PROTEINASES AND CELL FUSION

Q. F. AHKONG, A. M. J. BLOW, G. M. BOTHAM, J. M. LAUNDER, S. J. QUIRK* and J. A. LUCY
Department of Biochemistry and Chemistry, Royal Free Hospital School of Medicine, University of London, 8 Hunter Street, London WC1N 1BP, England

Received 6 September 1978

1. Introduction

It is widely thought that the aggregation of integral membrane proteins, visualized as intramembranous particles in freeze-fractured membranes, is involved in membrane fusion reactions. The distributions of these particles at sites of membrane fusion, reviewed in [1], variously support the hypothesis that fusion proceeds by the interdigitation of aggregated membrane proteins [2], or the alternative idea that fusion involves the intermingling of membrane lipids after the emergence of protein-free areas of lipid bilayer following protein aggregation [3].

The movement of integral membrane proteins 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 [4–6]. In this paper attention is drawn to a further possibility, namely that 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. This suggestion is based on observations that the fusion of human erythrocytes induced by oleoylglycerol (glycerol mono-oleate) is associated with the degradation of some membrane proteins, and that these cells are also fused by treatment with exogenous proteinases. Band 3 protein is degraded in both instances.

* Present address: Institute for Medical Research and Nuffield Department of Obstetrics and Gynaecology, John Radcliffe Hospital, Oxford, England

2. Materials and methods

Oleoylglycerol (99% purity), Tos–Phe–CH_2Cl (TPCK), phenylmethanesulphonyl fluoride (PMSF), subtilisin Carlsberg (protease type VIII), and subtilisin BPN′ (Nagarse, protease type VII) were from Sigma (London) Chemical Co. α-Chymotrypsin from bovine pancreas was from Serva Feinbiochemica, Heidelberg, and pronase from Koch-Light Laboratories Ltd. Acrylamide (specially purified for electrophoresis) was from BDH Chemicals Ltd.

In experiments with oleoylglycerol, human blood (O Rh^+) was withdrawn into a sterile solution of citrate anticoagulant; erythrocytes were prepared and treated with oleoylglycerol [7] on the day of collection. (The presence of a leukocyte in the preparations used was an extremely rare event.) The fusion index was:

$$\frac{\text{No. erythrocytes participating in fusion}}{\text{Total no. erythrocytes originally present as single cells}} \times 100$$

For cell fusion with proteinases, erythrocytes were prepared from fresh blood and from blood that had been stored in the cold for 4 weeks.

Erythrocyte ghosts were prepared using the hypoosmotic lysis method [8], as modified in [9], except that, after the cells had been washed twice, 1 mM PMSF was present in all subsequent steps to prevent proteolysis. Irreversible binding of haemoglobin to the erythrocyte membrane occurred (fig.1d) when the cells were heated to 47°C, and this treatment also made it difficult to recover intact ghosts. SDS–poly-

acrylamide gel electrophoresis was essentially by the method in [10].

Samples for electron microscopy were fixed at the temperatures specified in the text in a buffered solution of glutaraldehyde, and freezefractured preparations made as in [11].

3. Results and discussion

3.1. *Cell fusion induced by oleoylglycerol*

Alterations in membrane proteins in human erythrocytes during cell fusion induced by oleoylglycerol were investigated using electrophoresis of

