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Membrane Proteins in Human Erythrocytes during Cell Fusion induced by Oleoylglycerol

By SUSAN J. QUIRK,* QUET FAH AHKONG, GAYNOR M. BOTHAM, JAN VOS† and JACK A. LUCY
Department of Biochemistry and Chemistry, Royal Free Hospital School of Medicine, University of London, 8 Hunter Street, London WC1N 1BP, U.K.

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1. The fusion of human erythrocytes into multicellular bodies that is induced by microdroplets of oleoylglycerol was investigated by optical and electron microscopy, and by gel electrophoresis of membrane proteins. 2. At the highest concentrations of oleoylglycerol and Ca^{2+} used, at least 80% of the cells fused after 30min at 37°C and only about 5% of the cells had completely lysed; the shapes of fused multicellular bodies were usually retained in 'ghosts' prepared by hypo-osmotic lysis. 3. The rate of cell fusion was related to the concentration of Ca^{2+}, although some cells fused when no exogenous Ca^{2+} was present. 4. Interactions of microdroplets of oleoylglycerol with the cells led to abnormalities in the structural appearance of the erythrocyte membrane; subsequent membrane fusion occurred, at least in some instances, at the sites of the microdroplets. 5. The intramembranous particles on the P-fracture face of the treated cells were more randomly distributed, but not significantly increased in number by comparison with the control cells. 6. Gel electrophoresis of the proteins of 'ghosts' prepared from fused human erythrocytes showed a production of material of very high molecular weight, the development of a new component in the band-3 region, an increased staining of bands 4.3 and 4.5, and a new component moving slightly faster than band 6. 7. Bands 2.1–2.3 were altered, band 3 was decreased and band 4.1 was lost. 8. Most, but not all, of the changes in the membrane proteins appeared to result from the entry of Ca^{2+} into the cell. 9. 1-Chloro-4-phenyl-3-L-toluene-*p*-sulphonamidobutan-2-one partially inhibited both cell fusion and the associated decrease in band-3 protein. 10. The possibility that proteolytic degradation of membrane proteins may be involved in cell fusion induced by oleoylglycerol is considered, and some implications of this possibility are discussed.

Oleoylglycerol has previously been reported to fuse avian and mammalian erythrocytes (Ahkong *et al.*, 1973). This and other fusogenic lipids also interact with aqueous dispersions of phospholipids to alter their macromolecular organization, indicating that a primary effect of low-melting lipids in causing erythrocytes to fuse is on the organization of membrane phospholipids (Howell *et al.*, 1973). Investigations on mixed monolayers at the air/water interface of fusogenic lipids with choline-containing phospholipids have supported this concept. Thus negative deviations from ideality, in both surface area and surface potential, have been observed in mixed monolayers of non-ionizable (e.g. oleoylglycerol) and ionizable (e.g. oleic acid) fusogenic lipids with phosphatidylcholine (Maggio & Lucy, 1975, 1976). Similar deviations were not found with chemically related non-fusogenic lipids (e.g. stearoylglycerol, stearic acid).

As pointed out earlier, however, alterations in membrane lipids during the fusion process do not exclude changes from occurring in membrane proteins also (Howell *et al.*, 1973). In the present paper we report ultrastructural studies on human erythrocytes during fusion induced by oleoylglycerol, and parallel investigations on the behaviour of membrane proteins during the fusion process. Most, but not all, of the changes found in the gel-electrophoresis patterns of membrane proteins in the treated cells appear to be a consequence of the entry of Ca^{2+} into the cytoplasm. Observations are described which indicate

* Present address: Nuffield Institute for Medical Research and Nuffield Department of Obstetrics and Gynaecology, John Radcliffe Hospital, Headington, Oxford, OX3 9DU, U.K.

† Present address: Centre for Medical Electron Microscopy, University of Groningen, Groningen, The Netherlands.

Abbreviations used: Tos-Lys-CH_2Cl, 7-amino-1-chloro-3-L-tosylamidoheptan-2-one ('TLCK'); Tos-Phe-CH_2Cl, 1-chloro-4-phenyl-3-L-toluene-*p*-sulphonamidobutan-2-one ('TPCK').

that a proteolytic degradation of band-3 protein is involved in the fusion of human erythrocytes induced by oleoylglycerol.

Aspects of this work have previously been reported in a brief form (Lucy, 1977).

Materials and Methods

Erythrocytes

Human blood (O Rh^+) was withdrawn into a sterile solution of citrate anticoagulant (De Gowin *et al.*, 1949) and then used on the day of collection for electron microscopy and the isolation of 'ghosts', or otherwise stored at 4°C for up to 2 weeks. Before experiments, blood cells were washed twice by centrifugation and resuspension in NaCl (0.85%, w/v) with removal of the buffy layer, and then washed once with a modified Eagle's basal salt solution at pH7.4 (Ahkong *et al.*, 1973) with the concentration of Ca^{2+} adjusted when necessary. The erythrocytes (8×10^8 cells/ml) were resuspended in the basal salt solution, containing dextran (80mg/ml), and stored at 4°C until used. Inspection of the cells by optical microscopy showed that the erythrocytes were virtually free from leucocytes; the presence of a leucocyte was an extremely rare event.

For experiments with the ionophore A23187, the above procedure was followed except that the basal salt solution was a high-potassium medium (medium A) containing KCl (135mM), NaCl (5.4mM), penicillin 120μg/ml), streptomycin (100μg/ml), buffered at pH7.4 with sodium cacodylate (10mM). Also, after the washes with NaCl (0.85%, w/v), there was an extra wash with medium A supplemented with 0.5mM-EGTA to remove residual Ca^{2+}.

Reagents

All reagent chemicals used were commercially obtained and were of analytical grade. Dextran (average mol.wt. 60000–90000, clinical grade), oleoylglycerol (99% purity), Tos-Phe-CH_2Cl, Tos-Lys-CH_2Cl, phenylmethanesulphonyl fluoride and bovine albumin (fraction V powder, 96–99% albumin) were from Sigma (London) Chemical Co. (Kingston upon Thames, Surrey, U.K.). Kodak (Liverpool, U.K.) supplied acrylamide, *NN'*-methylenebisacrylamide and *NNN'N'*tetramethylethylenediamine. Coomassie Brilliant Blue R 250 (Colour Index 42660) and 2-mercaptoethanol were from BDH Chemicals (Poole, Dorset, U.K.). Glutaraldehyde was from Edward Gurr (London S.W.14, U.K.), and Araldite epoxy resin was from CIBA (A.R.L.) (Duxford, Cambridge, U.K.). The ionophore A23187 was the gift of Eli Lilly (Windlesham, Surrey, U.K.). Pyronin Y (Colour Index 45005) was from Searle Diagnostics (High Wycombe, Bucks., U.K.).

