is largely retained in human erythrocytes in 40% PEG 6000 but that the fluorophore immediately leaks from the cells when the poly(ethylene glycol) is diluted to 13%. La^{3+} ions (50 μM) are, however, highly effective in decreas-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ing this rapid leakage, and their presence allows the fluorophore to be used as a cytoplasmic marker for cell fusion. When a mixed monolayer of carboxyfluorescein-labelled erythrocytes and non-labelled cells (in the proportion of 1 : 9) is incubated for 1 h at 37°C in buffered saline or in 40% PEG 6000, none of the unlabelled erythrocytes becomes fluorescent. By contrast, Fig. 2 shows the changes observed when, after an initial incubation for 5 min in 40% poly(ethylene glycol) (Fig. 2a), the cells are incubated for 5 min in 13% poly(ethylene glycol) and then for a further 10 min in buffered saline (Fig. 2b). Many cells are now weakly fluorescent which, beforehand, were non-fluorescent (cf. the arrowed regions in Fig. 2a). Continued incubation for a further 50 min was found to increase the number of weakly fluorescent cells (Fig. 2c), and to yield cells that are seen by phase-contrast microscopy to be fused (Fig. 2d). When such cells are swollen by a 2-fold dilution of the buffer, very large polycells of irregular shape are observable by both fluorescence and phase-contrast microscopy (Figs. 2e and 2f).

The most probable explanation for these findings is that carboxyfluorescein is transferred to the previously unlabelled erythrocytes as a consequence of cell fusion. It is unlikely that any carboxyfluorescein (or its unhydrolysed acetate derivative), which leaks from the labelled erythrocytes, is taken up from high dilution in the medium by specific groups of unlabelled cells so effectively that they become fluorescent. Furthermore, when human erythrocytes are fused in the presence of 5 mM EGTA in place of 50 μM La^{3+} (i.e., under leaky conditions), cellular transfer of fluorescence is not seen and, at the end of the experiment, none of the fused cells is more fluorescent than the background. We conclude from these experiments that the fusion of erythrocytes does not occur when they are dehydrated in concentrated solutions of poly(ethylene glycol). Furthermore, although a few unlabelled erythrocytes become fluorescent when the poly(ethylene glycol) is diluted to a concentration of 13% (data not shown), it is apparent that fusion mostly occurs when water enters the dehydrated cells during their rehydration in the buffered saline. This behaviour differs from the fusion of multilamellar liposomes by poly(ethylene glycol), which occurs in concentrated solutions of the polymer and is accompanied by extensive structural rearrangements that give rise to larger, but still multilamellar, liposomes [27].

When human erythrocytes in suspension are treated with PEG 6000, they shrink and collapse into aggregates of tightly apposed cells. Some aggregates contain up to 20 cells, while others contain only a few (Fig. 3a). No evidence of cytoplasmic continuity between the apposed cells is seen until they are rehydrated in buffered saline. It is then apparent that some of the cells are fused via narrow, cytoplasmic bridges (Fig. 3c). These bridges probably derive from small regions of cellular contact, like that arrowed in Fig. 3a, and at higher magnification in Fig. 3b, since penetration of water between the rehydrating erythrocytes causes most regions of the tightly apposed plasma membranes to separate. Quite large spaces thus develop between the fused erythrocytes (Fig. 3c and d). As a consequence, the fused cells appear by light microscopy not to be fused, and it is doubtless for this reason that the weakly fluo-

Fig. 2. (a) A monolayer of a mixture of 6-carboxyfluorescein-labelled and non-labelled human erythrocytes was prepared as described in Materials and Methods and the cells were incubated for 5 min at 37°C in 1 ml of buffered salt solution that contained 40% (w/v) PEG 6000 and 50 μM La^{3+}. The fluorescent marker can be seen in individual erythrocytes. Arrows indicate the regions where cell fusion is subsequently apparent in (b) and (c). (b) 2 ml of the NaCl/Hepes buffer containing 50 μM La^{3+} was added to dilute the poly(ethylene glycol) to 13%, and the cells in (a) were then incubated for 5 min at 37°C. The whole volume of poly(ethylene glycol) solution (3 ml) was then withdrawn and replaced by 2 ml of the NaCl/Hepes buffer containing 50 μM La^{3+}, and the cells were incubated for a further 10 min at 37°C. The fluorescent marker has now been transferred by cell fusion to adjacent cells. (c) The cells in (b) after incubation for a further 50 min at 37°C, showing more extensive fusion. (d) A phase-contrast micrograph of the cells in (c), from which it is apparent that fusion is revealed much more effectively at this stage by the fluorescent cytoplasmic marker than by direct visual observation. (e) Cells like those in (c) after a 2-fold dilution of the buffer to cause swelling of the fused cells. (f) A phase-contrast micrograph of the cells in (e) showing that, as a consequence of the random distribution of the cells that were initially labelled with the fluorescent marker, a larger number of fused cells can now be seen by phase-contrast microscopy than are apparent by fluorescence microscopy. Magnification: ×350.

