Calcium-activated thiol-proteinase activity in the fusion of rat erythrocytes induced by benzyl alcohol

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1. Rat erythrocytes were fused by incubation with benzyl alcohol and Ca^{2+} . 2. Cell fusion was inhibited by EGTA, *N*-ethylmaleimide, tetrathionate, iodoacetamide, cystamine, Tos-Lys- CH_2Cl , and to a lesser extent by Tos-Phe- CH_2Cl . Phenylmethanesulphonyl fluoride, Tos-Arg-OMe and histamine did not inhibit cell fusion. 3. Gel electrophoresis of membrane proteins from 'ghosts' of the erythrocytes treated with benzyl alcohol showed that a high-molecular-weight polymer was present; this was consistent with the entry into the cells of Ca^{2+} and the activation of a transglutaminase enzyme. 4. In the treated cells the proteins corresponding to bands 2 and 3 in human erythrocytes were decreased, and a polypeptide with a slightly greater mobility than band 3 was produced. 5. These changes were inhibited by EGTA, *N*-ethylmaleimide, tetrathionate, iodoacetamide, cystamine and Tos-Lys- CH_2Cl , but not by phenylmethanesulphonyl fluoride, Tos-Arg-OMe, or histamine. 6. The intramembranous particles of the P-fracture face of cells treated with benzyl alcohol to induce fusion were decreased in number and were susceptible to cold-induced aggregation; both of these phenomena were markedly inhibited by EGTA, and partially inhibited by Tos-Lys- CH_2Cl and *N*-ethylmaleimide. 7. These several observations indicate that a Ca^{2+} -activated thiol-proteinase, which acts to degrade membrane proteins and to give freedom of lateral movement to intramembranous particles, may be an essential feature of membrane fusion in this system. 8. It is suggested that this proteinase may act to degrade spectrin-binding proteins that attach band-3 protein to the erythrocyte cytoskeleton.

Our earlier work has shown that oleoylglycerol induces cell fusion with avian and mammalian erythrocytes, including those of the rat (Ahkong *et al.*, 1973). Studies on model membrane systems have demonstrated that a primary site of action of this and other fusogenic lipids is membrane phospholipids (Howell *et al.*, 1973; Maggio & Lucy, 1975, 1976), and it has been shown that oleoylglycerol increases the permeability of erythrocyte membranes to Ca^{2+} (Blow *et al.*, 1979). However, investigations on human erythrocytes during cell fusion induced by oleoylglycerol indicated that the proteolytic degradation of certain membrane proteins may also be involved in the fusion process

(Quirk *et al.*, 1978; Ahkong *et al.*, 1978). Thus the proteinase inhibitor Tos-Phe- CH_2Cl inhibited both cell fusion induced by oleoylglycerol and the loss of band 3 from the membrane proteins that was found to be associated with the fusion process. As the fusion of human erythrocytes by oleoylglycerol is much enhanced by Ca^{2+} , it has been suggested that perturbation of the structure of the plasma membrane by this fusogen may expose a normally latent Ca^{2+} -activated proteinase in the interior of the membrane or on its cytoplasmic surface (Quirk *et al.*, 1978).

In the present paper, experiments are reported on the extensive fusion of rat erythrocytes induced by benzyl alcohol. These experiments demonstrate that changes in membrane proteins are not a special feature of the fusion of human erythrocytes by oleoylglycerol. The membranes of rat erythrocytes treated with benzyl alcohol in the presence of Ca^{2+} exhibited particularly extensive changes in the regions

Abbreviations used: Tos-Arg-OMe, *N*-tosyl-L-arginine methyl ester ('TAME'); 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 correspond on gel electrophoresis to the positions of bands 2 and 3 in human cells. Cell fusion and the changes in these proteins were markedly inhibited by *N*-ethylmaleimide and other thiol reagents, as well as by EGTA. These observations thus develop our earlier brief report that *N*-ethylmaleimide inhibits the fusion of hen and human erythrocytes induced by a number of chemical fusogens, including oleoyl-glycerol (Hart *et al.*, 1975).

Parallel ultrastructural studies in the present work have shown that clustering of the intramembranous particles of rat erythrocyte membranes occurs at 0°C in the benzyl alcohol-treated cells. This behaviour is similar to that of human erythrocytes when cell fusion is induced by subtilisin, and it is thought to provide support for our suggestion (Ahkong *et al.*, 1978) that proteolytic activity in membranes may, in general, be associated with an increased freedom of lateral movement of integral proteins that facilitates membrane fusion.