Incubation of erythrocytes

Emulsions of oleoylglycerol were prepared by sonication as previously described by Ahkong *et al.* (1973), except that stock solutions were stored in ethanol at −15°C, and that a stream of N_2 was unnecessary during sonication. The freshly prepared emulsion of lipid (0.167mg/ml) in basal salt solution at pH7.4, adjusted to the required Ca^{2+} concentration, and containing dextran (80mg/ml) was mixed with the erythrocyte suspension and incubated at 37°C for the desired periods. The concentrations of erythrocytes, oleoylglycerol and Ca^{2+} were as indicated in Table 1 unless otherwise stated. The progress of the incubation was followed by phase-contrast microscopy (Standard WL Research Microscope, Carl Zeiss, Degenhardt and Co., London W1P 8AP, U.K.). Samples were removed and fixed (see below) for electron microscopy, or for determination of the fusion index.

In experiments with the ionophore A23187, erythrocytes (8×10^8 cells/ml) were incubated with ionophore A23187 (7.5μg/ml) for up to 70min at 37°C. The stock solution of ionophore A23187 (1mg/ml) was in ethanol; control incubations without ionophore therefore contained ethanol. Calcium was added to give final concentrations of 0, 0.2, 2, 5 and 20mM. If a subsequent incubation with oleoylglycerol was to be carried out, the erythrocytes were diluted appropriately to give 2.4×10^8 cells/ml (and Ca^{2+} and dextran adjusted to the required concentrations) before addition of the lipid emulsion.

Stock solutions of the proteinase inhibitors Tos-Phe-CH_2Cl and Tos-Lys-CH_2Cl were prepared in methanol at 0.1 and 0.2M respectively. Phenylmethanesulphonyl fluoride (0.1M) was dissolved in ethanol. Inhibitor, or ethanol, or methanol, was added to washed erythrocytes in the dextran-containing basal salt solution (Ahkong *et al.*, 1973) at 37°C. Incubation was carried out for 20min (unless otherwise stated), oleoylglycerol was then added and the cells were incubated under conditions B or E (Table 1).

Determination of the fusion index

Incubated samples were fixed as for electron microscopy (see below), and the cells then counted by using phase-contrast microscopy. The occurrence of cell-to-cell fusion was determined by inspection of cell size and shape (e.g. dumb-bell-shaped cells). Multicellular bodies containing up to four cells were recognized, but when more than four cells had fused into one entity, the fusion index determined was underestimated, since it was not then possible to determine accurately the number of participating cells. This proviso applied particularly to condition E (see below) where the highest fusion indices were obtained. In each sample, 800–1000 cells were

counted, and random checks on the reproducibility of the counting method were carried out by counting 800–1000 cells of certain samples several times. The fusion index rarely differed by more than 5% in counts undertaken on any one sample. If it did, these samples were re-counted.

The fusion index was expressed as the percentage of erythrocytes which had fused, as follows. Cell bodies were counted and scored as being composed of either two, three, four or more than four cells (or unfused single cells). The total number of erythrocytes that would have been originally present was obtained by correction from these data. (The number of erythrocytes that had participated in fusion)/(the total number of erythrocytes that were present originally as single cells)$\times$100 was the fusion index.

Isolation of 'ghosts'

'Ghosts' were prepared from human erythrocytes by using the hypo-osmotic lysis method described by Dodge *et al.* (1963) modified by Elgsaeter & Branton (1974) except that, after the cells had been washed twice, 1mM-phenylmethanesulphonyl fluoride was present in all subsequent steps to prevent proteolysis. (However, preparations made in the absence of phenylmethanesulphonyl fluoride gave no indication that proteolysis had occurred.) The 'ghost' pellets were observed by phase-contrast microscopy and frozen on the day of preparation. Protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as the standard.

Polyacrylamide-gel electrophoresis in sodium dodecyl sulphate

The method of Fairbanks *et al.* (1971) was followed by using gels of polyacrylamide concentration 5.6% (w/v), except that sodium dodecyl sulphate (0.2% w/v) in the electrophoresis buffer and gels replaced the 1% (w/v) specified by Fairbanks *et al.* (1971), and 2-mercaptoethanol (5%, v/v) replaced dithiothreitol in the sample preparation. All stock buffers were filtered through glass microfibre filters (GF/C; Whatman Biochemicals, Maidstone, Kent, U.K.).

Samples were prepared for electrophoresis by adding 'ghosts' to a solution containing (final concentration) 10mM-Tris/HCl/1mM-EDTA (pH8), 2-mercaptoethanol (5%, v/v), sodium dodecyl sulphate (2%, w/v), glycerol (5%, v/v) and Pyronin Y (10μg/ml) at a temperature above 70°C. The samples were heated in a boiling-water bath for a further 2min. After preparation, samples were usually electrophoresed the same day, but they could be stored at −15°C and subsequently reheated at 100°C before loading: 40–50μl containing 40–50μg of protein was loaded per gel. The weight of protein applied per gel was constant between samples in the same run. Each run included (*a*) 'ghosts' isolated from unincubated, untreated erythrocytes, and (*b*) 'ghosts' prepared from appropriate controls incubated in the absence of oleoylglycerol, or ionophore or Ca^{2+}, depending on the experiment. Electrophoresis was carried out in an apparatus similar to that of Davis (1964) and proceeded at 3mA/gel for 20min, then at 7mA/gel until the Pyronin Y marker was within 0.5cm of the end of the gel.

Gels were fixed overnight, with stirring and one solvent change, in propan-2-ol/acetic acid/water (5:2:13, by vol.) and stained the next day for 6h at 37°C in Coomassie Blue (0.2%, w/v) in the same solvent. Gels were destained in acetic acid/methanol/water (14:7:79, by vol.) at 37°C for 2–3h and then overnight with several changes. The Coomassie Blue-stained protein bands were identified according to the designation of Steck (1974). In this nomenclature, spectrin comprises bands 1 and 2, the major erythrocyte protein is band 3, the actin-like polypeptide is band 5, and band 6 is glyceraldehyde 3-phosphate dehydrogenase. The slowest-moving component of the broad complex band designated by Steck (1974) as band 4.5 has been re-numbered here as band 4.3. Gels were photographed, or scanned with an SP.1809 scanning densitometer in an SP.1800 spectrophotometer (Pye–Unicam, Cambridge, U.K.).

Electron microscopy

Samples (1vol.) for electron microscopy were fixed at 0°C for 1h in a solution (2vol.) of glutaraldehyde (2%, v/v) in 0.1M-sodium cacodylate/HCl buffer, pH7.4 (Glauert, 1975), and studied in thin sections and in freeze-fractured preparations as previously described (Vos *et al.*, 1976). Intramembranous particles were counted in appropriate but randomly chosen areas of 5cm^2 per photograph, in a randomly chosen series of ten photographs. The number of particles per μm^2 was calculated, and corrected for small inherent faults of magnification in the electron microscope and photographic equipment.