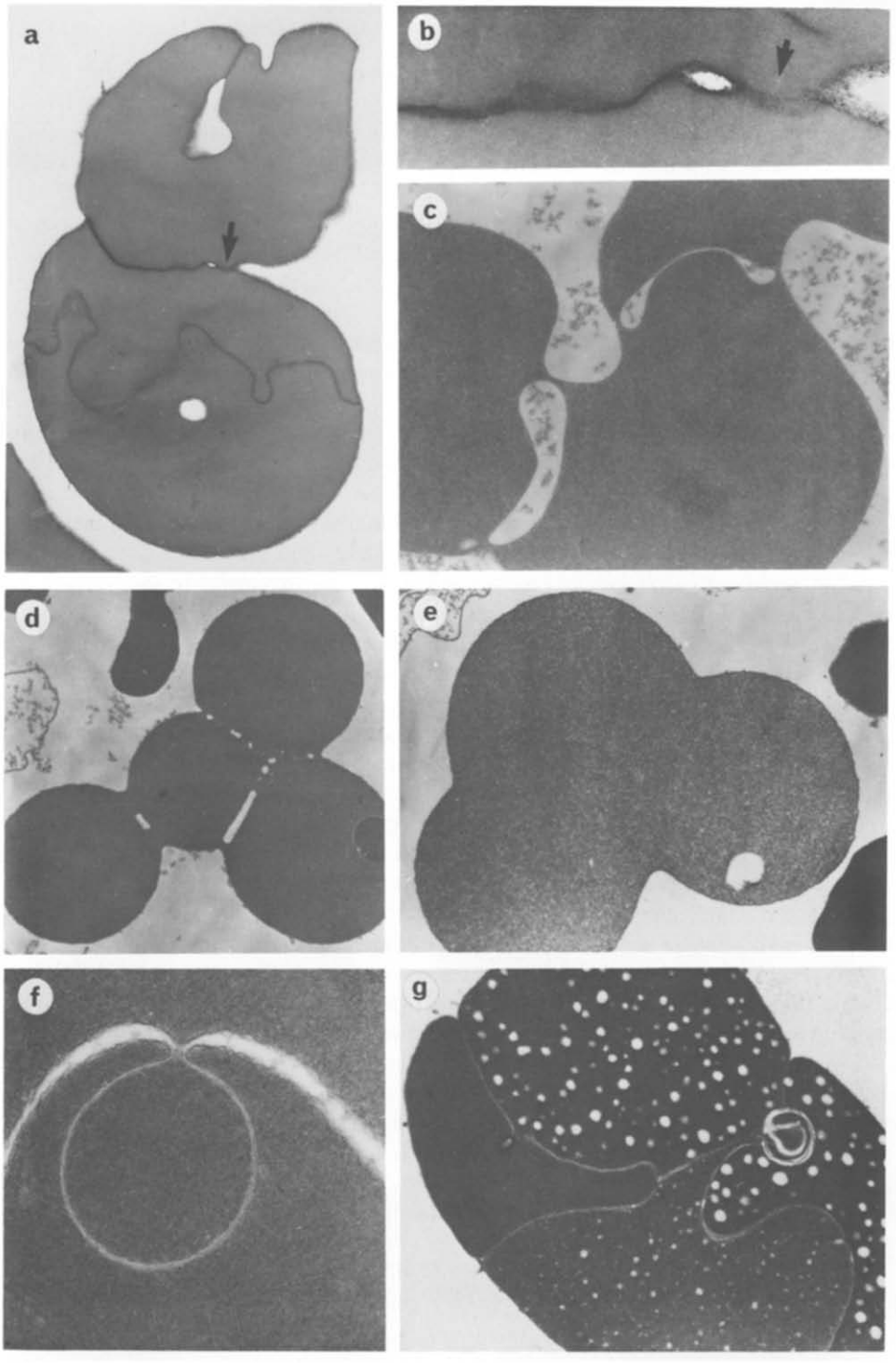

Fig. 3. Electron micrographs of thin sections of human erythrocytes. (a) and (b) Cells that were incubated in suspension with 40% PEG 6000 in the NaCl/Hepes buffer and 5 mM EGTA for 5 min at 37°C, and then fixed in an equal volume of the same poly(ethylene glycol) solution containing 2% glutaraldehyde. (c)–(f). For these micrographs, the cells were treated with 40% poly(ethylene glycol) as in (a). The poly(ethylene glycol) solution was then diluted to 13% with NaCl/Hepes buffer containing 5 mM EGTA. After 5 min at 37°C, the cells were centrifuged, resuspended in NaCl/Hepes buffer with 5 mM EGTA (without poly(ethylene glycol)), incubated for 10 min at 37°C, and then fixed by addition of an equal volume of 2% glutaraldehyde in buffer alone. (g) Cells that were treated with 40% poly(ethylene glycol) as in (a), then brought into 13% poly(ethylene glycol) solution, and fixed immediately with 2% glutaraldehyde in the NaCl/Hepes buffer containing 13% poly(ethylene glycol). Magnifications: (a), (c) and (g) ×11,000, (b) ×45,000, (d) and (e) ×5,500, (f) ×30,000.

rescent cells of Figs. 2b and 2c (which are attached to a positively charged surface) seem to be separate and not fused, until they are hypotonically swollen (Figs. 2e and 2f). The fusion of human erythrocytes by small cytoplasmic bridges which is induced by non-haemolytic Sendai virus is similarly not detectable by light microscopy until after the fused cells have been swollen with a hypotonic buffer [30,31]. Like the cells in Figs. 2e and 2f, hypotonically swollen, virally fused cells that are attached to a surface do not become round. By contrast, the erythrocytes that were fused in suspension by poly(ethylene glycol) became round without the addition of hypotonic buffer. With these cells, the swelling that results in the absence of 50 μM La^{3+} from permeabilisation of the plasma membrane by the treatment with 40% poly(ethylene glycol) [27] (cf. the size of the cells in Figs. 3d and 3e) was eventually sufficient to enable the fused erythrocytes to round up completely after the cytoplasmic bridges between the cells had enlarged.

The magnitude of the osmotic forces generated in erythrocytes during rehydration and swelling is indicated by the manner in which vesicles, like that shown in Fig. 3f are extruded from one cell and forced into the cytoplasm of an adjacent cell in the absence of fusion. Fig. 3g shows cytoplasmic vacuoles in erythrocytes which are formed when PEG 6000 is diluted to 13%, and which subsequently disappear when the cells are resuspended in isotonic buffer. These vacuoles are also seen by light microscopy, and they are thought to be droplets of aqueous poly(ethylene glycol) that are immiscible with proteins [32]. This observation extends previous reports that PEG 1000 [33] and PEG 1500 [34] enter cells. Since PEG 4000 precipitates proteins most effectively [35], we suggest that precipitation of components of the membrane skeleton by PEG may facilitate the osmotically driven cell fusion. In relation to changes in the membrane skeleton, it is relevant to note that the electron micrographs of Fig. 3 clearly show that the fusion of human erythrocytes by poly(ethylene glycol), unlike fusion of mouse and hen erythrocytes by the polymer [10,36] is independent of extracellular Ca^{2+} [34].

Electrically induced cell fusion

When erythrocytes are exposed to a short high-voltage pulse, their membranes are permeabilized and this causes colloid osmotic swelling and lysis [37–39]. The cells are also fused [40], and we have investigated the effects of osmotic forces on the fusion process.