Materials and methods

Erythrocytes

Rat blood was obtained from Sprague-Dawley rats by heart puncture or by decapitation and mixed with cold citrate anticoagulant (De Gowin *et al.*, 1949). The blood was washed three times in a salt solution containing NaCl (116 mM), glucose (1 mg/ml), penicillin G (120 µg/ml) and streptomycin sulphate (100 µg/ml), buffered at pH 7.4 with sodium cacodylate (10 mM), the buffy coat being carefully removed with each washing. The erythrocytes (approx. 1.3×10^9 cells/ml) were resuspended in the buffered salt solution, containing dextran (80 mg/ml), and stored at 4°C until used on the same day. Inspection of the cells by optical microscopy showed that the erythrocytes were virtually free from leucocytes.

Reagents

All reagent chemicals used were commercially obtained and were of analytical grade. Dextran (average mol.wt. 60 000–90 000, clinical grade), Tos-Phe-CH₂Cl, Tos-Lys-CH₂Cl, Tos-Arg-OMe, phenylmethanesulphonyl fluoride, *N*-ethylmaleimide, iodoacetamide, cystamine dihydrochloride, histamine dihydrochloride, EGTA, DL-dithiothreitol and agarose were from Sigma (London) Chemical Co. (Poole, Dorset, U.K.). E. Merck (Darmstadt, Germany) supplied sodium tetrathionate. Benzyl alcohol, sodium cacodylate, acrylamide (specially purified for electrophoresis), sodium dodecyl sulphate (specially pure) and Coomassie Brilliant Blue R250 (colour index 42660) were from BDH Chemicals (Poole, Dorset, U.K.). Glutaraldehyde (Gurr) was supplied by Hopkin and Williams (Chadwell Heath, Essex, U.K.), and Pyronin Y

(colour index 45005) was from Searle Diagnostics (High Wycombe, Bucks., U.K.).

Incubation of erythrocytes with benzyl alcohol

A dispersion of benzyl alcohol in the salt solution containing dextran, described above, was made by sonication as described by Ahkong *et al.* (1973). Rat erythrocytes (approx. 5×10^8 cells/ml) in the salt solution containing dextran were preincubated for 10 min at 37°C with or without various proteinase inhibitors or thiol reagents, Ca²⁺ or EGTA was added to final concentrations of 4 mM, and the cells were then mixed with freshly dispersed benzyl alcohol (final concentration 100 mM). The treated cells were then incubated at 37°C for the desired period, and samples removed at frequent intervals for examination by phase-contrast microscopy.

Stock solutions of the proteinase inhibitors Tos-Phe-CH₂Cl, Tos-Lys-CH₂Cl and phenylmethanesulphonyl fluoride were prepared in methanol at 0.1 M. Stock solutions of Tos-Arg-OMe (20 mM), sodium tetrathionate (100 mM), *N*-ethylmaleimide (50 mM), iodoacetamide (100 mM), cystamine (100 mM) and histamine (200 mM) were prepared in the dextran-containing buffered salt solution and the pH was adjusted to pH 7.4.

Electron microscopy

Samples (1 vol.) for electron microscopy were fixed at the indicated temperature for 1 h in a solution (2 vol.) of glutaraldehyde (2%, v/v) in 0.1 M-sodium cacodylate/HCl buffer, pH 7.4 (Glauert, 1975). Freeze-fractured preparations were made as previously described (Vos *et al.*, 1976).

Isolation of 'ghosts'

'Ghosts' were prepared from the treated rat erythrocytes by using the hypo-osmotic lysis method of Hanahan & Ekholm (1974), except that EGTA (1 mM) was included in the first two of the four hypo-osmotic washes used to prepare the erythrocyte membranes. The presence of EGTA was necessary to prevent proteolysis of membrane proteins during the washing procedure. The 'ghost' preparation was resuspended in an equal volume of distilled water, and samples were immediately taken for gel electrophoresis and for the determination of total phosphorus as P_i (Baginski *et al.*, 1967).