Results

Morphology and kinetics of cell fusion: role of Ca^{2+} ions

Human erythrocytes incubated at 37°C with oleoylglycerol, in the presence of Ca^{2+} and dextran, changed shape from biconcave discs (Plate 1*a*), via cup-shaped cells, to spheres (Plate 1*b*). The cells also aggregated. Necks formed between the aggregated cells which progressed with time from a bicellular dumb-bell shape (Plate 1*c*) to bi-, tri- and multicellular bodies that were often spherical (Plates 1*c*, 1*d* and 2*a*). With condition E (Table 1), the total population of single cells was spherical after about

Table 1. *Fusion indices of human erythrocytes incubated with different concentrations of oleoylglycerol and Ca^{2+}, and the changes observed in the protein components of 'ghosts' prepared from the treated erythrocytes*

Human erythrocytes were incubated, under the conditions shown in the Table, as described in the Materials and Methods section. Incubation times were 30 min except in condition A which was 50 min. Fusion indices were determined on fixed cells; the values given are for single experiments under conditions A and D, for two independent experiments under condition C, and for four and nine independent experiments (mean ± S.D.) for conditions B and E respectively. At the end of the incubations, 'ghosts' were prepared and 40–50 μg of protein was subjected to electrophoresis as described in the Materials and Methods section. The alterations in the protein component of 'ghosts' are expressed relative to the pattern of proteins seen with 'ghosts' prepared from erythrocytes that were incubated in medium containing 5 mM-Ca^{2+}, but without oleoylglycerol.

Conditions of incubation ...	A	B	C	D	E	Control
Erythrocytes (10^8 × cells/ml)	2.6	2.4	2.4	2.16	2.16	2.16
Oleoylglycerol (μg/10^8 cells)	32.0	42.0	42.0	47.0	47.0	—
Ca^{2+} (mM)	1.8	1.8	5.0	1.8	5.0	5.0
Fusion index ([8])	30 (1)	43.9 ± 2.9 (4)	47, 74	75 (1)	75.8 ± 10.8 (9)	—
	Major alterations* in protein components of 'ghosts'					
High mol.wt.	Present	Present	Present	Present	Present	Absent
Band 2.3	No change	No change	No change	No change	Decreased	Present
Band 3	Decreased†	Decreased†	Decreased	Decreased	Decreased	Present
Prominent new component in band-3 region	Absent	Absent	Present	Present	Present	Absent
Band 4.1	Absent	Absent	Absent	Absent	Absent	Present
Band 4.3	Increased	Increased	Increased	Increased	Increased	Increased†

* Additional changes discussed in the text.
† Slight.

7 min of incubation, and 50–80% of the erythrocytes had undergone bi- or multi-cellular fusion after 30 min (mean of fusion index, 75.8%; S.D. ± 10.8 in nine experiments). The remaining cells stayed single; about 5% of them had completely lysed. The shapes of the 'ghosts' prepared from human erythrocytes treated with oleoylglycerol were similar to those of the treated cells (Plate 1*e*); such 'ghosts' may be compared with those prepared from untreated erythrocytes (cf. Plate 1*f*).

The kinetics of fusion depended on the concentrations of oleoylglycerol and Ca^{2+}. Thus with condition A (the lowest concentrations of oleoylglycerol and Ca^{2+} used; Table 1) large multicellular bodies did not form and the shape of each erythrocyte participating in fusion was still apparent after 50 min. Most of the fused cells were then bicellular and the fusion index was 25%.

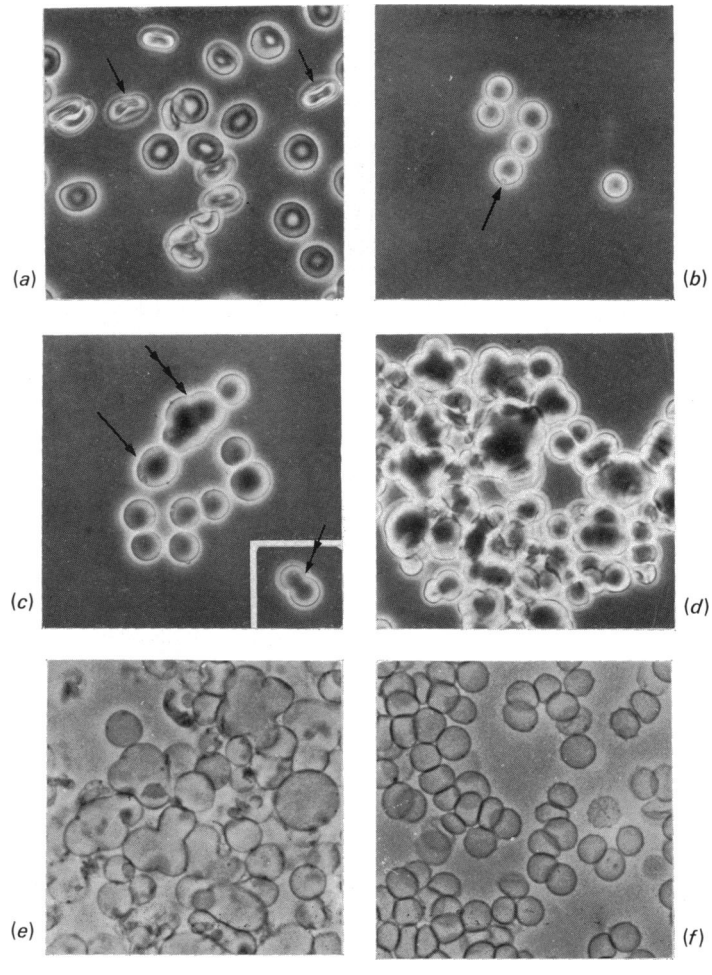
None of the above changes was observed when the cells were incubated for 30 min in the absence of oleoylglycerol. Table 1 summarizes the observations made, and it illustrates the increase in fusion index that occurred with increasing concentrations of Ca^{2+} or oleoylglycerol.

When no exogenous Ca^{2+} was present, some fusion still occurred. Furthermore, the presence of 1 mM-EDTA did not inhibit fusion completely. In three experiments in which erythrocytes were washed with EDTA or EGTA (1 mM) and incubated for 30 min with the concentration of oleoylglycerol as in condition E, but without Ca^{2+}, the fusion was 24.9% (range 22–30%) of that found for erythrocytes incubated for 30 min under condition E.