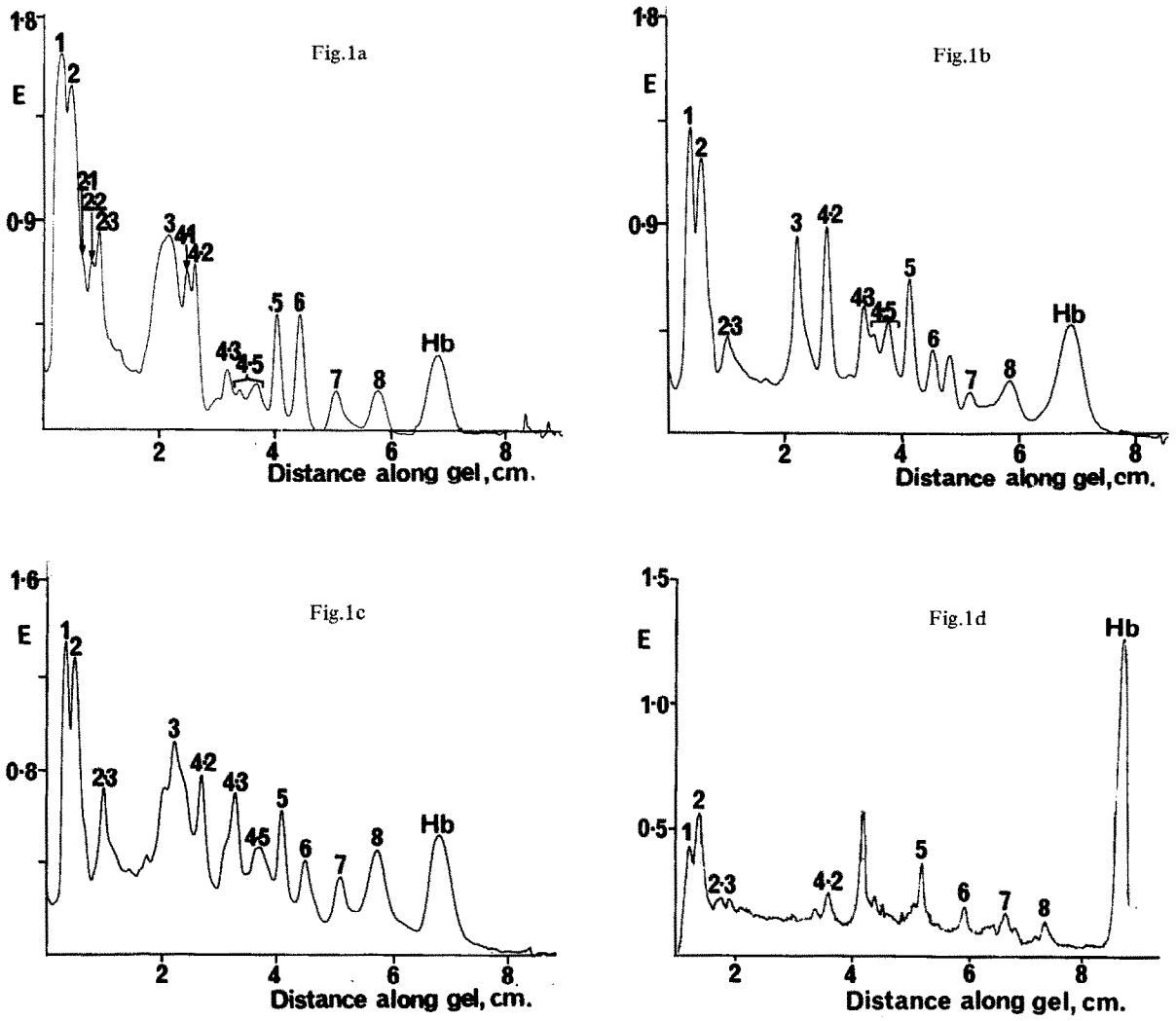

Fig.1. Densitometry recordings of the electrophoretic patterns of polypeptide ghosts prepared from human erythrocytes. (a) Freshly isolated erythrocytes (2.2 × 10^8 cells/ml) were incubated (without oleoylglycerol) at 37°C with Ca^{2+} (5 mM) at pH 7.4 for 30 min in the basic medium containing dextran used in [7]. (b) Cells were incubated as for (a) but with oleoylglycerol (47 μg/10^8 cells); under these conditions extensive cell fusion occurred (fusion index, 76%; SD ± 11 in 9 experiments). (c) Cells were incubated as for (b) after pre-treatment with TPCK (1 mM) for 20 min at 37°C. (d) Cells were incubated as for (a) but with subtilisin Carlsberg (1 mg/ml) at 47°C for 5 min. At the end of each incubation, ghosts were prepared; electrophoresis was undertaken on 40–50 μg protein and densitometric recordings made. Traces (a–c) are from cylindrical gels; trace (d) is from a slab gel in which the polypeptides migrated greater distances than in (a–c).