Polyacrylamide-gel electrophoresis in sodium dodecyl sulphate

Samples were prepared for electrophoresis by the addition of an equal volume of a modified Laemmli's sample buffer, Tris/HCl (125 mM) at pH 6.8, containing sodium dodecyl sulphate (1.25%, w/v), dithiothreitol (40 mM), glycerol (12.5%, v/v), EDTA (2 mM) and Pyronin Y (100 µg/ml) (Laemmli, 1970), followed immediately by heating in a boiling-water

EXPLANATION OF PLATE 1

Morphological behaviour of rat erythrocytes on treatment with benzyl alcohol

Rate erythrocytes were incubated for 15 min at 37°C in a salt solution at pH 7.4, containing dextran (80 mg/ml) as described in the Materials and methods section. (a) Benzyl alcohol (100 mM) and Ca^{2+} (4 mM) were present during the incubation, and the cells fused into the giant cellular bodies shown. (b) Cell fusion by benzyl alcohol (100 mM) was markedly inhibited when EGTA (4 mM) was present in the absence of added Ca^{2+} ; cell aggregation was also inhibited. (c) Cells that were incubated with Ca^{2+} (4 mM) in the absence of benzyl alcohol did not fuse. In (d)–(f), benzyl alcohol (100 mM) and Ca^{2+} (4 mM) were present. (d) Cell fusion, but not cell aggregation, was inhibited by preincubation with Tos-Lys- CH_2Cl (1 mM). (e) Preincubation with Tos-Arg-OMe (2 mM) enhanced cell fusion. (f) Preincubation with *N*-ethylmaleimide (1 mM) inhibited cell fusion but not cell aggregation. Phase-contrast microscopy: magnification $\times 600$.

bath for 5 min. They were usually stored overnight at -15°C and subsequently reheated for 5 min at 100°C , and then cooled to room temperature before loading; $30\text{--}40\mu\text{l}$, containing $0.03\mu\text{mol}$ of total phosphate, was loaded per lane. Loading of equal amounts of total phosphate in erythrocyte-membrane preparations was preferred to the loading of equal protein contents, since (as indicated in the text) some of the membrane preparations studied had adsorbed cytosolic proteins.

Electrophoresis was undertaken in the buffer system of Laemmli (1970) by using slab gels. The electrophoresis apparatus was based on that of Reid & Bielecki (1968), but allowed two slab gels to be run simultaneously. The 3 mm-thick slabs consisted of a linear gradient of 3–15% (w/v) acrylamide for the separating gel, surmounted by a layer of agarose gel (1 cm) (0.8%, w/v) prepared in Tris/HCl buffer (125 mM) at pH 6.8 containing sodium dodecyl sulphate (0.1%, w/v) and EDTA (2 mM). Electrophoresis was conducted until the tracking dye reached the bottom of the slab gel.

The proteins were detected by staining the gels with Coomassie Blue, essentially as described by Fairbanks *et al.* (1971). Gels were photographed, or scanned with an SP.1809 scanning densitometer in an SP.1800 spectrophotometer (Pye Unicam, Cambridge, U.K.).

Results

Cell fusion and its inhibition

A high concentration of benzyl alcohol (100 mM) was required to induce cell fusion by comparison with other fusogenic lipids, e.g. oleoylglycerol, oleic acid and retinol ($350\text{--}400\mu\text{M}$) (Ahkong *et al.*, 1973). Like the other lipids, benzyl alcohol was haemolytic as well as being fusogenic, but, under the experimental conditions used here, cell fusion occurred before lysis. EGTA (4 mM) inhibited both cell fusion and cell lysis.

Plate 1(a) illustrates the giant cellular bodies formed in 15 min at 37°C by the fusion of rat erythrocytes treated with benzyl alcohol (100 mM) in the presence of Ca^{2+} (4 mM). The inhibitory effect on cell fusion of EGTA (4 mM) in the absence of Ca^{2+} is shown in Plate 1(b), from which it appears that even cell aggregation was minimal in the presence of EGTA. No fusion was observed with cells incubated with Ca^{2+} (4 mM) in the absence of benzyl alcohol (Plate 1c).

Tos-Lys- CH_2Cl (1 mM) was inhibitory (Plate 1d), but Tos-Phe- CH_2Cl (1 mM) permitted some benzyl alcohol-induced cell fusion to occur (result not shown). The behaviour of these two alkylating proteinase inhibitors thus differed from their effects on the fusion of human erythrocytes induced by oleoylglycerol, where Tos-Phe- CH_2Cl inhibited fusion but Tos-Lys- CH_2Cl did not (Quirk *et al.*, 1978). As with human erythrocytes fused by oleoylglycerol, the serine proteinase inhibitor phenylmethanesulphonyl fluoride (1 mM) had no effect on the fusion of rat erythrocytes by benzyl alcohol. Tos-Arg-OMe (2 mM), a substrate analogue for serine proteinases, actually facilitated the fusion of rat cells by benzyl alcohol (Plate 1e).

