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INTENSE, REVERSIBLE AGGREGATION OF INTRAMEMBRANE PARTICLES IN NON-HAEMOLYZED HUMAN ERYTHROCYTES

A FREEZE-FRACTURE STUDY

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Acridine orange, a strongly cationic, membrane-penetrating dye, caused intense aggregation of intramembrane particles in non-haemolyzed human erythrocytes at 5 mM concentration. Simultaneously with the particle aggregation, large, empty, intramembrane particle-free or particle-depleted vesicles were detached from the red cells. Washing the erythrocytes after Acridine orange treatment resulted in complete disaggregation of the intramembrane particles. Less cationic acridine dyes (9-aminoacridine, 5-aminoacridine and Quinacrine) caused much less conspicuous alterations. Rivanol (ethacrine lactate), on the one hand, caused intramembrane particle aggregation in human red cells as well as ribosome aggregation in rabbit reticulocytes when it was dissolved in lactate-buffered sucrose. On the other, Rivanol dissolved in phosphate-buffered saline failed to induce these alterations. Neuraminidase treatment had no effect on the intensity of Acridine orange-induced particle aggregation, but impeded disaggregation. Our results indicate that, in contrast to previous observations, intense and reversible clustering of intramembrane particles is certainly possible in non-haemolyzed erythrocytes. The intramembrane particle aggregation may be due primarily to perturbation of the inner red cell surface by strongly cationic dyes and the presence of sialic acid residues on the outer red cell surface seems to be essential for the reversibility of the process.

Introduction

It is generally accepted that intramembrane particles representing the main integral proteins of the erythrocyte membrane cannot aggregate significantly in intact, non-haemolyzed red cells [1–3]. A submembranous cytoskeletal meshwork composed of spectrin, actin and band 4.1 protein plays an important role in determining the shape and deformability of the erythrocytes and hinders the lateral motion of the intramembrane particles via spectrin-ankyrin-band 3 complexes [4,5]. Although both intramembrane particles and their sialic acid residues can aggregate at different rates in fresh as well as in spectrin-depleted ghosts [6–12], the conditions indicated to induce aggregation cannot be achieved in non-haemolyzed erythrocytes, because neither antispectrin IgG nor basic proteins can penetrate the plasma membrane. Moreover, red cells are haemolyzed by the non-physiological decrease in the intracellular pH that might induce intramembrane particle aggregation.

It would be of significance to known whether

Trivial names: Quinacrine, 6-chloro-9(4-diethylamino-1-methylbutylamino)-2-methoxyacridine; Rivanol, 6,9-diamino-2-ethoxyacridine.

intramembrane particles can be induced to cluster in red cells whose cytoskeletons are not modified by different ghosting procedures and the intracellular haemoglobin concentration is also maintained at the physiological level. Although some authors have already reported on intramembrane particle aggregation in red cells [13–15], truly convincing ultrastructural evidence of marked particle aggregation in non-haemolyzed erythrocytes has not yet been published.

In this paper we describe a simple, rapid and easily reproducible method using acridine dyes which leads to very significant and reversible intramembrane particle aggregation in non-haemolyzed human erythrocytes.

This work was presented in part at the Second International Congress on Cell Biology, Berlin (West), August 31–September 5, 1980 [16], at the XIth Conference on Membrane Transport, Sümeg (Hungary), May 12–15, 1981 [17] and at the Joint Congress of ISH-ISBT, Budapest (Hungary), August 1–7, 1982.

Materials and Methods

Acridine orange (Chroma), 9-aminoacridine hydrochloride (Serva), 5-aminoacridine hydrochloride (British Drug Houses), Quinacrine hydrochloride (Sigma), Rivanol (Serva) and Brillantcresyl blue (Merck) were dissolved in isotonic phosphate-buffered saline (pH 7.4). In addition to phosphate-buffered saline, Rivanol was also dissolved in isotonic sucrose buffered with 10 mM lactic acid/sodium hydroxide (pH 5.5). Solutions of 2-fold concentrations from 1.25 mM to 10 mM were prepared. The solutions were filtered and used immediately after preparation.