The exposure of erythrocytes, aligned by dielectrophoresis (Fig. 4a), to a breakdown pulse in 150 mM erythritol yields spherical fused cells and chains of fused cells within 3 min. Thus, in Fig. 4b many fused cells are seen. As a consequence of colloid osmotic swelling [37–39], many of the cells that are exposed to the breakdown pulse (both unfused and fused) subsequently lyse and, in Fig. 4b, a short chain of lysed single cells (arrowed) and one lysed fused cell (arrowhead) can be seen only 3 min after the breakdown pulse. A proportion (10%) of the cells in Fig. 4a contained carboxyfluorescein (illustrated in Fig. 4c) and, when the breakdown pulse was applied, a number of the adjacent unlabelled cells instantaneously became fluorescent (Fig. 4d). Short chains of four or five (and sometimes more) fluorescent cells are thus formed, and these fluorescent chains give rise within a few minutes to fused cells that are observable by phase-contrast microscopy. We therefore conclude that the transfer of carboxyfluorescein, which occurs instantaneously with the breakdown pulse under these conditions, results from the fusion of erythrocytes via the small, cytoplasmic bridges that have been suggested to mediate electrically induced cell fusion [40].

In contrast to the observations made with erythrocytes in 150 mM erythritol, when less swollen cells in 200 mM erythritol were investigated, carboxyfluorescein was instantaneously transferred only to single cells that were immediately adjacent to the fluorescent cells (Fig. 4e). Chains of fluorescent cells were not formed, and few fused cells were subsequently seen by phase-contrast microscopy. Furthermore, the instantaneous transfer of fluorescence was negligible with cells in 400 mM erythritol, and no fused cells were produced. This behaviour indicates that osmotic forces play a rôle in the electrically-induced fusion of erythrocytes.