Plate 1(f) shows the marked inhibition of benzyl alcohol-induced fusion of rat erythrocytes that was observed in experiments with *N*-ethylmaleimide (1 mM). Iodoacetamide (10 mM) was also inhibitory.

Cell fusion was inhibited by pre-treatment with tetrathionate (10 mM). This reagent reacts reversibly with thiol groups (Deuticke *et al.*, 1978) and, by washing the cells and then incubating them with cysteine (5 mM) for 15 min at 37°C before adding benzyl alcohol, the inhibition was reversed. Inhibition by cystamine (10 mM) was similarly reversible, but not that by Tos-Lys- CH_2Cl , Tos-Phe- CH_2Cl or *N*-ethylmaleimide.

Membrane proteins: transglutaminase activity

Fig. 1 shows an electrophoresis slab gel that

EXPLANATION OF PLATE 2

Ultrastructural behaviour of rat erythrocyte membranes on treatment with benzyl alcohol

Electron micrographs of P-faces of freeze-fractured preparations of rat erythrocyte membranes that were fixed and prepared for electron microscopy as described in the Materials and methods section. (a) Cells were incubated for 70 min at 37°C in salt solution at pH 7.4, containing Ca^{2+} (4 mM) and dextran (80 mg/ml), and then fixed at 37°C . (b) Cells that were incubated for 15 min with benzyl alcohol (100 mM) and Ca^{2+} (4 mM) and then fixed at 37°C . (c) Cells that were treated as in (b), but were incubated for 40 min at 37°C before being fixed at 37°C , exhibited a loss of intramembranous particles. The 'plaques' seen represent E-faces of the membranes of adherent cells that may be in the process of fusion. (d) Cells were incubated for 15 min as in (b), but maintained at 0°C for 5 min before being fixed at 0°C ; the aggregation of the intramembranous particles is seen. (e) Cells were incubated for 60 min at 37°C with Ca^{2+} (4 mM) but without benzyl alcohol, maintained at 0°C for 10 min and then fixed at 0°C ; the intramembranous particles showed little decrease in number, and they were not aggregated. (f) Cells were incubated for 15 min at 37°C with benzyl alcohol (100 mM) and EGTA (4 mM) without added Ca^{2+} , maintained at 0°C for 10 min and then fixed at 0°C ; the loss of particles and their aggregation was inhibited by comparison with (d). The direction of shadowing, indicated by the arrowhead, was the same for (a)–(f).

illustrates the changes seen in the membrane proteins of rat erythrocytes which were incubated with benzyl alcohol (100 mM) and Ca^{2+} (4 mM) to induce cell fusion (lane *d*), by comparison with the protein pattern of cells incubated with Ca^{2+} (4 mM) alone (lane *k*). Erythrocytes incubated with benzyl alcohol and Ca^{2+} contained a polymeric material of high molecular weight that was unable to enter the gel (lane *d*). By contrast, in membranes from cells incubated with Ca^{2+} alone, the polymeric material was absent (lane *k*).

Human erythrocytes contain a transglutaminase that is activated by the entry of exogenous Ca^{2+} into the cytoplasm (Lorand *et al.*, 1979). The formation by this enzyme of a protein polymer in human erythrocytes is accompanied by the total disappearance of band 4.1 and a decrease in the quantities of protein in the spectrin and band-3 regions. Treatment of erythrocytes with benzyl alcohol allows Ca^{2+} to enter the cells (Blow *et al.*, 1979) and the protein pattern in Fig. 1 (lane *d*) is consistent with

the activation by exogenous Ca^{2+} of a comparable transglutaminase in rat erythrocytes. Thus the production of a high-molecular-weight polymer was accompanied by some diminution of the staining of the two bands that correspond to spectrin, and a loss of the protein (mol.wt. approx. 100 000) that appears to correspond to band 3. There was also decreased staining of a wide band (mol.wt. 70 000–80 000) that may correspond to the band-4 polypeptides in human cells.