Freshly drawn heparinized human blood samples from healthy donors were washed three times in isotonic NaCl, then incubated in the dyes for 30 min at room temperature. The haematocrit of all suspensions was 0.05 l/l. In experiments using Rivanol dissolved in sucrose, red blood cells were also washed before incubation in lactate-buffered sucrose.

Since Acridine orange, Rivanol and Brillantcresyl blue caused agglutination of red blood cells, care was taken not to agitate the erythrocytes by pipetting during incubation. After spontaneous sedimentation, the cells were occasionally stirred gently with a thin glass rod. After 30 min, the incubating solutions were aspirated off the agglutinated red blood cells, leaving only a small portion of dye above the pellets. 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) was added to the pellets which were then dispersed into small red blood cell clusters in the fixative. After 30 min, the erythrocytes were washed several times in cacodylate buffer, then glycerinated and frozen in Freon 12 cooled by liquid nitrogen.

In other experiments, red blood cells incubated in dye were washed twice in phosphate-buffered saline (this resulted in disagglutination of the erythrocytes), fixed, washed and processed for freeze-fracture.

It is important to note that Brillantcresyl blue-treated erythrocytes remained dark blue even after very thorough washing. Moreover, ghosts prepared from Brillantcresyl blue or Acridine orange-treated, washed erythrocytes also preserved their blue or orange colour although they were washed many times and stored for days in a refrigerator (Lelkes et al., unpublished data). This indicates that some dye is irreversibly retained in the erythrocyte membrane.

In some experiments, red blood cells were incubated for 30 min in solutions of Acridine orange or Rivanol containing 25% glycerol. These erythrocytes were frozen in Freon 12 without washing and fixation.

Neuraminidase treatment was carried out according to Nicolson [8] with minor modifications. 0.5 ml of packed red cells were incubated in 9.5 ml Tris-maleate-buffered NaCl (pH 5.5) containing 0.1 U neuraminidase (from *Vibrio cholerae*; Behringwerke, Marburg). Neuraminidase-treated erythrocytes were washed twice in phosphate-buffered saline, then incubated for 30 min in 2.5 and 5 mM Acridine orange, respectively. Neuraminidase treatment prevented almost completely the Acridine orange-induced red cell agglutination. After 30 min, the cells were centrifuged, fixed with glutaraldehyde without washing or fixed after two washings in phosphate-buffered saline. The cells were then washed, glycerinated and freeze-fractured.

Freeze-fracture was carried out in a Balzers 510 type freeze-etch apparatus. Replicas were cleaned

with household bleach, washed in distilled water and mounted on uncoated 300 mesh grids.

Reticulocyte-rich rabbit red blood cell suspension was prepared as follows: 12–14 ml blood/kg was taken per day by venipuncture from rabbits for 4 days. On the seventh day the rabbits were bled by cardiac puncture. Heparinized blood was centrifuged and the cells were resuspended in serum in order to obtain an 85% suspension. This suspension was centrifuged for 30 min at $700 \times g$. In the upper layer, 80–90% of the cells were reticulocytes. Reticulocytes were washed in phos-