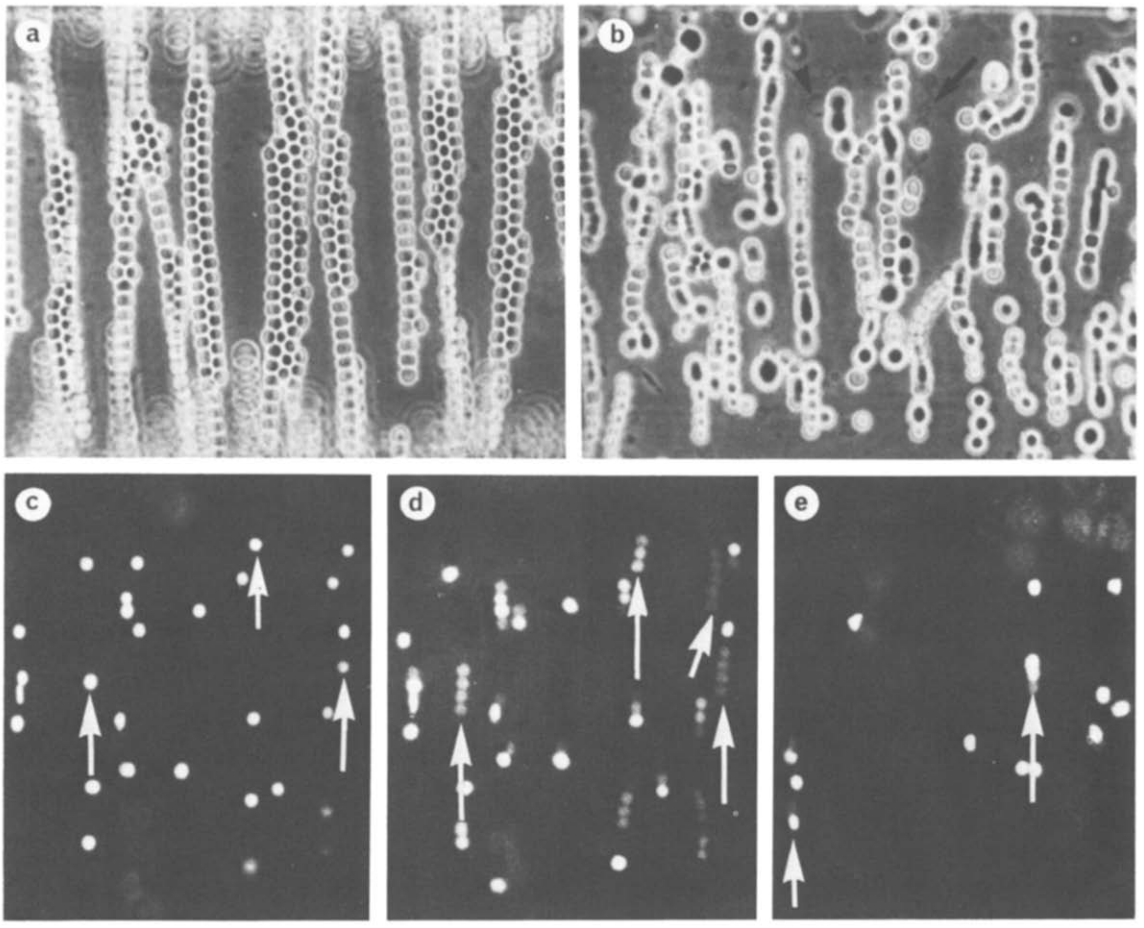

Fig. 4. Human erythrocytes were labelled with carboxyfluorescein, and a mixture of labelled and non-labelled cells (1:9) was resuspended in a solution of 150 mM erythritol and 10 mM histidine. The medium also contained 1 $mg \cdot ml^{-1}$ pronase [49]. (a) 50 μl of cell suspension was placed in the electrode chamber, and the cells were aligned by dielectrophoresis. (b) A square-wave d.c. pulse of 3.5 $kV \cdot cm^{-1}$ and 20 μs duration was applied to the cells. Cell fusion, and lysis of both fused (arrowhead) and unfused (arrow) cells, was apparent after 3 min at room temperature. (c) A fluorescent micrograph of the cells shown in (b) immediately before the breakdown pulse. (d) The same field as in (b) and in (c), 20 s after the breakdown pulse. The fluorescent label has leaked into the medium from the electropermeabilised cells (as indicated by the lighter background), and weakly fluorescent chains of cells have been formed by the fusion of individual fluorescent cells with three or four unlabelled cells (arrows). (e) The appearance of a preparation of cells in a medium containing a higher concentration of erythritol (200 mM) 15 s after the breakdown pulse. Carboxyfluorescein was transferred only to cells that were immediately adjacent to fluorescent cells (arrows) and no chains of fluorescent cells were formed. Magnification: ×315.

Discussion

In early work we concluded that cell swelling by colloid osmosis plays an essential part in chemically induced cell fusion [1]. Subsequently we attempted to fuse erythrocytes merely by causing them to swell (unpublished work with Dr D. Fisher and Dr A.H. Goodall) but, by contrast with the present work, the experiments were unsuccessful because the restraining influence of the membrane skeleton was not then apparent. It has nevertheless been shown that unilamellar proteoliposomes containing phosphatidylserine, which cease to fuse in the presence of Ca^{2+} after the liposomes have grown to diameters of about 100 nm, can be induced to fuse further by an osmotic gradient (internal osmotic pressure higher than external) and form single-walled liposomes with diameters