ghost membranes on SDS–polyacrylamide gels. By comparison with the electrophoretic pattern of ghosts prepared from control erythrocytes (fig.1a), the most striking finding made with ghosts from cells treated with the fusogenic lipid was a loss of band 3 protein (fig.1b). Additionally, the production of a new sharp band within the region of band 3 was observed in the polyacrylamide gels of membranes from the treated cells. Interestingly, the proteinase inhibitor TPCK partially inhibited both cell fusion induced by oleoylglycerol, and the associated decrease in band 3 protein (fig.1c).

A number of other changes in membrane proteins were also observed in the treated erythrocytes. These included a loss of band 4.1, increased staining in the region of bands 4.3 and 4.5, and the appearance of a new component moving slightly faster than band 6 (fig.1b). These changes are quite similar to the effects of pronase and of chymotrypsin on intact erythrocytes [12,13]. In view of reports [14,15] of proteolytic activity in human erythrocytes, possibly activated or released by Ca^{2+}, the proteolytic degradation of certain membrane proteins may possibly be important in the fusion of human erythrocytes induced by oleoylglycerol.

3.2. *Cell fusion induced by exogenous proteinases*

If the activities of membrane-bound proteinases are, in fact, involved in the chemically-induced cell fusion of erythrocytes, it should be possible under appropriate circumstances to induce these cells to fuse by treating them with exogenous proteinases. Treatment of human erythrocytes with pronase, or chymotrypsin, induced a limited degree of cell fusion. Subtilisin Carlsberg and subtilisin BPN′ (Nagarse) were considerably more effective but fusion was observable only after several hours at 37°C. Cellular aggregation, resulting from treatment with subtilisin Carlsberg for 25 min in the presence of Ca^{2+} (fig.2a), was followed by extensive cell fusion after 60 min at 47°C (fig.2b) particularly with erythrocytes from blood that had been stored in the cold for 4 weeks. Both aggregation and fusion were inhibited by PMSF (fig.2c); EGTA inhibited fusion but not aggregation (fig.2d). Freshly isolated human erythrocytes were less susceptible to fusion by subtilisin (fig.2e). Interestingly, although fresh human erythrocytes do not fuse when treated with ionophore A23187 and Ca^{2+} at either 37°C or 47°C [11], the cells fused extensively when treated simultaneously with the ionophore, Ca^{2+} and subtilisin (fig.2f).

In related work with hen erythrocytes, exogenous proteinases induced these cells to fuse also. Other workers have shown that pretreatment of hen erythrocytes with a proteinase preparation enhances fusion induced by poly(ethylene glycol) [16]. Digestion with Nagarse has been reported to cause fusion of sarcoplasmic reticulum vesicles but it did not fuse human erythrocyte ghosts [17].

The P-fracture faces of intact human erythrocytes from stored blood were studied in control cells that were incubated at 47°C for 60 min and then cooled at 0°C for 10 min, prior to fixation at 0°C and subsequent freeze-fracturing. P-Fracture faces of cells treated in this way showed the characteristic appearance of ghost membranes prepared from freshly isolated human erythrocytes [18] (fig.3a). Treatment of human erythrocyte ghosts with proteolytic enzymes is known to decrease the number of intramembranous particles in freeze-fractured preparations [17,19], and digestion of intact erythrocytes with subtilisin Carlsberg at 47°C to induce fusion in the present experiments caused a progressive decrease in the number of intramembranous particles on the P-fracture faces (fig.3b).