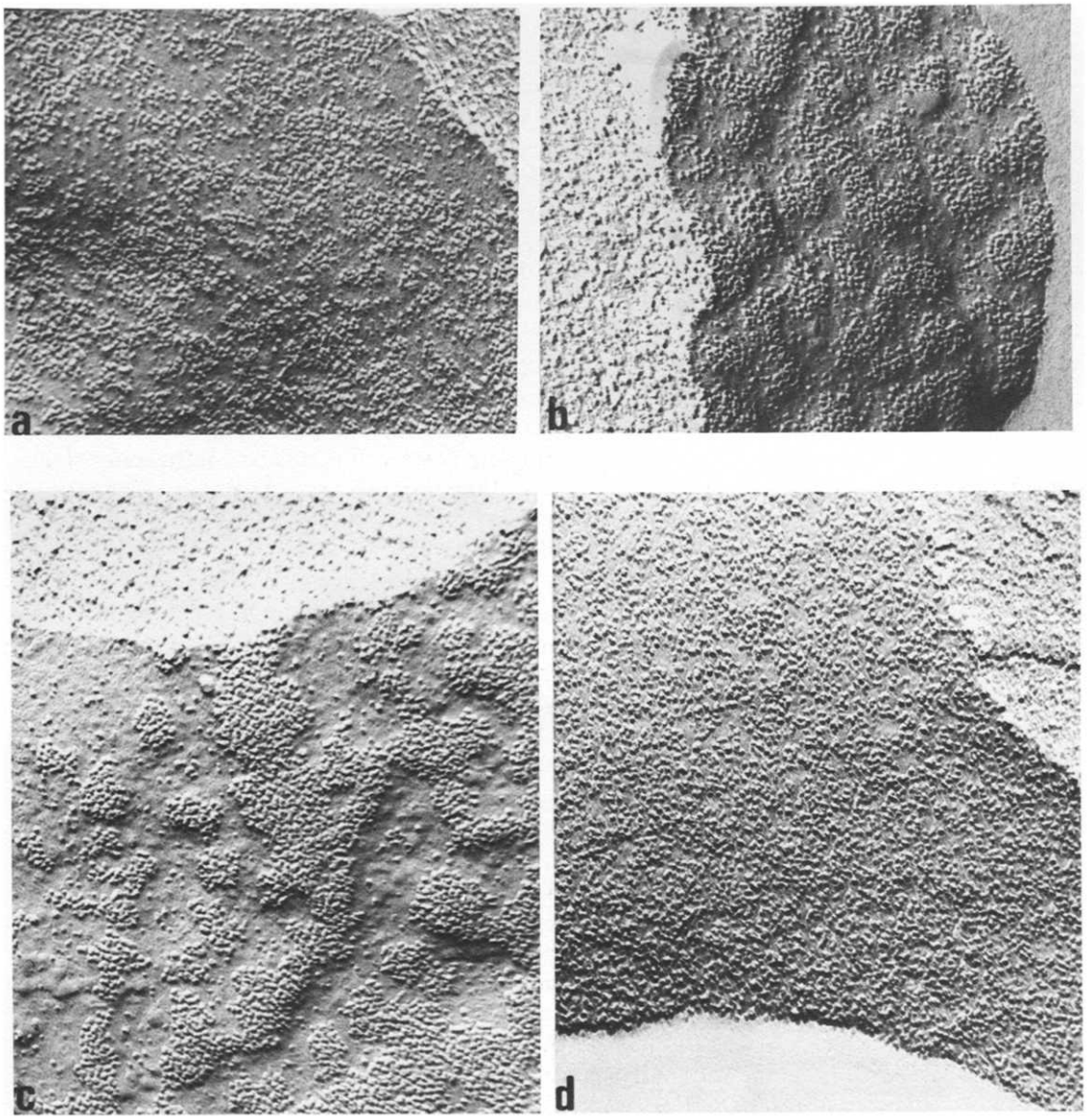

Fig. 1. Freeze-fracture electron micrographs of membranes of erythrocytes which had been treated for 30 min with Acridine orange and then were fixed in the presence of the dye (a, b, c) or after washing (d). (a) Moderate aggregation occurs in the presence of 2.5 mM Acridine orange; (b and c) 5 mM Acridine orange caused strong particle aggregation; (d) washing of erythrocytes after Acridine orange treatment resulted in complete disaggregation of the particles. In addition to P fracture faces, all micrographs show adjacent cytoplasmic areas revealing compact structure. Magnification, 80000×.

phate-buffered saline or lactate-buffered sucrose. Reticulocytes were incubated in 5–10 mM Acridine orange dissolved in phosphate-buffered saline and in 5–10 mM Rivanol in phosphate-buffered saline and in lactate-buffered sucrose, respectively. After 30 min, the cells were fixed with glutaraldehyde as described above, postfixed in 1% osmium tetroxide, stained en bloc with 0.5% aqueous uranyl acetate, dehydrated in graded ethanol and embedded in Spurr's low viscosity medium. Thin sections were made with an LKB Ultrotome III type ultramicrotome and stained with uranyl acetate and lead citrate.

Both replicas and sections were investigated in a Philips EM 300 electron microscope at 60 kV accelerating voltage.