Is the loss of band 3 (Fig. 1, lane *d*) in benzyl alcohol-treated cells to be attributed solely to the activity of a transglutaminase enzyme? That this is probably not so is indicated by experiments with histamine (20 mM), which behaves as an alternative substrate for the human transglutaminase (Lorand *et al.*, 1979). Lane (*j*) of Fig. 1 shows that, although the high-molecular-weight polymer was scarcely formed in the presence of histamine, benzyl alcohol and Ca^{2+} , a considerable loss of band 3 nevertheless still occurred. This was accompanied by the pro-

Fig. 1. Electrophoretic patterns of 'ghosts' prepared from rat erythrocytes

Rat erythrocytes were incubated and 'ghosts' then prepared and subjected to electrophoresis in a slab gel as described in the Materials and methods section. The 'ghosts' in the lanes (a)–(j) were from cells incubated with benzyl alcohol (100 mM); in lane (k) the material was from cells incubated with Ca^{2+} (4 mM) in the absence of benzyl alcohol. Ca^{2+} (4 mM) was present in all incubations except for that of lane (e), where benzyl alcohol (100 mM) and EGTA (4 mM) were present but Ca^{2+} was omitted. Other conditions for erythrocytes incubated with benzyl alcohol (100 mM) and Ca^{2+} (4 mM) were as follows: (a) preincubation with Tos-Lys- CH_2Cl (1 mM), (b) preincubation with Tos-Phe- CH_2Cl (1 mM), (c) preincubation with Tos-Arg-OMe (2 mM), (d) preincubation with buffer alone, (f) preincubation with tetrathionate (10 mM), (g) preincubation with *N*-ethylmaleimide (1 mM), (h) preincubation with iodoacetamide (10 mM), (i) preincubation with cystamine (10 mM), (j) preincubation with histamine (20 mM). All preincubations were for 10 min at 37°C; subsequent incubations were 15 min at 37°C. Arrows at the side of the Figure indicate the positions of molecular-weight markers, from top to bottom as follows: phosphorylase *a* (mol.wt. 94 000), bovine serum albumin (68 000), carbonic anhydrase (29 000) and cytochrome *c* (12 384).

duction of a diffuse band moving slightly faster than band 3. Since extensive cell fusion also occurred in the presence of 20mM-histamine, the transglutaminase activity thus seems unlikely either to be involved in cell fusion in this system, or to be entirely responsible for the observed changes in the band-3 region.

Proteinase activity

By contrast with the observations made with histamine, both the loss of band 3 and the production of the diffuse band moving ahead of band 3 seen in cells treated with benzyl alcohol and Ca^{2+} (Fig. 1, lane *d*) were prevented by Tos-Lys- CH_2Cl (1mM) (Fig. 1, lane *a*). This paralleled the inhibition of cell fusion by the inhibitor (Plate 1*d*). Tos-Phe- CH_2Cl (1mM) was less effective in preventing the changes in the band-3 region (Fig. 1, lane *b*), and it allowed some cell fusion to occur. When the effects of EGTA and Tos-Arg-OMe are compared, it is seen that the former completely inhibited the changes in the protein pattern (Fig. 1, lane *e*) and inhibited cell fusion (Plate 1*b*), whereas the latter did not inhibit the protein changes (Fig. 1, lane *c*) and actually enhanced cell fusion (Plate 1*e*). Phenylmethanesulphonyl fluoride (1mM), which did not inhibit cell fusion, was unable to prevent changes in the band-3 polypeptides (results not shown).

In addition to alterations in the band-3 region, a decrease in the intensity of a polypeptide in the region corresponding to band 2 in human erythrocytes was observed when rat erythrocytes were fused with benzyl alcohol and Ca^{2+} (Fig. 2*e*) and when fusion was enhanced by Tos-Arg-OMe (Fig. 2*f*), by comparison with cells treated with Ca^{2+} alone (Fig. 2*b*). This change was prevented by Tos-Lys- CH_2Cl (1mM) (Fig. 2*d*) and by EGTA (4mM) (Fig. 2*a*), but not by histamine (20mM) (Fig. 2*g*). The decrease in band 2 in the fused cells is therefore thought to have been due to proteolysis rather than to cross-linking by a transglutaminase.