Of particular interest was the clustering and the bare areas seen in the P-face of treated cells that were cooled to 0°C prior to freeze-fracturing (fig.3c). This behaviour closely resembles that of hen erythrocytes when cell fusion is induced by treatment with the ionophore A23187 and Ca^{2+} [11,20], indicating that freedom of the particles to move is associated with cell fusion. Human erythrocytes, by contrast, do not fuse on treatment with the ionophore and Ca^{2+} nor do the intramembranous particles cluster on cooling [11]. In ghosts of human erythrocytes, the immobility of the intramembranous particles is well known and aggregation, similar to that of fig.3c, is seen only after partial removal of spectrin [21]. When intact human erythrocytes were fused by oleoylglycerol, a loss of micro-aggregation in the intramembranous particles of the treated cells implied that the particles were at least partially free to move [22].

Figure 3d shows that the proteinase inhibitor PMSF, which inhibited cell fusion induced by subtilisin Carlsberg (fig.2c), also inhibited the loss of intra-

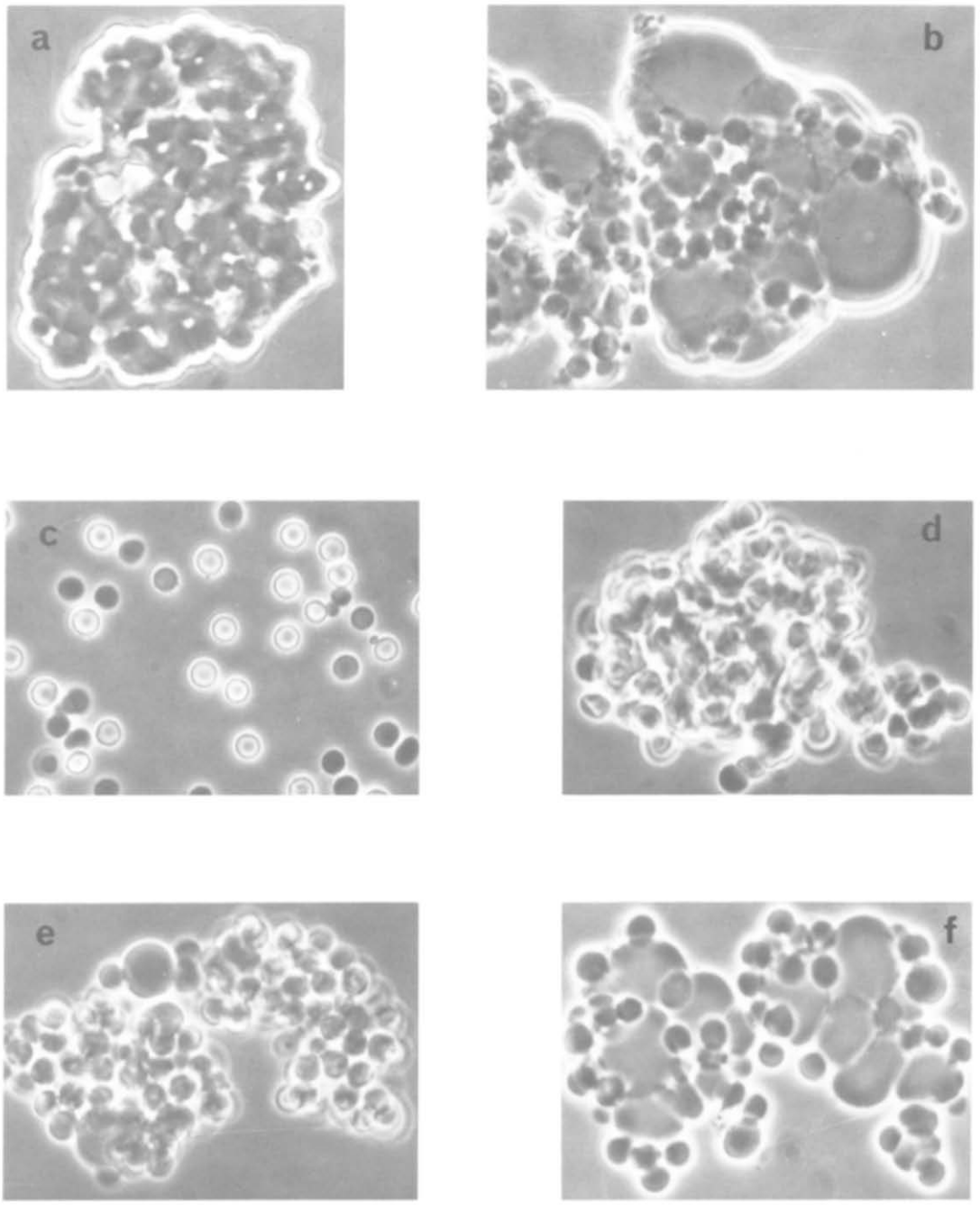