Results

When intact human erythrocytes were incubated in solutions of different supravital dyes, profound alterations in the usual, random distribution of the intramembrane particles was observed. It was found that Acridine orange, a penetrating, strongly cationic dye, caused significant intramembrane particle aggregation. The aggregation began at about 2.5 mM Acridine orange concentration (Fig. 1a). It was intense at 5 mM (Figs 1b and 1c), while higher concentrations of Acridine orange (up to 10 mM) did not significantly enhance intramembrane particle aggregation seen in Fig. 1c, but more fracture faces showed this rate of aggregation.

While almost all fracture faces showed intramembrane particle aggregation in the presence of Acridine orange, washing the erythrocytes after Acridine orange treatment resulted in complete disaggregation of the intramembrane particles (Fig. 1d). Simultaneously with aggregation, large empty, particle-free or particle-depleted vesicles were detached from the red cells (Figs. 2a and 2b). The rate of aggregation and vesiculation was similar to or even higher than that which had formerly been found by Elgsaeter et al. [11] in erythrocyte ghosts.

Rivanol caused neither aggregation nor vesiculation in phosphate-buffered saline (Fig. 3a), while in lactate-buffered sucrose it was as effective as Acridine orange (Fig. 3b).

Among the less cationic acridine dyes, 5-aminoacridine, as well as 9-aminoacridine, caused much less conspicuous alterations (minimal aggregation at 5 mM concentration), while Quinacrine induced no aggregation at all.

Brillantcresyl blue was also less effective than Acridine orange: only higher concentrations of this dye (1%) caused intramembrane particle aggregation and vesiculation.

5–10 mM Acridine orange, as well as 5–10 mM Rivanol dissolved in lactate-buffered sucrose produced intense ribosome aggregation in rabbit reticulocytes (Fig. 4a), but Rivanol dissolved in phosphate-buffered saline failed to induce such alteration (Fig. 4b).

Neuraminidase treatment had no effect on the intensity of Acridine orange-induced particle aggregation but prevented disaggregation of the particles after washing (Figs. 5a and 5b).

In spite of the significant intramembrane particle aggregation induced by Acridine orange, neither partially nor completely haemolyzed forms were obtained when red cells were incubated in this dye (Figs 4a and 6). Under the experimental conditions employed by us, only discocyte → spherocyte transformation was seen (Fig. 6).

Discussion

The aggregation phenomenon of the intramembrane particles in erythrocyte ghosts has been extensively studied [6,7,10–12]. According to Elgsaeter et al. [10,11], the intramembrane particle aggregation induced by low pH, Ca^{2+} and basic proteins is primarily due to the precipitation of spectrin molecules attached to the cytoplasmic poles of intramembrane particles. In spectrin-depleted ghosts the aggregation is maximal, while in fresh, leaky ghosts the aggregation is limited and is accompanied by release of particle-free lipidic vesicles. The formation of these vesicles was explained by the compressive effect of the shrinking spectrin meshwork on the lipid bilayer. Other researchers [12] have also pointed out that low pH and Ca^{2+} may induce intramembrane particle aggregation in alkali-treated, stripped ghosts which are almost completely devoid of spectrin. The intramembrane particle clustering observed in these experiments could best be explained by lateral phase separation of anionic phospholipids induced by low pH and Ca^{2+}. Both studies imply that