Lipid–membrane and membrane–membrane interactions

Microdroplets of dispersed oleoylglycerol, which were up to approx. 1 μm in diameter and were easily seen by optical microscopy, rapidly adhered to the erythrocytes during the incubation (condition E) (Plates 1*b* and 1*c*). In general, the shapes of lipid droplets were retained in thin sections of treated cells that were fixed for electron microscopy, but the lipid itself was extracted during the dehydration procedure. After adhering to the cell surface, the microdroplets were largely engulfed by invagination of the plasma membrane (Plates 2*a* and 3*d*).

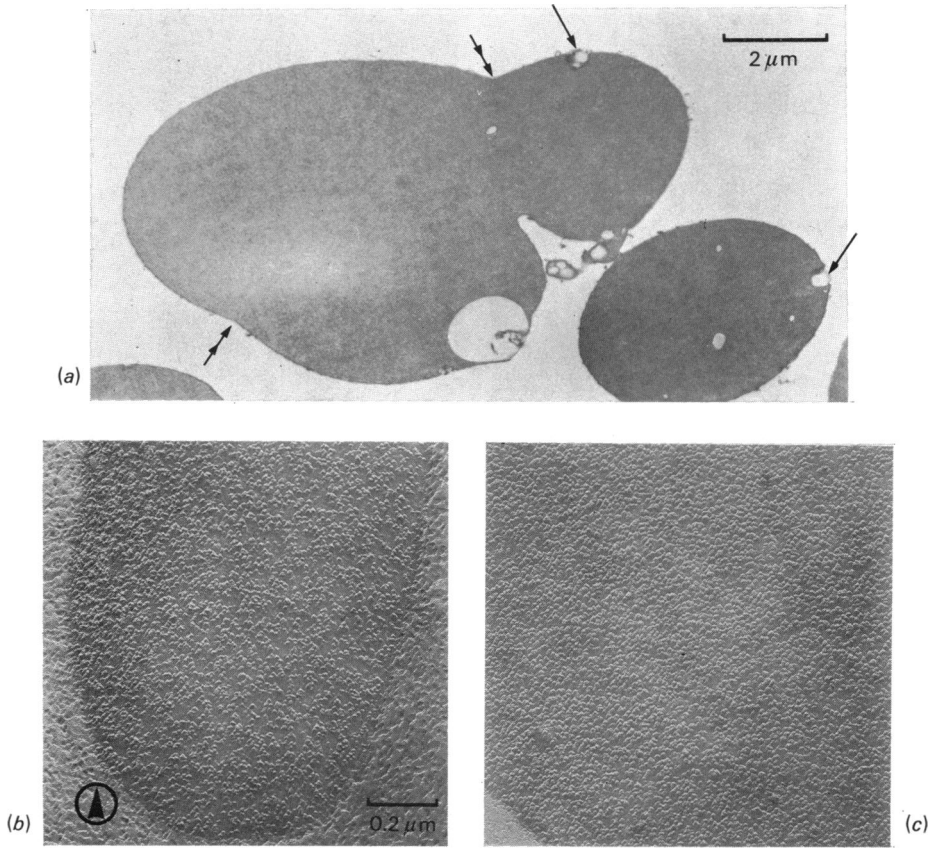
By comparison with an untreated membrane (Plate 3*a*), abnormalities appeared in the plasma membranes of human erythrocytes in the first few seconds of treatment with oleoylglycerol (condition E) (Plate 3*b*). These changes may result from the initial interaction of a microdroplet of lipid with the membrane, and they may conceivably be related to the alterations in membrane proteins that are reported below. Later, adjacent cells fused around the site of a microdroplet (Plate 3*e*). Membrane



EXPLANATION OF PLATE 1

Morphological behaviour of human erythrocytes on treatment with oleoylglycerol

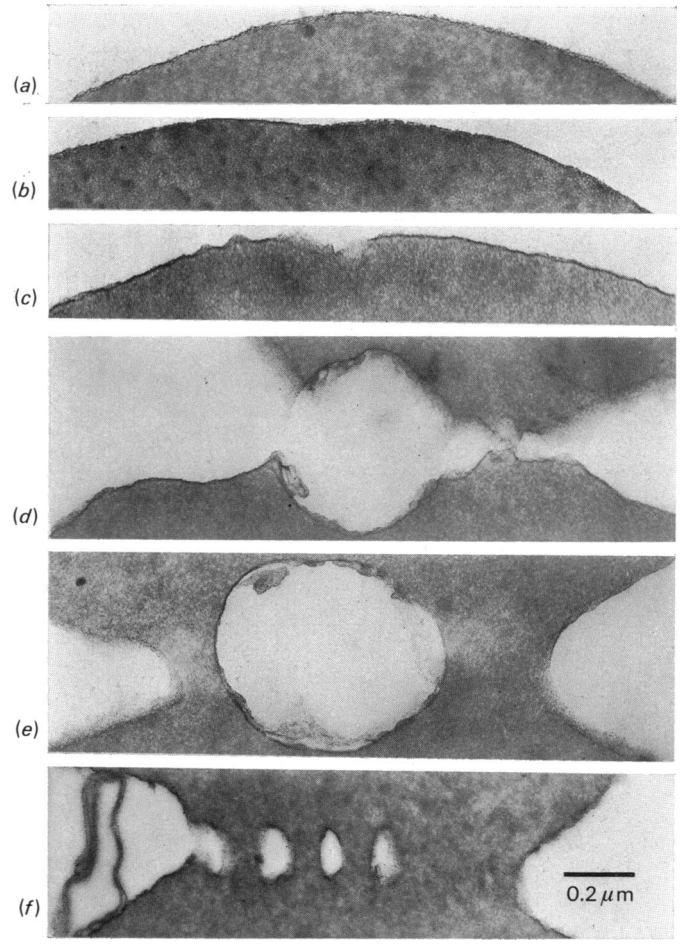
(*a*) Human erythrocytes were incubated for 30min in Eagle's basal salt solution at pH 7.4, containing dextran (80mg/ml): these cells retained their biconcave shape (arrow). In (*b*)–(*d*), the cells were treated with oleoylglycerol under condition E (cf. Table 1) and then fixed, as described in the Materials and Methods section. (*b*) After treatment with oleoylglycerol for 2min, the cells were spherical; a microdroplet of oleoylglycerol was attached to a cell (arrow). (*c*) After 7min a spherical bicellular body (arrow), a dumb-bell-shaped bicellular body (double arrow) and a multi-cellular body (triple arrow) were present. (*d*) After 30min, extensive cell fusion had occurred. The 'ghosts' in (*e*) were prepared from erythrocytes treated with oleoylglycerol for 30min under condition E, as described in the Materials and Methods section. Some ghosts had the lobed shape of the treated cells and others were spherical. More haemoglobin was retained than in the 'ghosts' shown in (*f*), which were prepared from erythrocytes incubated as in (*a*). Phase-contrast microscopy: magnification × 357.