Fig.2. Light micrographs (phase contrast × 600) of human erythrocytes. (a) Cells prepared from stored blood were incubated (3×10^8 cells/ml) for 25 min at 47°C with subtilisin Carlsberg (1 mg/ml). (b) Cells treated as in (a) but incubated with enzyme for 60 min at 47°C. (c) Cells treated with enzyme as in (b) but in the presence of PMSF (1 mM). (d) Cells treated with enzyme as in (b) but with EGTA (4 mM) in the absence of exogenous Ca^{2+}. (e) Freshly prepared erythrocytes treated with enzyme as in (b). (f) Freshly prepared erythrocytes treated with enzyme as in (b) but in the presence of ionophore A23187 (10 μg/ml). In these experiments the buffered saline used, containing dextran, was as in [4] except that the $CaCl_2$ was 8 mM.

membranous particles and their thermally-induced, lateral movement in the membranes of enzyme-treated cells. EGTA (4 mM) in the absence of exogenous Ca^{2+} had similar effects to PMSF. However, after 2 h at 47°C with subtilisin Carlsberg in the presence of PMSF or EGTA, some fusion was observed that was accompanied by aggregation of the intramembranous particles on cooling.

It is pertinent to note that band 3 protein was depleted in the cells fused by oleoylglycerol (fig.1b), and in those fused on treatment with subtilisin (fig.1d). The new polypeptide between bands 4.2 and