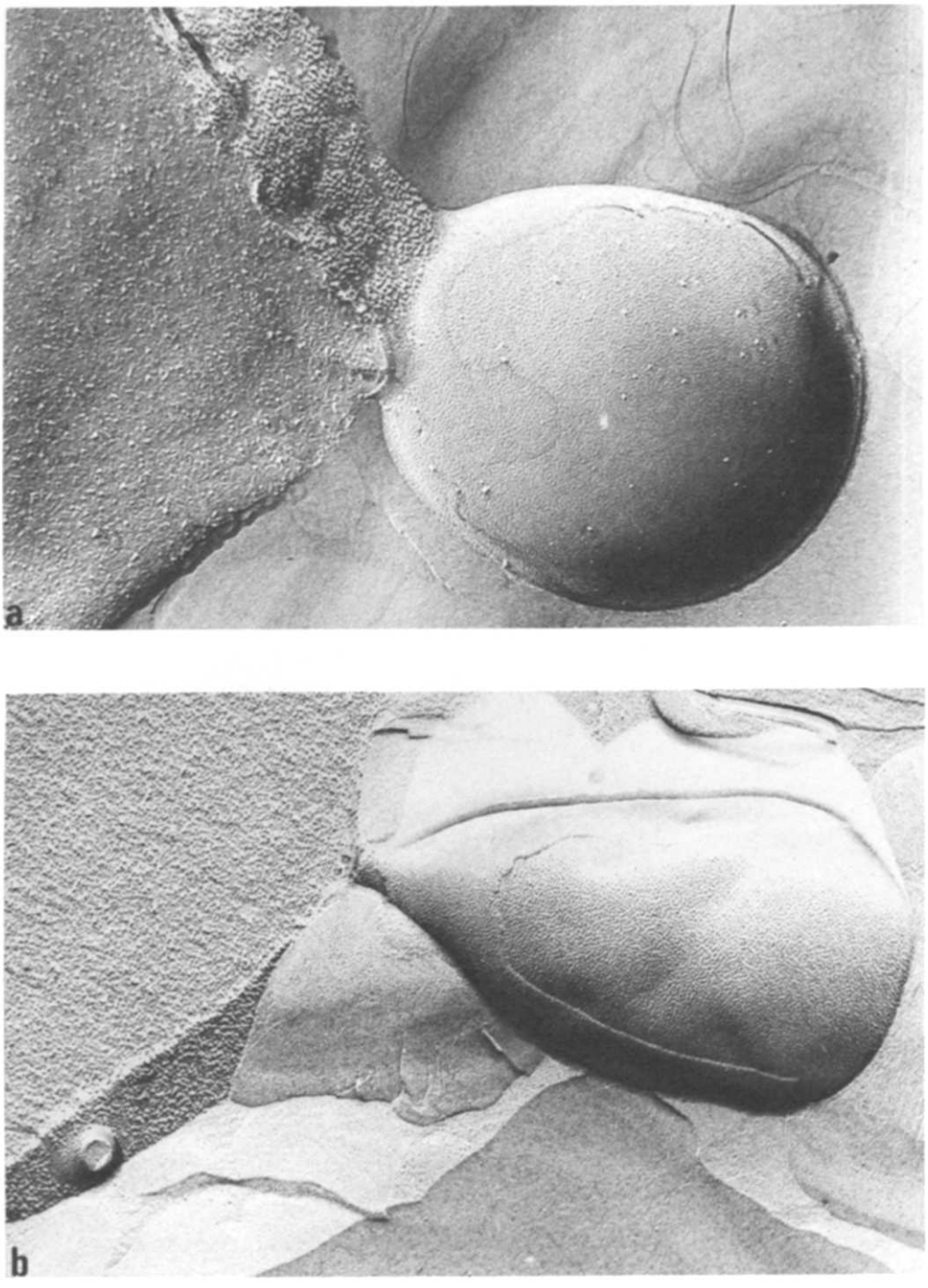

Fig. 2. Freeze-fracture electron micrographs of erythrocytes showing vesiculation after Acridine orange treatment. (a) Red cells had been incubated in 5 mM Acridine orange containing 25% glycerol and then were frozen without washing and fixation; observe the plasma membrane showing particle aggregation and the large vesicle strongly depleted of particles. (b) When red cells were washed after incubation in Acridine orange, the particle aggregation disappeared but a few vesicles remained attached to the erythrocytes. Unfixed, glycerinated sample. Note the compact structure of cytoplasm of both erythrocytes. Magnification, 54000×.