EXPLANATION OF PLATE 2

Ultrastructural behaviour of human erythrocytes on treatment with oleoylglycerol

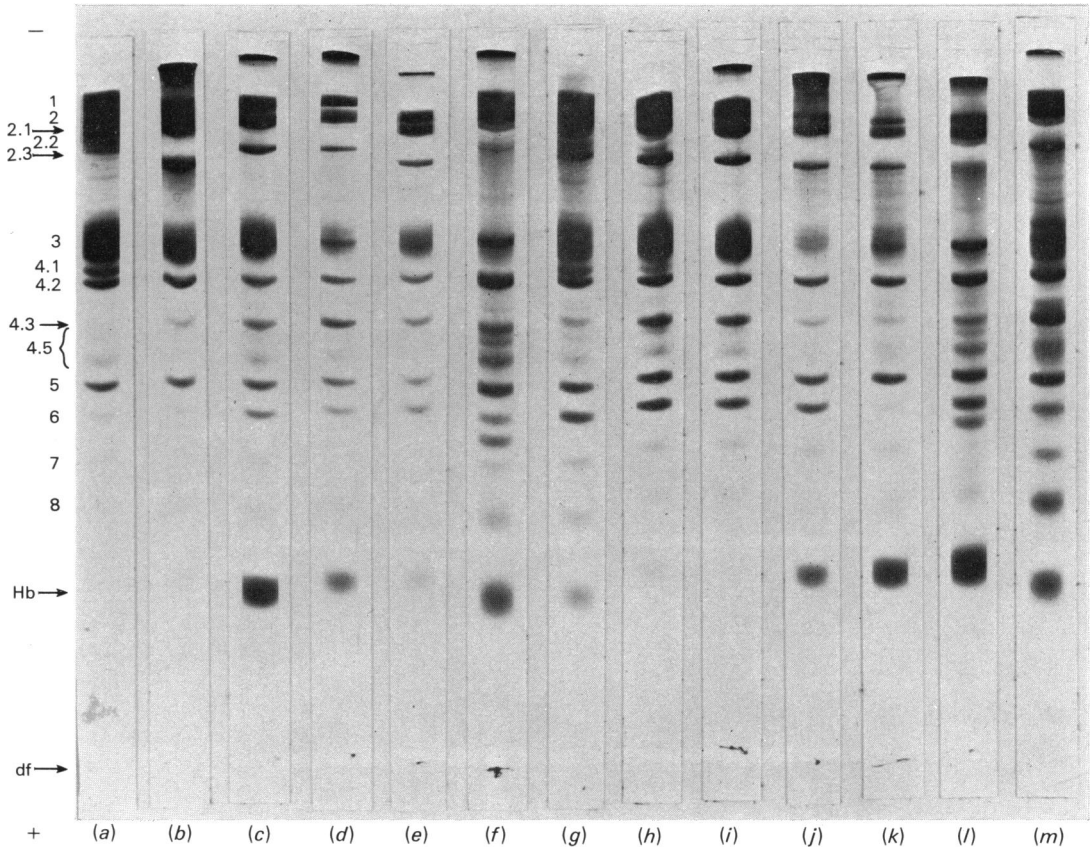
(*a*) An electron micrograph of a thin section of human erythrocytes incubated with oleoylglycerol for 30min under condition E (cf. Table 1) and then fixed, as described in the Materials and Methods section. Microdroplets of oleoylglycerol are seen that have been engulfed by invagination of the plasma membrane (single arrows). A multicellular body is present: double arrows indicate the lines of cell fusion, which for the right-hand arrow is marked by intracellular membranous vesicles. (*b*) and (*c*) Electron micrographs of the P-faces of freeze-fractured preparations of erythrocyte membranes. In (*b*) cells were incubated for 30min under condition E, but without oleoylglycerol, and the intramembranous particles exhibit micro-aggregation. In (*c*) cells were incubated with oleoylglycerol for 30min under condition E, and the intramembranous particles are more randomly distributed. The direction of shadowing, and the magnification, are the same in (*b*) and (*c*).



EXPLANATION OF PLATE 3

Ultrastructure of cell fusion in human erythrocytes treated with oleoylglycerol

(*a*)–(*f*) Electron micrographs of thin sections of human erythrocytes prepared as described in the Materials and Methods section. (*a*) The plasma membrane of a cell after incubation for 25min under condition E (Table 1), but without oleoylglycerol. (*b*)–(*f*) The membranes of cells incubated for various periods of time with oleoylglycerol under condition E. (*b*) The abnormal appearance of the membrane of a cell fixed immediately after the addition of oleoylglycerol. (*c*)–(*e*) Cells fixed after 10min of incubation with oleoylglycerol. In (*c*) the membrane appears increasingly abnormal. In (*d*) a microdroplet of oleoylglycerol is largely engulfed by two adjacent cells. In (*e*) two cells have fused around the site of a microdroplet. (*f*) Cells fixed after 30min of incubation with oleoylglycerol, showing a line of membranous vesicles at the site of cell fusion.



EXPLANATION OF PLATE 4

Electrophoretic patterns of 'ghosts' prepared from human erythrocytes

Human erythrocytes were incubated under the conditions described in Table 1; the 'ghosts' were then prepared and subjected to electrophoresis as described in the Materials and Methods section. The band designations shown are those of Steck (1974), except that the slowest-moving component of the broad, complex band designated as band 4.5 has been renumbered here as 4.3. The positions of haemoglobin (Hb), and the dye front (df) which appears as a black line, are indicated. The conditions and length of incubation time of the erythrocytes before the isolation of 'ghosts' were: (*a*) no incubation (Dodge 'ghosts'), (*b*) condition A for 50min, (*c*) condition B for 30min, (*d*) condition C for 30min, (*e*) condition D for 30min, (*f*) condition E for 30min, (*g*) condition E, but in the absence of oleoylglycerol, for 30min, (*h*) ionophore A23187 and 0.2mM-Ca^{2+} for 70min, (*i*) ionophore A23187 and 2.0mM-Ca^{2+} for 70min, (*j*) ionophore A23187 and 5.0mM-Ca^{2+} for 70min, (*k*) ionophore A23187 and 20mM-Ca^{2+} for 70min, (*l*) ionophore A23187 and 5.0mM-Ca^{2+} for 40min, followed by incubation under condition E for 30min, as described in the Materials and Methods section, (*m*) condition E for cells treated with 1mM-Tos-Phe-CH_2Cl, 30min incubation.

fusion also occurred over large areas of membrane, with the line of fusion being indicated by membranous vesicles (Plates 3*f* and 2*a*). It is not clear, however, whether or not such fusion was initially associated with one or more microdroplets of lipid.