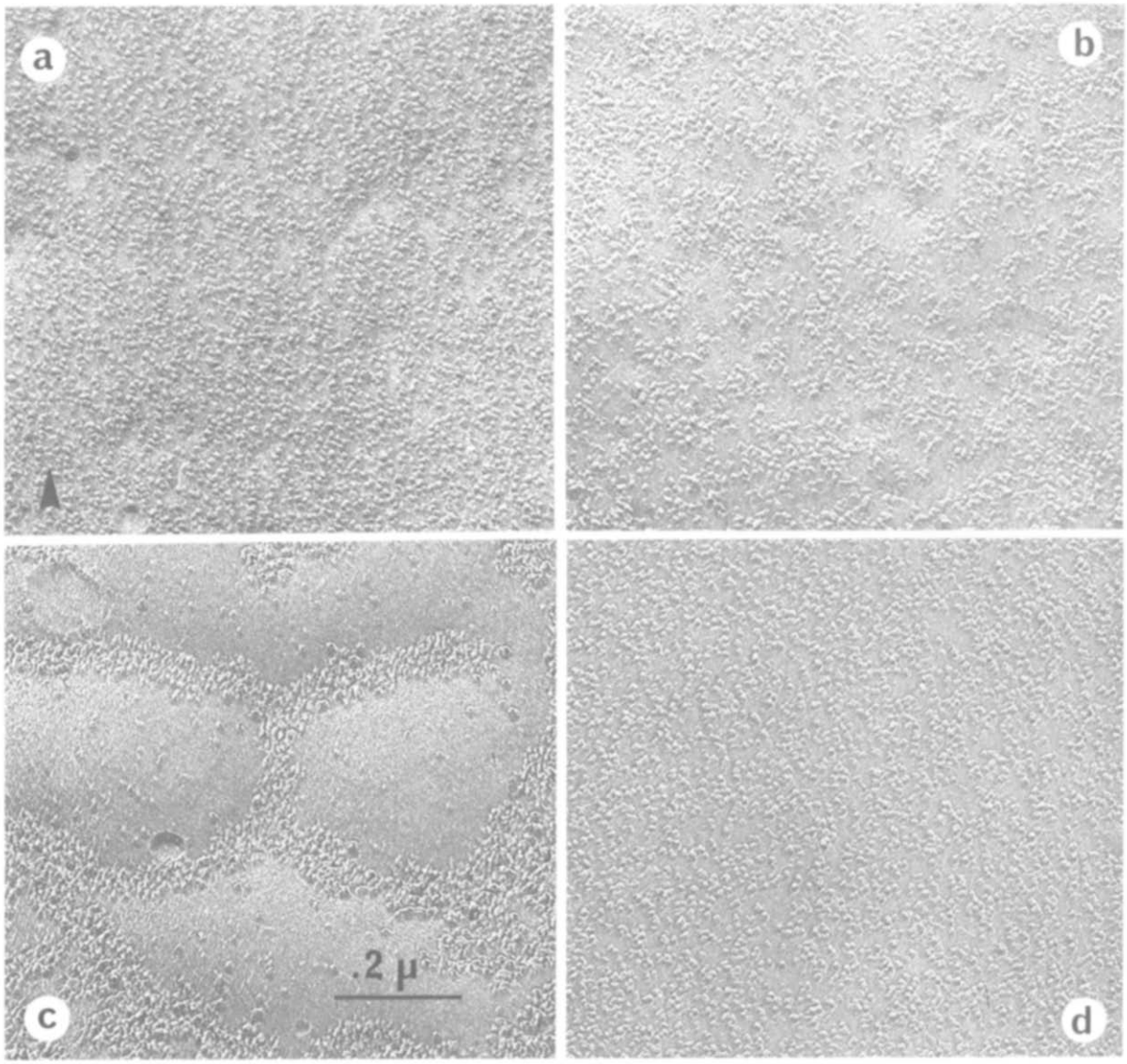

Fig.3. Electron micrographs of the P-fracture faces of human erythrocytes. The cells were prepared from stored blood and handled as in fig.2. (a) Cells were incubated for 60 min at 47°C and then cooled for 10 min at 0°C, prior to fixation at 0°C. (b) Cells were incubated with subtilisin Carlsberg (1 mg/ml) for 60 min at 47°C, and fixed at 47°C. (c) Cells treated with enzyme as in (b) but cooled to 0°C for 10 min before fixation at 0°C. (d) Cells incubated with enzyme as in (b), but with PMSF (1 mM), and fixed after cooling as in (c). The direction of shadowing for each micrograph is as indicated by the arrow on (a).

5 in fig.1d has mol. wt 60 000 ± 2000, and it is probably the digestion product referred to as 'fragment e' that is produced by degradation of band 3 protein with a number of proteinases, including subtilisin [23]. Similar fragments of band 3 protein may be responsible for the apparently increased staining of bands 4.3 and 4.5 in the membranes of cells treated with oleoylglycerol (fig.1b). Changes involving band 3 protein may thus be particularly important in the fusion of erythrocyte membranes. It is relevant that this integral protein is apparently eliminated from the region of membrane fusion in the microvesiculation of human erythrocytes that occurs during storage, or on treatment with Ca^{2+} and ionophore A23187 [24].