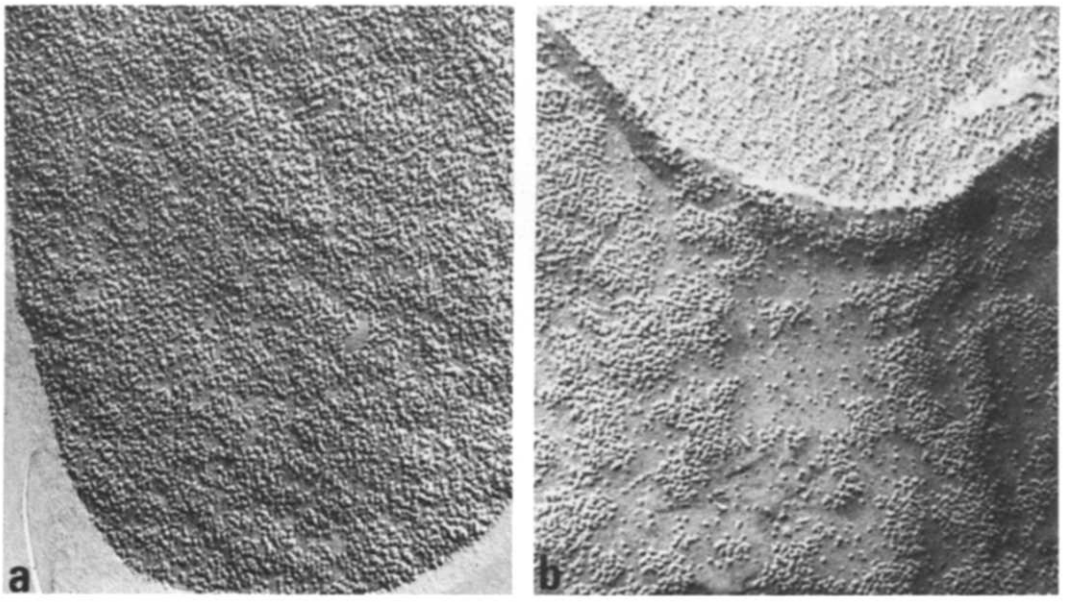

Fig. 3. Freeze-fracture electron micrographs of membranes of erythrocytes treated with 5 mM Rivanol. (a) No aggregation was induced when Rivanol was dissolved in phosphate-buffered saline; (b) Rivanol dissolved in lactate-buffered sucrose caused the same degree of aggregation as Acridine orange. Magnification, 80000×.