Intramembranous particles

The appearance of the E- and P-fracture (Plate 2*b*) faces of freshly prepared human erythrocytes was consistent with observations reported in the literature (Weinstein, 1974). (For the nomenclature of the faces, see Branton *et al.*, 1975.) On treatment of the erythrocytes with oleoylglycerol for 7–30min (condition E), however, the appearance of the P-fracture face of the cells was changed as compared with control cells, in that there was no clear evidence of micro-aggregation of the intramembranous particles in the treated cells. This alteration, of which an example is given in Plate 2(*c*), was observable in areas of the specimen that were more or less flat (local angle of shadowing approx. 45°). The altered appearance of this face was found to be highly reproducible at the various time intervals of treatment with oleoyl-glycerol (7, 15 and 30min) and in successive experiments.

In erythrocytes incubated without oeloylglycerol, the number of particles per μm^2 of P-fracture face was 2534 ± 168 (mean $\pm$ S.D., $n=10$), which is consistent with the values of 2579, 2600 and 2658 reported for the human erythrocyte by Chevalier (1974), Weinstein (1974) and Kirk & Tosteson (1973) respectively. The number of particles per μm^2 of P-fracture face in cells incubated with oleoylglycerol for 7–30min (condition E) was 2791 ± 242 (mean $\pm$ S.D., $n=30$). The two values for treated and untreated cells do not differ significantly. There is even less difference between them if they are corrected for changes in surface area, which was calculated to be minimally (approx. 8%) decreased in the treated, spherical cells as compared with the untreated discocytes.

On the basis of the above findings it is concluded that the intramembranous particles on the P-fracture faces of the treated cells were more randomly distributed, but not significantly altered in number by comparison with the control cells.

Erythrocyte proteins

The electrophoresis pattern of proteins of 'ghosts' from unincubated erythrocytes is shown in Plate 4(*a*). Gel electrophoresis was undertaken on 'ghosts' that were prepared from erythrocytes after incubation with or without oleoylglycerol. Plate 4 (gels *b–f*) shows the electrophoretic patterns of the proteins of 'ghosts' prepared from erythrocytes that were fused under conditions A–E. Several distinctive changes (see Table 1 for summary) are apparent on comparing these patterns with that of 'ghosts' prepared from erythrocytes that were incubated in the absence of oleoylglycerol (Plate 4*g*). The appearance of the latter was identical with that seen with 'ghosts' from unincubated erythrocytes (Plate 4*a*).

Proteins of high molecular weight, and band 4.1

With conditions A–E, high-molecular-weight material that barely entered the gel was observed after 30–50min of incubation with oleoylglycerol, and band 4.1 was absent (Plate 4, gels *b–f*). These alterations were not seen in 'ghosts' prepared from erythrocytes sampled at 0min, but they were apparent within 15min of incubation (results not shown). The observed changes may have been caused by the entry of Ca^{2+} into the cells, since a similar pattern was seen in 'ghosts' prepared from erythrocytes that were incubated with the bivalent cation ionophore A23187 and Ca^{2+}. Thus with ionophore and a low concentration of Ca^{2+} (0.2mM), band 4.1 was decreased slightly after 70min incubation (Plate 4*h*); with ionophore and 2mM-Ca^{2+}, band 4.1 was absent and high-molecular-weight material was present after 70min of incubation (Plate 4*i*). Ionophore alone, in the absence of Ca^{2+}, was without effect on the electrophoretic pattern.

Bands 4.3, 4.5 *and the region of band* 6

Band 4.3 was more prominent in 'ghosts' prepared from erythrocytes treated under conditions A–E (Plate 4, gels *b–f*) than in 'ghosts' prepared from untreated erythrocytes (Plate 4*a*). Increased staining was also apparent with condition E after 30min in the region of band 4.5, and another band was seen that moved slightly faster than band 6 (Plate 4*f*). Treatment of erythrocytes with ionophore A23187 and 0.2 or 2mM-Ca^{2+} also led to an increase in band 4.3 in 'ghosts' prepared from these erythrocytes (Plate 4, gels *h* and *i*); with 5 and 20mM-Ca^{2+} the increase was less marked (Plate 4, gels *j* and *k*). It was noticeable that most preparations of 'ghosts' from fused cells exhibited a markedly increased staining in the region of haemoglobin. This was even apparent to some extent in zero-time samples from oleoylglycerol-teated erythrocytes. Additional washes of 'ghosts' did not decrease the staining. In view of the increased retention of haemoglobin in our experiments it could be suggested that band 4.3 was increased because of a generally increased retention of cytoplasmic proteins during the preparation of 'ghosts' from oleoylglycerol-treated erythrocytes. However, increased staining was often seen in the region of haemoglobin, but not in band 4.3, with zero-time samples of 'ghosts' prepared from oleoyl-glycerol-treated erythrocytes in the absence of cell

fusion. In addition, 'ghosts' prepared from cells treated with ionophore A23187 and 0.2 or 2mM-Ca^{2+} retained relatively little haemoglobin, but nevertheless exhibited a marked increase in band 4.3 staining (Plate 4, gels *h* and *i*).

In 'ghosts' prepared from fused cells, an apparent increase in haemoglobin may be due to proteins other than haemoglobin, possibly proteolytic products, because the gel used will not resolve components with molecular weights of less than about 20000. It is possibly relevant that Triplett & Carraway (1972) and Cabantchik & Rothstein (1974) found an increased retention of material in the haemoglobin region, which they assumed was haemoglobin, in 'ghosts' obtained from Pronase-treated erythrocytes.

Band 3

The most marked alteration in the electrophoretic pattern of 'ghosts' prepared from cells fused with oleoylglycerol was a diminution in the intensity of staining of band 3, the major erythrocyte protein. This was most pronounced when the fusion index was high, as in conditions C, D and E (Plate 4, gels *d–f*). With condition E, this finding was made in five independent experiments. The loss of band 3 was also apparent, but was not as marked, after only 15min of incubation (results not shown).

Concomitant with a marked decrease in band 3 was the appearance, or possible unmasking, of a new very sharp band within the broad region where band 3 is normally located (Plate 4, gels *d–f*). It should be noted here, since the oleoylglycerol-treated preparations contained both fused and unfused cells, that one might expect band 3 to be less affected and no new band to be discernible within the band-3 region, when the fusion index is low as under conditions A and B (Plate 4, gels *b* and *c*).