The observations reported here lead us to suggest that the proteolytic degradation of membrane proteins may conceivably be of general significance in membrane fusion reactions including, for example, secretion by exocytosis, endocytosis, lysosomal fusion reactions, and cell division. It also seems possible that proteolytic activity in membranes may allow an increased lateral movement of integral proteins that facilitates membrane fusion. Finally, our findings additionally indicate that exogenous proteinases may be a useful experimental tool in the production of cell hybrids in vitro.

Acknowledgements

This work was supported by grants from the Medical Research Council and from the Wellcome Trust.

References

[1] Poste, G. and Nicolson, G. L. eds (1978) Membrane Fusion; Cell Surface Reviews, vol. 5, Elsevier/North-Holland Biomedical Press, Amsterdam, New York.
[2] Poste, G. and Allison, A. C. (1973) Biochim. Biophys. Acta 300, 421–465.
[3] Ahkong, Q. F., Fisher, D., Tampion, W. and Lucy, J. A. (1975) Nature 253, 194–195.
[4] Ahkong, Q. F., Tampion, W. and Lucy, J. A. (1975) Nature 256, 208–209.
[5] Lucy, J. A. (1978) in: Membrane Fusion (Poste, G. and Nicolson, G. L. eds) Cell Surface Reviews, vol. 5, pp. 267–304, Elsevier/North-Holland Biomedical Press, Amsterdam, New York.
[6] Papahadjopoulos, D. (1978) in: Membrane Fusion (Poste, G. and Nicolson, G. L. eds) Cell Surface Reviews, vol. 5, pp. 765–790, Elsevier/North-Holland Biomedical Press, Amsterdam, New York.
[7] Ahkong, Q. F., Fisher, D., Tampion, W. and Lucy, J. A. (1973) Biochem. J. 136, 147–155.
[8] Dodge, J. T., Mitchell, C. and Hanahan, D. J. (1963) Arch. Biochem. Biophys. 100, 119–130.
[9] Elgsaeter, A. and Branton, D. (1974) J. Cell Biol. 63, 1018–1036.
[10] Fairbanks, G., Steck, T. L. and Wallach, D. F. M. (1971) Biochemistry 10, 2606–2617.
[11] Vos, J., Ahkong, Q. F., Botham, G. M., Quirk, S. J. and Lucy, J. A. (1976) Biochem. J. 158, 651–653.
[12] Bender, W. W., Garan, H. and Berg, H. C. (1971) J. Mol. Biol. 58, 783–797.
[13] Cabantchik, Z. I. and Rothstein, A. (1974) J. Membr. Biol. 15, 227–248.
[14] Tökés, Z. A. and Chambers, S. M. (1975) Biochim. Biophys. Acta 389, 325–338.
[15] King, L. E. and Morrison, M. (1977) Biochim. Biophys. Acta 471, 162–168.
[16] Hartmann, J. X., Galla, J. D., Emma, D. A., Kao, K. N. and Gamborg, O. L. (1976) Can. J. Genet. Cytol. 18, 503–512.
[17] Yamanaka, N. and Deamer, D. W. (1976) Biochim. Biophys. Acta 426, 132–147.
[18] Weinstein, R. S. (1974) in: The Red Blood Cell (Surgenor, D. MacN. ed) vol. 1, pp. 213–268, Academic Press, New York.
[19] Branton, D. (1971) Phil. Trans. Roy. Soc. (Biol. Sci.) 261, 133–138.
[20] Volsky, D. and Loyter, A. (1977) Biochim. Biophys. Acta 471, 243–259.
[21] Elgsaeter, A., Shotton, D. M. and Branton, D. (1976) Biochim. Biophys. Acta 426, 101–122.
[22] Quirk, S. J., Ahkong, Q. F., Botham, G. M., Vos, J. and Lucy, J. A. (1978) Biochem. J. 174, in press.
[23] Jenkins, R. E. and Tanner, M. J. A. (1975) Biochem. J. 147, 393–399.
[24] Shukla, S. D., Berriman, J., Coleman, R., Finean, J. B. and Michell, R. H. (1978) FEBS Lett. 90, 289–292.