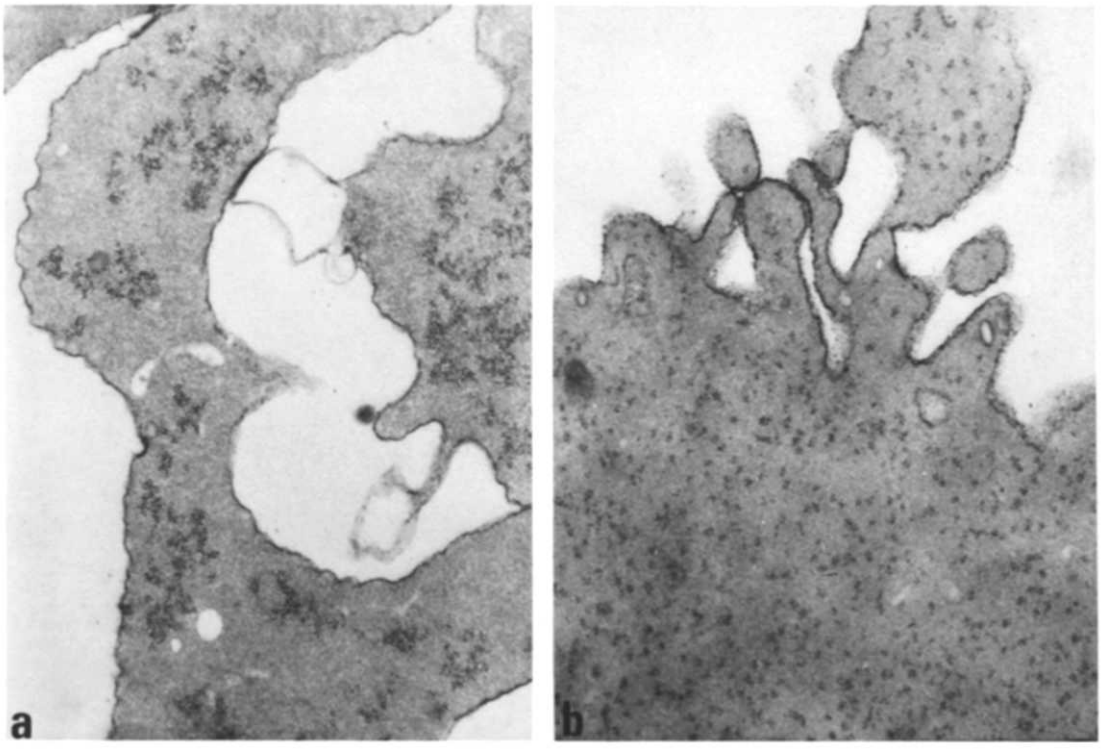

Fig. 4. Thin-section electron micrographs of rabbit reticulocytes incubated in acridine dyes for 30 min. (a) Ribosomes were strongly aggregated when reticulocytes were incubated in 5 mM Acridine orange, indicating that the dye penetrated the plasma membrane. Note the presence of haemoglobin-free vesicles. Similar results were obtained with Rivanol when it was dissolved in lactate-buffered sucrose. No sign of haemolysis can be seen. Magnification, 18000×. (b) When reticulocytes were incubated in 5 mM Rivanol dissolved in phosphate-buffered saline no ribosome aggregation was obtained. Magnification, 24000×.

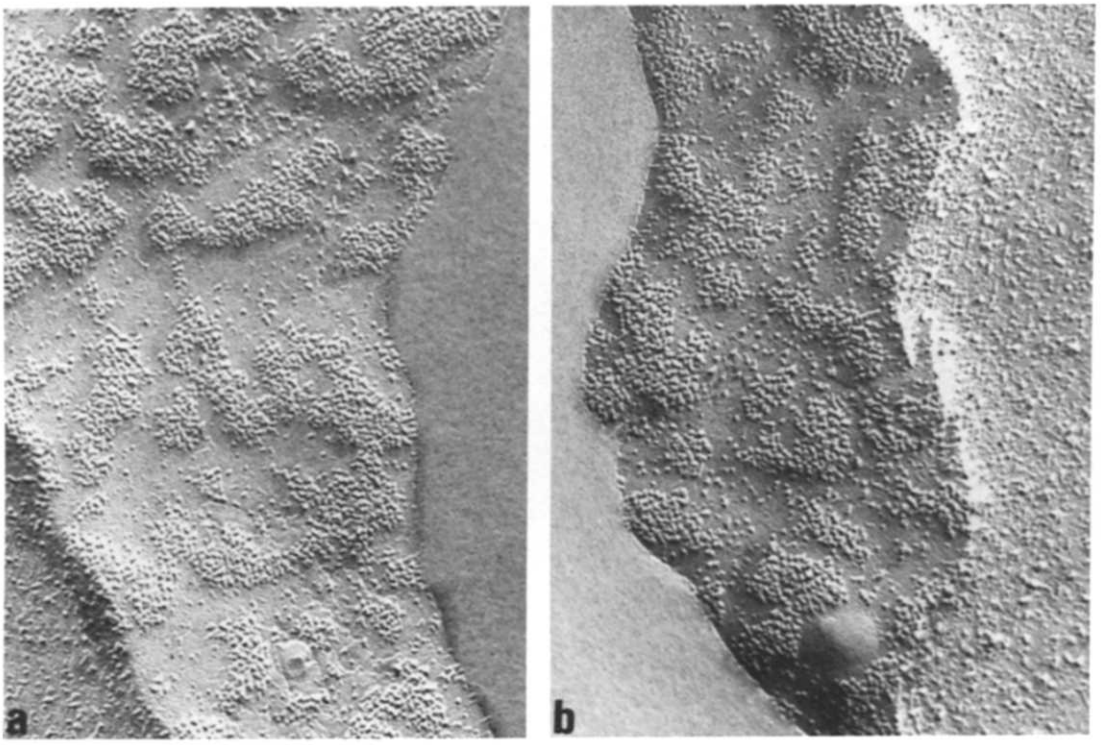

Fig. 5. Freeze-fracture electron micrographs of membranes of neuraminidase-treated erythrocytes which were incubated in 5 mM Acridine orange. (a) Red cells were fixed in the presence of the dye. The rate of aggregation is essentially the same as in erythrocytes not treated with the enzyme. (b) Red cells were fixed after washing. Note that neuraminidase treatment prevents particle disaggregation. Magnification, 80000×.