N-Ethylmaleimide and other thiol reagents

The extensive inhibition of benzyl alcohol-induced cell fusion by *N*-ethylmaleimide (1mM) (Plate 1*f*) was paralleled by an apparently complete inhibition of the loss of band 3 (Fig. 1, lane *g*), the decrease in band 2 (Fig. 2*c*) and the production of diffuse material moving slightly faster than band 3. By contrast histamine (20mM), the alternative substrate for human transglutaminase, did not inhibit cell fusion or the changes in the band-2 and band-3 regions (Fig. 1, lane *j*; Fig. 2*g*). Thus, although the transglutaminase of human erythrocytes has a thiol group at its active centre (Brenner & Wold, 1978; Lorand *et al.*, 1979) and the rat enzyme is therefore likely to be inhibited by *N*-ethylmaleimide, it seems reasonable to conclude

Fig. 2. Densitometric scans of sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of 'ghosts' obtained from rat erythrocytes treated with benzyl alcohol

These scans were derived from the gels shown in Fig. 1. The preparations were obtained as described in the Materials and methods section. The bar in the absorbance scale of each scan represents an absorbance increment of 1.0. The 'ghosts' subjected to electrophoresis in (a) were from cells incubated with benzyl alcohol (100mM) and EGTA (4mM). (b) Cells were incubated with Ca^{2+} (4mM) in the absence of benzyl alcohol. (c) Cells were preincubated with *N*-ethylmaleimide (1mM), and then incubated with benzyl alcohol (100mM) and Ca^{2+} (4mM). (d) Cells were preincubated with Tos-Lys- CH_2Cl (1mM), and then incubated with benzyl alcohol (100mM) and Ca^{2+} (4mM). (e) Cells were incubated with benzyl alcohol (100mM) and Ca^{2+} (4mM). (f) Cells were preincubated with Tos-Arg-OMe, and then incubated with benzyl alcohol (100mM) and Ca^{2+} (4mM). (g) Cells were preincubated with histamine (20mM), and then incubated with benzyl alcohol (100mM) and Ca^{2+} (4mM). All preincubations were for 10 min at 37°C; subsequent incubations were 15 min at 37°C. Arrows at the top of the Figure indicate the positions of molecular-weight markers, from left to right as follows: phosphorylase *a* (mol.wt. 94 000), bovine serum albumin (68 000), carbonic anhydrase (29 000) and cytochrome *c* (12 384).

that *N*-ethylmaleimide is not merely acting to inhibit the transglutaminase, but is also behaving (like Tos-Lys-CH₂Cl) as an inhibitor of a thiol-proteinase.

Cystamine (10mM) (Fig. 1, lane *i*) was more effective than histamine (but less so than *N*-ethylmaleimide) in inhibiting changes in the band-3 region. This would be consistent with the fact that cystamine apparently inactivates thiol enzymes by disulphide exchange (Lorand *et al.*, 1979) as well as functioning as an alternative substrate for the transglutaminase. Tetrathionate (10mM) (Fig. 1, lane *f*) and iodoacetamide (10mM) (Fig. 1, lane *h*) also markedly inhibited the changes in the band-3 and band-4 regions. Cystamine, tetrathionate and iodoacetamide all inhibited the decrease in band 2 (results not shown).

Band 2 was decreased and a new band moving slightly faster than band 3 was formed when rat erythrocytes were fused directly by benzyl alcohol and Ca²⁺ (Fig. 2*e*), and on fusion by benzyl alcohol and Ca²⁺ after preincubation with Tos-Arg-OMe (Fig. 2*f*) or histamine (Fig. 2*g*). Conversely, when cell fusion was not induced (Fig. 2*b*) or was inhibited by EGTA (Fig. 2*a*), Tos-Lys-CH₂Cl (Fig. 2*d*), *N*-ethylmaleimide (Fig. 2*c*), cystamine, tetrathionate or iodoacetamide, neither of these changes occurred in the polypeptide patterns. Luna *et al.* (1979) have presented data on human erythrocytes which indicate that band 3' (a polypeptide remaining in the lower portion of band 3 after treatment of the cells with chymotrypsin) is proteolytically derived from the spectrin-binding protein, band 2.1. It therefore seems likely that the new diffuse material that migrated in front of band 3 in the present studies on rat erythrocytes was produced by the proteolytic degradation of polypeptide(s) in the band-2 region that may correspond to band 2.1 in human erythrocytes.

A notable feature of the polypeptide patterns from experiments in which *N*-ethylmaleimide and other thiol reagents were used was the presence of many additional bands in the gels. This phenomenon, which has also been seen by others with human erythrocytes (Allen & Cadman, 1976) and rat erythrocytes (Tanaka, 1978), may result from the binding of cytoplasmic proteins to erythrocyte membranes.