exceeding 1 μm as well as multilayered vesicles [41]. More recently it has been established that phospholipid vesicles can be fused in a model system with a phospholipid bilayer by an osmotic gradient alone [5]. Also, as solutions of high osmolarity inhibit exocytosis, it has been suggested that osmotic swelling is important for membrane fusion in exocytosis [2–4], although the basis for such chemiosmotic behaviour is not yet clear [42]. This recent work has stimulated us to reinvestigate the role of osmotic forces in artificially induced cell fusion.

As far as we are aware, the experiments described here constitute the first demonstration that osmotic forces can drive cell fusion when steps are taken to free the plasma membrane from the restraining influence of its membrane skeleton. It is relevant to the present studies that, as well as behaving like a simple osmometer at very low tonicities, human erythrocytes also lose their restricted response to changes in tonicity in the physiological range after heating to 50°C. This may explain our earlier finding that the swelling of hen erythrocytes, which occurs on heating to 48°–50°C in the absence of exogenous fusogenic lipids, is accompanied by cell fusion [1].

In the present work we have demonstrated that the fusion of erythrocytes, which is induced by treatment with 40% poly(ethylene glycol), occurs primarily when water enters the cells as they are rehydrated in an isotonic buffer. There is evidence that endogenous, Ca^{2+}-dependent, thiol-proteinases degrade the membrane skeleton of mouse erythrocytes treated with poly(ethylene glycol) [10]. Ca^{2+} is also required for the fusion of hen erythrocytes by poly(ethylene glycol) [36]. Some of the human erythrocytes studied here were, however, fused in the presence of EGTA. It is possible that, as with human erythrocytes fused by treatment with chlorpromazine, the poly(ethylene glycol)-induced fusion of these cells is independent of Ca^{2+} because the ankyrin component of the membrane skeleton is degraded by a serine proteinase that does not require Ca^{2+} for its activity [11]. However, as erythrocytes in 40% PEG 6000 are apparently permeable to the polymer, the precipitation of spectrin and of other membrane proteins by poly(ethylene glycol) may facilitate cell fusion.