Further experiments with ionophore A23187 were undertaken in an attempt to determine whether the diminution of band 3 was yet another apparent facet of the entry of Ca^{2+} into the cellular cytoplasm, or whether other factors were involved. As previously reported, human erythrocytes (unlike hen erythrocytes) do not fuse into multinucleated cells on treatment with the ionophore in the presence of Ca^{2+} (Vos *et al.*, 1976). Changes in membrane proteins occurring during the fusion of human erythrocytes induced by oleoylglycerol that cannot be simulated by ionophore treatment are thus presumably a feature, or a consequence, of cell fusion itself. With ionophore and 0.2 or 2mM-Ca^{2+} (70min at 37°C), there was little or no diminution of the staining of band 3 (Plate 4, gels *h* and *i*). On increasing the concentration of Ca^{2+} to 5mM (70min at 37°C), band 3 was diminished (Plate 4*j*). On further increasing the concentration of Ca^{2+} to 20mM in the presence of ionophore (70min at 37°C), the intensity of band-3 staining was similar to that seen with 5mM-Ca^{2+}, but a faint new band was visible within the band-3 region (Plate 4*k*). When erythrocytes were preincubated for 40min with ionophore and 5mM-Ca^{2+}, and then treated for 30min at 37°C with the concentration of oleoylglycerol corresponding to condition E, extensive cell fusion resulted. This was accompanied by a loss of band 2.3, a further decrease in band 3, and the appearance of a new sharp band within the band-3 region (Plate 4*l*, cf. Plate 4*j*: both 70min total incubation). Changes in bands 4.3, 4.5, and between bands 6 and 7 that were observed with condition E were also seen.

We conclude from these results that the entry of Ca^{2+} into human erythrocytes can reproduce some of the effects that are observed when erythrocytes are fused by treatment with oleoylglycerol, including some diminution of band 3. However, although a new narrow band appeared within region 3 on treating the cells with 20mM-Ca^{2+} and ionophore (in the absence of cell fusion), this was very faint by comparison with the corresponding band seen with the high indices of cell fusion occurring with oleoylglycerol under condition E, where the Ca^{2+} concentration was only 5mM.

Band 2 *region*

Close inspection of the gels revealed an increased prominence of band 2.3 after 30min with conditions A–D and a loss of minor bands 2.1 and 2.2 (Plate 4, gels *b–e*, cf. gel *g*); this was also seen in 'ghosts' prepared at earlier times with condition E (results not shown). After 30min with condition E, band 2.3 had itself disappeared almost completely (Plate 4*f*). The 'ghosts' from cells treated with ionophore A23187 and Ca^{2+} (0.2–20mM) also showed a loss of minor bands between 2 and 2.3 but no marked loss of band 2.3 itself (Plate 4, gels *h–k*).

Proteinase inhibitors

The effects of the presence of inhibitors of proteinase activity, phenylmethanesulphonyl fluoride, Tos-Lys-CH_2Cl and Tos-Phe-CH_2Cl, on cell fusion and on membrane proteins were studied to see whether they prevented the loss of band-3 protein that was associated with the fusion process.

Phenylmethanesulphonyl fluoride (2mM) had no effect on the fusion index or on the electrophoretic pattern of 'ghosts' prepared from erythrocytes treated with oleoylglycerol under condition E.

In trial experiments Tos-Lys-CH_2Cl did not inhibit cell fusion induced by oleoyglycerol. Instead, it activated fusion by 155, 126 and 117% at concentrations of 2, 1 and 0.2mM respectively, perhaps by affecting the permeability of the cells to Ca^{2+}. This

substance had no action at lower concentrations (0.01–0.1 mM).

The effect of Tos-Phe-CH_2Cl was also examined: a range of concentrations from 0.005 to 2.00 mM was tested under condition B. Concentrations of 0.005 and 0.01 mM activated cell fusion by 128% and 143% compared with control incubations without Tos-Phe-CH_2Cl. Higher concentrations (0.5, 1 and 2 mM) gave fusion that was 76, 50 and 30% respectively of the control values in 15 min of incubation at 37°C. For inhibition to occur, preincubation of Tos-Phe-CH_2Cl with erythrocytes at 37°C for at least 5 min was necessary before the addition of oleoylglycerol. Preincubation at 0°C with Tos-Phe-CH_2Cl was ineffective in inhibiting fusion, as was the simultaneous addition of Tos-Phe-CH_2Cl and oleoylglycerol. In six experiments with condition B, and a preincubation of 20 min at 37°C with 1 mM-Tos-Phe-CH_2Cl, the fusion was 47 ± 8% (mean ± S.D.) of that observed in the absence of inhibitor. At early sampling times, little or no inhibition was observed, but inhibition was established by 15 min.

These experiments with condition B, with its relatively low fusion indices, gave little discernible loss of band 3. We therefore tested the effects of Tos-Phe-CH_2Cl under conditions when band 3 was more conclusively diminished. The action of Tos-Phe-CH_2Cl on cell fusion was much less marked with condition E, and Tos-Phe-CH_2Cl-treated cells exhibited fusion that was 80% of that shown by the control cells. Gel electrophoresis of 'ghosts' prepared from these treated cells showed that band 3 was diminished less than usual and that a new band was just apparent within the band-3 region (Plate 4*m*; cf. gel *f*). With condition D, band 3 was also only slightly diminished after 30 min in the Tos-Phe-CH_2Cl-treated cells, and a new band was just apparent within the band-3 region (results not shown). 'Ghosts' prepared from erythrocytes incubated with 1 mM-Tos-Phe-CH_2Cl but without oleoylglycerol, showed no differences from control 'ghosts'. Thus it seems that Tos-Phe-CH_2Cl partially inhibited the loss of band 3 which was so striking under condition E, but it was unable to prevent the appearance of a new band within this region. It is possible that inhibition of the loss of band 3 was responsible for the partial inhibition of fusion that was observed. Other band alterations were similar to those found under condition E in the absence of Tos-Phe-CH_2Cl, except that the band moving slightly faster than band 6 was absent.

Discussion

It has been suggested that oleoylglycerol induces cell fusion by increasing the proportion of hydrocarbon chains in the membrane that are in a relatively liquid state (Ahkong *et al.*, 1973), and this proposal was supported by the results of a spectrofluorimetric study of the interaction of oleoylglycerol with human erythrocyte 'ghosts' (Kennedy & Rice-Evans, 1976). It is interesting that Plate 3(*e*) indicates that some of the treated cells may fuse as their membranes flow around microdroplets of oleoylglycerol. An incorporation of oleoylglycerol from such a droplet into plasma membranes will probably not only alter membrane fluidity in the region of the droplet, but might also induce other changes in the physical properties of membrane lipids (Maggio & Lucy, 1975, 1976) that aid fusion of the membranes of the treated cells. It is also relevant that Lentz *et al.* (1976) have proposed that regions of high curvature in biomembranes may be associated with the lateral segregation of lipids into different domains. This may further facilitate fusion between plasma membranes that come into contact around microdroplets of oleoylglycerol.