Intramembranous particles

Freeze-fractured preparations of the membranes of rat erythrocytes treated with benzyl alcohol (100mM) and Ca²⁺ (4mM) revealed that the appearance of the intramembranous particles of the protoplasmic (P) face was altered with increasing time of incubation at 37°C of the cells. Plate 2(*a*) shows the particles in the P-fracture face of cells incubated with Ca²⁺ alone for 70min at 37°C. This

may be compared with Plate 2(*b*), which illustrates the decreased uniformity in particle density observed in cells incubated for 15min at 37°C with benzyl alcohol and Ca²⁺ (corresponding to the cell fusion shown in Plate 1*a*). After 40min of incubation, extensive areas that were almost free of intramembranous particles were commonly seen (Plate 2*c*).

A similar decrease in the number of intramembranous particles was seen when human erythrocytes were incubated with exogenous subtilisin (Ahkong *et al.*, 1978). Also, as with human erythrocytes treated with subtilisin to induce cell fusion, treatment of rat erythrocytes with benzyl alcohol and Ca²⁺ for 15min at 37°C led to the particles on the P-fracture face becoming susceptible to aggregation when the cells were cooled to 0°C before freeze-fracturing (Plate 2*d*). Particle aggregation was not observed in cells that had been incubated with Ca²⁺ in the absence of benzyl alcohol (Plate 2*e*). Nor did it occur to any significant extent when the fusogenic action of benzyl alcohol was inhibited (cf. Plate 1*b*) by 4mM-EGTA (Plate 2*f*).

N-Ethylmaleimide and Tos-Lys-CH₂Cl also inhibited the loss of intramembranous particles, and the cold-induced aggregation of particles in the membranes of benzyl alcohol-treated cells. They were, however, less effective in these respects than EGTA.

Discussion

Benzyl alcohol has a relatively low partition coefficient for membranes (Colley *et al.*, 1971) and, by comparison with oleoylglycerol and retinol, a high aqueous concentration (approx. 100mM) is required to fuse erythrocytes (Fisher, 1975). However, benzyl alcohol, like other lipid-soluble fusogenic substances, has been shown by Blow *et al.* (1979) to increase the permeability of erythrocytes to Ca²⁺, and it appears that an increase in the concentration of intracellular Ca²⁺ initiates events leading to the chemically induced fusion of erythrocytes.

Previous studies on the fusion of human erythrocytes by oleoylglycerol indicated that a Ca²⁺-activated proteinase was involved (Quirk *et al.*, 1978), and this possibility is supported by the present work on rat erythrocytes treated with benzyl alcohol. The present studies additionally indicate that a Ca²⁺-activated thiol-proteinase is concerned, since cell fusion and the associated change in the band-3 region were prevented by *N*-ethylmaleimide and other thiol reagents. Conceivably, a similar enzyme may be involved in the fusion of hen and human erythrocytes induced by oleoylglycerol, as cell fusion in these systems was also inhibited by *N*-ethylmaleimide (Hart *et al.*, 1975). It is relevant to

note, in this connection, that Lenard (1970) has commented on the large degree of homology between the proteins of human and rat erythrocytes. The fact that the fusion of human erythrocytes by oleoylglycerol was inhibited by Tos-Phe-CH₂Cl, whereas fusion of rat cells induced by benzyl alcohol was inhibited by Tos-Lys-CH₂Cl, is not necessarily incompatible with the involvement of thiol-proteinases, since both of these compounds are known to inhibit papain by reacting with an active thiol group (Whitaker & Perez-Villasenor, 1968).

Our finding that histamine did not inhibit the fusion of rat erythrocytes induced by benzyl alcohol is compatible with the work of Palek *et al.* (1978), who have proposed that the cross-linking activity of the transglutaminase enzyme inhibits the fusion of ATP-replete human erythrocytes treated with ionophore A23187 and Ca²⁺. ATP-depleted human erythrocytes fuse with ionophore A23187 and 10mM-Ca²⁺, and in these cells the formation of a high-molecular-weight polymer is decreased by comparison with comparably treated fresh cells (Palek *et al.*, 1978), probably because the transglutaminase enzyme requires ATP as a stabilizing factor (Brenner & Wold, 1978).