It has recently been reported, in experiments with leukaemic lymphoblasts aligned by dielectrophoresis, that vitally staining dyes within one cell could move to the partner cells several minutes after exposure to electric pulses [43]. In our work, by contrast, the transfer of 6-carboxyfluorescein from labelled to unlabelled human erythrocytes was virtually instantaneous with the breakdown pulse, and this observation parallels a similar finding made with erythrocyte ghosts that were labelled with fluorescein isothiocyanate-dextran [44]. It has also been shown that the electrically-induced fusion of NS1 myeloma cells is effectively instantaneous with the breakdown pulse (unpublished work with Susan Brown), and it seems likely that the apparently slower rate of fusion with lymphoma cells that has been reported by Okada et al. [43] may be due to fusion being less readily detectable with vital stains than with a water-soluble, fluorescent label.

In their studies on osmotically driven fusion in model membrane systems, Zimmerberg et al. [45] reported that phospholipid vesicles that are preswollen fuse more readily with a phospholipid bilayer than more flaccid vesicles under the same osmotic gradient. It is therefore interesting that when human erythrocytes were aligned by dielectrophoresis in a range of concentrations of erythritol (150–400 mM), the cells in 150 mM erythritol fused instantaneously into long chains of cells, while fusion of the cells in 400 mM erythritol was negligible. Since erythrocytes are permeabilised by the high-voltage pulse and immediately begin to swell, it was suggested in a preliminary communication on our work that the more extensive swelling of permeabilised erythrocytes in 150 mM erythritol may be responsible for their fusing more reasily than cells in solutions of higher osmolarity [13]. This possibility is being investigated, and it cannot be completely excluded from consideration at the present time. It is known, however, that the breakdown potential of the cell membrane decreases with increasing turgor pressure in *Valonia utricularis* [46] and with increasing hydrostatic pressure in erythrocytes [47]. It thus seems probably that the extent of erythrocyte fusion in the present work was governed by a combination of the electrical-compressive force, due to the breakdown pulse, and the osmotic force applied prior to electrical breakdown.

In conclusion we suggest, in the light of our experiments, that osmotic forces may provide the driving force for many fusion reactions in biological systems. It has been proposed that such reactions may depend on closely apposed, phospholipid bilayers being driven by osmotic swelling into a transient, single bilayer, which is then ruptured as a consequence of continued osmotic swelling [13]: further consideration is given elsewhere to this possibility [48].