Our observations on the gel-electrophoresis patterns of membrane proteins of 'ghosts' obtained from cells treated with oleoylglycerol show that alterations in protein structure are also associated with cell fusion in this system. Thus the major changes seen (e.g. the loss of band 3 and the formation of a new band in this region) were most marked when the fusion index was high as a consequence of treating cells with high concentrations of oleoylglycerol and of Ca^{2+}. Some of the observed alterations in membrane proteins may be attributable to entry of Ca^{2+} into the cytoplasm of the treated cells, and this would be consistent with work by Blow *et al.* (1978), which has shown that isotopically labelled Ca^{2+} rapidly enters erythrocytes on incubation at 37°C with oleoylglycerol. For example, the very high-molecular-weight material produced in the presence of oleoylglycerol resembled that formed when 'ghost' membranes were prepared by hypo-osmotic lysis in the presence of Ca^{2+} (Triplett *et al.*, 1972; Carraway *et al.*, 1975). In addition, band 4.1 disappeared and new material was seen in a region equivalent to band 2.3 when human erythrocytes were lysed in Ca^{2+}-containing medium (Triplett *et al.*, 1972). The transamidase activity that has been found by Lorand *et al.* (1976) to be activated by Ca^{2+} in lysed human erythrocytes is probably responsible for the production of the very high-molecular-weight material in our experiments, and possibly also for the loss of band 4.1. In our experiments these changes were unaffected by the presence of Tos-Phe-CH_2Cl.

The most striking observations in our experiments were the loss of the broad band 3, the major protein of these erythrocytes, and the production of a sharp new band within the region of band 3. These changes were more marked on treatment of erythrocytes with oleoylglycerol and were observed at lower Ca^{2+} concentrations (1.8 mM), than when cells were treated with ionophore and Ca^{2+}. With ionophore, 5 mM

Ca^{2+} was required before band 3 was markedly decreased. Even with 20mM-Ca^{2+} and ionophore, the new band in this region was much less apparent than with cells that were treated with oleoylglycerol and 5mM-Ca^{2+}, or with cells that were pre-treated with ionophore A23187 and 3mM-Ca^{2+} and then further subjected to treatment with oleoylglycerol; We therefore conclude that some, if not all, of the changes in the electrophoretic pattern of 'ghosts' prepared from cells treated with oleoylglycerol can be reproduced by the use of ionophore A23187 and Ca^{2+}, but to a markedly lesser extent for the major alterations observed. It is relevant to note that Weidekamm *et al.* (1977) have suggested that some loss of band 3 occurs, together with an increase in band 4.3, if 4.5mM-Ca^{2+} is present when human erythrocytes are lysed. Depletion of band 3 has also been found in the microvesicles that are formed from human erythrocytes in response to an increase concentration of intracellular Ca^{2+} in 1-day-old (but not in 1-week-old) preparations of erythrocytes (Allan *et al.*, 1976).

Band 3 may possibly be a substrate for proteinase activity in erythrocytes treated with oleoylglycerol; this is indicated by the inhibitory effect of Tos-Phe-CH_2Cl on the loss of band 3. At least two enzymes that are active in the proteolytic self-digestion of human erythrocyte membranes have been reported by Tökés & Chambers (1975). King & Morrison (1977) have reported that incubation of isolated membranes from human erythrocytes results in a Ca^{2+}-dependent loss of bands 3 and 4.1 together with increased staining of bands in the 2.2 and 4.5 regions, and the production of lower-molecular-weight components below band 5. They attributed these changes to the effects of a Ca^{2+}-activated proteinase. Perturbation of the structure of the plasma membrane by oleoylglycerol may conceivably expose a normally latent Ca^{2+}-dependent proteinase that is located in the interior of the membrane or on its cytoplasmic surface. Should this be so, band 3 may then be degraded. Conceivably, the proteolytic degradation of certain membrane proteins may be an important feature of cell fusion induced by oleoylglycerol. This would be consistent with the finding that subtilisin Carlsberg (Sigma) (1mg/ml) induces fusion of human and hen erythrocytes that is inhibited by 0.1mM-phenylmethanesulphonyl fluoride (Q. F. Ahkong & J. A. Lucy, unpublished work).

It is interesting that the disappearance of band 3 in our experiments is concomitant with an increase in band 4.3. With high fusion indices we also detected new bands, one in the band 4.5 region and another with a slightly greater mobility than band 6 at a mol.wt. of approx. 30000, which may have resulted from the proteolytic degradation of band 3. Polypeptides of comparable molecular weight are formed when Pronase and chymotrypsin act extracellularly on intact human erythrocytes. Digestion of band 3 (95000 mol.wt.) in erythrocytes by these enzymes gives rise to smaller polypeptides with mol.wts. of 35000 (below band 6) and of 65000 (in the region of band 4.3), which remain in the membrane and are detectable by gel electrophoresis (Bender *et al.*, 1971; Cabantchick & Rothstein, 1974; Drickamer, 1976). Band-3 protein may be a component of the intramembranous particles observed in freeze-fractured human erythrocyte membranes (cf. Yu & Branton, 1976). In our experiments there was no major change in the appearance of distribution of the particles in the membranes of erythrocytes treated with oleoylglycerol, as might be expected if band 3 were degraded by proteolytic action and subsequently lost from the membrane. We did observe, however, a loss of the micro-aggregation of intramembranous particles that is normally a characteristic feature of human erythrocyte membranes.

The new sharp band within the band-3 region was the most pronounced alteration associated with cell fusion: this was barely seen in the presence of ionophore, even with 20mM-Ca^{2+}. It is possible that proteolysis of band 2.3 is responsible for the new band, since a concomitant diminution in the staining of this component was observed, but further work is necessary to resolve this question.

Whether the observed changes in membrane proteins occurring during cell fusion induced by oleoylglycerol are a cause or a consequence of cell fusion is not clear from our experiments, although the inhibitory effect of Tos-Phe-CH_2Cl on cell fusion and on the loss of band 3 show that the two phenomena are related. Further work is also needed to determine whether alterations in membrane occur only in the system of cell fusion studied here or whether they are of wider significance in membrane-fusion reactions. A number of observations (Chi *et al.*, 1976; Zakai *et al.*, 1976; Lawson *et al.*, 1977; Friend *et al.*, 1977; Orci *et al.*, 1977) have supported the suggestion by Ahkong *et al.* (1975) that membrane fusion proceeds by the intermingling of membrane lipids after the emergence of protein-free areas of lipid bilayer, after protein aggregation. However, the immobility of the intramembranous particles in fresh 'ghosts' from erythrocytes is well known (Elgsaeter *et al.*, 1976), and the fusion of human erythrocytes induced by oleoylglycerol that is reported here was not associated with a demonstrable development of protein-free areas of lipid bilayer. The absence of the micro-aggregation of intramembranous particles in the treated cells implies nevertheless that the proteins are at least partly free to move. Furthermore, it seems possible that proteolytic activity in the membrane may result in an increased freedom of movement of membrane proteins and that this could be an important feature of membrane fusion.

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