Although the loss of band-3 protein in the present work was accompanied by the production of a diffuse band moving slightly ahead of band 3 in the membranes of cells fused with benzyl alcohol, it does not necessarily follow that the latter polypeptide was formed by the degradation of band 3. Indeed, as indicated above, it seems likely that it was derived from proteins in the band-2 region. This interpretation would be consistent with our previous finding that a loss of band 2.1 and the development of a sharp band at the front of band 3 occurred in human erythrocytes that were fused with oleoylglycerol (Quirk *et al.*, 1978); in retrospect, it would appear that the latter polypeptide was probably band 3' (cf. Luna *et al.*, 1979). Digestion of band 3 itself in human erythrocytes by Pronase and chymotrypsin gives rise to polypeptides with mol.wts. of 35 000 and 65 000, which are detectable by gel electrophoresis (Bender *et al.*, 1971; Cabantchik & Rothstein, 1974; Drickamer, 1976). It is noteworthy that the decrease in band 3 in the present work was accompanied by the formation of two small peaks with mol.wts. of approx. 30 000 (Figs. 2e, 2f and 2g), which may thus represent degradation products of band 3.

Shenstone (1978) has presented brief data which suggest that the membranes of human and hen erythrocytes contain a proteinase that is active only after treatment with thiol compounds. It is also pertinent that a Ca²⁺-dependent proteinase is present in human platelets, and that the activity of this enzyme towards endogenous platelet polypeptides is inhibited by iodoacetamide and by *N*-ethyl-

maleimide (Phillips & Jakabova, 1977). The platelet enzyme did not hydrolyse Tos-Arg-OMe. We have previously proposed that the proteolytic degradation of membrane proteins may conceivably be of general significance in membrane-fusion reactions, including secretion by exocytosis (Ahkong *et al.*, 1978). Interestingly, Phillips & Jakabova (1977) have commented that one possible function of the platelet enzyme might be in the secretory function of these cells, a process that may involve membrane fusion (cf. Henson *et al.*, 1978). Ca²⁺-activated thiol-proteinase activities have also been reported to occur in dog heart preparations (Mellgren, 1980), squid axoplasm (Pant *et al.*, 1979) and human skeletal muscle (Suzuki *et al.*, 1979).

It was initially suggested by Poste & Allison (1973) that the aggregation of membrane proteins is important in membrane fusion, including cell fusion. Subsequently it was proposed that membrane fusion proceeds by the intermingling of membrane lipids after the emergence of protein-free areas of lipid bilayer after protein aggregation (Ahkong *et al.*, 1975). Considerable circumstantial evidence has been documented in recent years in support of these two concepts (cf. Poste & Nicolson, 1978). The cold-induced clustering of intramembranous particles observed here cannot itself show that the hypotheses are correct, although it does demonstrate that the particles are free to move in the plasma membranes of cells that are undergoing fusion. The ability of the intramembranous particles to move and the altered behaviour of the membrane proteins on gel electrophoresis that are reported here also support our previous suggestion that the proteolytic degradation of membrane proteins may be of major importance in membrane-fusion reactions (Ahkong *et al.*, 1978). More recent work has indicated that in human erythrocytes band 2.1 links molecules of spectrin, at the cytoplasmic surface, to the major trans-membrane protein, band 3 (Bennett & Stenbuck, 1979). As losses of band 2.1 in human erythrocytes (Quirk *et al.*, 1978) and of material in the band-2 region in rat erythrocyte (together with the production of a polypeptide at or near the front of band 3) are associated with the fusion of these cells by lipid fusogens, it now seems likely that the degradation of spectrin-binding proteins in the band-2 region may be responsible for the increased freedom of intramembranous particles in erythrocytes treated with lipid-soluble fusogens. The accompanying proteolysis of band-3 proteins themselves in these cells may thus not be directly related to the cell fusion process.

It should, however, be recorded that we cannot at present exclude the possibility that the entry of Ca²⁺ into benzyl alcohol-treated cells, the degradation of membrane proteins, and the increased freedom of movement of the intramembranous particles may

conceivably accompany or perhaps even be consequences of cell fusion rather than being concerned in the initiation of the membrane-fusion reaction. Nevertheless it is noteworthy, in relation to our present observations, that the fusion of phagocytic vesicles with lysosomes in polymorphonuclear leucocytes is inhibited by *N*-ethylmaleimide and by *p*-chloromercuribenzoate (Amano *et al.*, 1979). Also, the fusion of Ehrlich ascites-tumour cells induced by Sendai virus is inhibited by proteinase inhibitors (Asano *et al.*, 1974), although Tos-Phe-CH₂Cl is more effective (62% inhibition) than antipain (18% inhibition) in this system.

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