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References

1 Ahkong, Q.F., Cramp, F.C., Fisher, D., Howell, J.I., Tampion, W., Verrinder, M. and Lucy, J.A. (1973) Nature New Biol. 242, 215–217
2 Pollard, H.B., Pazoles, C.J., Creutz, C.J., Scott, J.H., Zinder, O. and Hotchkiss, A. (1984) J. Biol. Chem. 259, 1114–1121
3 Hampton, R.Y. and Holz, R.W. (1983) J. Cell Biol. 96, 1082–1088
4 Zimmerberg, J. and Whitaker, M. (1985) Nature 315, 581–584
5 Akabas, M.H., Cohen, F.S. and Finkelstein, A. (1984) J. Cell Biol. 98, 1063–1071
6 Quirk, S.J., Ahkong, Q.F., Botham, G.M., Vos, J. and Lucy, J.A. (1978) Biochem. J. 176, 159–167
7 Ahkong, Q.F., Botham, G.M., Woodward, A.W. and Lucy, J.A. (1980) Biochem. J. 192, 829–836
8 Thomas, P., Limbrick, A.R. and Allan, D. (1983) Biochim. Biophys. Acta 730, 351–358
9 Kosower, N.S., Glaser, T. and Kosower, E.M. (1983) Proc. Natl. Acad. Sci. USA 80, 7542–7546
10 Nakorchai, S., Sathitudsahakorn, C., Chongchirasiri, S. and Yuthavong, Y. (1983) J. Cell. Sci. 63, 147–154
11 Lang, R.D.A., Wickenden, C., Wynne, J. and Lucy, J.A. (1984) Biochem. J. 218, 295–305
12 Donath, E. and Arndt, R. (1984) Gen. Physiol. Biophys. 3, 239–249
13 Lucy, J.A. (1986) Biochem. Soc. Trans., in the press
14 Nermut, M.V. and Williams, L.D. (1980) J. Microsc. 118, 453–461
15 Heubusch, P., Jung, C.Y. and Green, F.A. (1985) J. Cell Physiol. 122, 266–272
16 Vos, J., Ahkong, Q.F., Botham, G.M., Quirk, S.J. and Lucy, J.A. (1976) Biochem. J. 158, 651–653
17 Ahkong, Q.F., Tampion, W. and Lucy, J.A. (1975) Nature 256, 208–209
18 Allan, D. and Michell, R.H. (1978) Biochim. Biophys. Acta 508, 277–286
19 Ahkong, Q.F., Fisher, D., Tampion, W. and Lucy, J.A. (1973) Biochem. J. 136, 147–155
20 Goodall, H. and Johnson, M.H. (1982) Nature 295, 524–526
21 Lieber, M.R. and Steck, T.L. (1982) J. Biol. Chem. 257, 11660–11666
22 Hauser, H., Phillips, M.C., Levine, B.A. and Williams, R.J.P. (1975) Eur. J. Biochem. 58, 133–144
23 Hammoudah, M.M., Nir, S., Bentz, J., Mayhew, E., Stewart, T.P., Hui, S.W. and Kurland, R.J. (1981) Biochim. Biophys. Acta 645, 102–114
24 Majumdar, S., Baker, R.F., and Kalra, V.K. (1980) Biochim. Biophys. Acta 598, 411–416
25 Blow, A.M.J., Botham, G.M., Fisher, D., Goodall, A.H., Tilcock, C.P.S. and Lucy, J.A. (1978) FEBS Lett. 94, 305–310
26 Boni, L.T., Stewart, T.P., Alderfer, J.L. and Hui, S.W. (1981) J. Membrane Biol. 62, 65–70
27 Aldwinkle, T.J., Ahkong, Q.F., Bangham, A.D., Fisher, D. and Lucy, J.A. (1982) Biochim. Biophys. Acta 689, 548–560
28 Knutton, S. (1979) J. Cell Sci. 36, 61–72
29 Krähling, H. (1981) Acta Histochem. Suppl. 23, S219–223
30 Knutton, S. (1979) J. Cell Sci. 36, 85–96
31 Knutton, S. and Bächi, T. (1980) J. Cell Sci. 42, 153–167
32 Edmond, E. and Ogston, A.J. (1968) Biochem. J. 109, 569–576
33 Szabó, G., Kiss, A. and Trón, L., (1982) Cytometry 3, 59–63
34 Krähling, H., Schinkewitz, U., Barker, A. and Hülser, D.F. (1978) Cytobiologie 17, 51–61
35 Ingham, K.C., (1978) Arch. Biochem. Biophys. 186, 106–113
36 Maggio, B., Ahkong, Q.F., and Lucy, J.A. (1976) Biochem. J. 158, 647–650
37 Zimmermann, U., Pilwat, G., Holzapfel, C. and Rosenheck, K. (1976) J. Membrane Biol. 30, 135–152
38 Kinosita, K. and Tsong, T.Y. (1977) Nature 268, 438–441
39 Schwister, K. and Deuticke, B. (1985) Biochim. Biophys. Acta 816, 332–348
40 Zimmermann, U. (1982) Biochim. Biophys. Acta 694, 227–277
41 Miller, C., Arvan, P., Telford, J.N. and Racker, E. (1976) J. Membrane Biol. 30, 271–282
42 Baker, P.F. and Knight, D.E. (1984) Biosci. Rep. 4, 285–298
43 Ohno-Shosaku, T. and Okada, Y. (1985) J. Membrane Biol. 85, 269–280
44 Sowers, A.E. (1984) J. Cell Biol. 99, 1989–1996
45 Zimmerberg, J., Cohen, F.S. and Finkelstein, A. (1980) Science 210, 906–908
46 Zimmermann, U., Beckers, F. and Coster, H.G.L. (1977) Biochim. Biophys. Acta 464, 399–416
47 Zimmermann, U., Pilwat, G., Pequeux, A. and Gilles, R. (1980) J. Membrane Biol. 54, 103–113
48 Lucy, J.A. and Ahkong, Q.F. (1986) FEBS Lett. 199, 1–11
49 Zimmermann, U., Pilwat, G. and Richter, H-P. (1981) Naturwissenschaft 68, S. 577–578