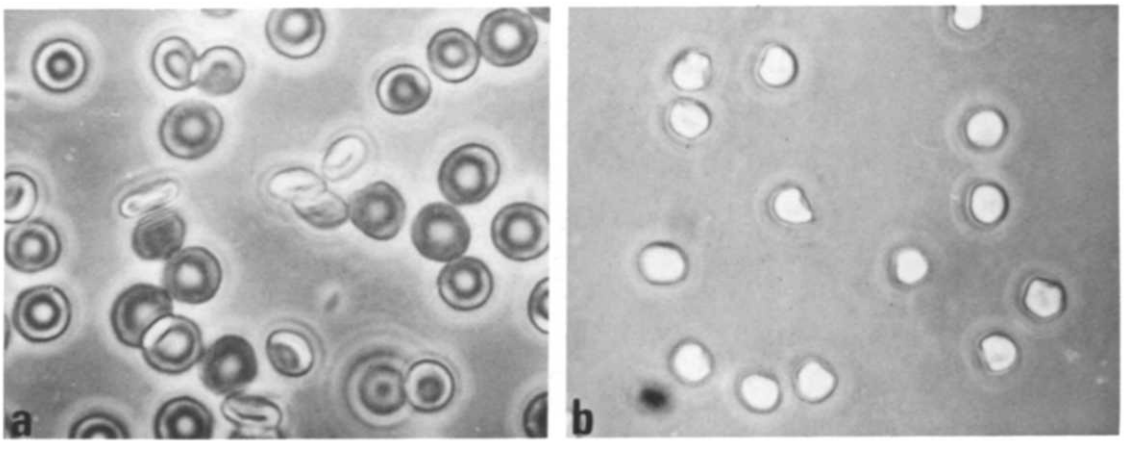

Fig. 6. Phase-contrast micrographs of control erythrocytes (a) and red cells incubated in 5 mM Acridine orange for 30 min (b). Note the discocyte → microspherocyte transformation and the absence of haemolyzed erythrocytes. Magnification, 800×.

physicochemical modification of the electrostatic properties of the inner red cell surface may lead to particle aggregation in ghosts.

The aim of our investigation was to explore whether modification of the inner surface of intact red cells might result in intramembrane particle aggregation without haemolysis. Since agents known to induce intramembrane particle aggregation in ghosts [11,12] cannot penetrate the intact red cell membrane, we tried to find such penetrating agents which could fulfil this modification. Most of the supravital dyes are cationic, readily penetrating compounds. Both Acridine orange and Brillantcresyl blue are capable of linkage to anionic intracellular structures such as ribosomes, thereby causing their aggregation [18,19]. The acridine derivates (mainly the Rivanol) have long been used for the precipitation of anionic plasma proteins [20]. The isoelectric point of spectrin is at pH 4.8 [11] and this is very near the isoelectric point of albumin, which is readily precipitable by Rivanol [21]. Since the local concentration of spectrin at the cytoplasmic surface is extremely high [4] and because the anionic phospholipids are preferentially localized in the inner leaflet of the bilayer [13], we assumed that penetrating acridine dyes might cause intramembrane particle aggregation by modifying the inner red cell surface at relatively low concentration without immediate haemolysis.

We have found, in fact, concentration-dependent and reversible intramembrane particle aggregation when intact red cells were incubated in Acridine orange (Fig. 1). At 5 mM concentration, this dye caused very significant intramembrane particle aggregation (Figs. 1b and 1c) which was similar to or even more intense than that which had formerly been found in fresh leaky ghosts after protamine treatment [11]. The vesiculation observed in our experiments (Fig. 2) was also identical with that induced in fresh ghosts [11].

Since penetrating, but less cationic derivatives (5-aminoacridine, 9-aminoacridine and Quinacrine) caused little or no intramembrane particle aggregation, we conclude that the aggregation observed is a function not only of the concentration but also of the basicity of the dye. The importance of permeation properties of the applied substances in inducing intramembrane particle aggregation could be demonstrated by experiments using Rivanol. Rivanol, which is a strong cation, showed different behaviour in phosphate-buffered saline and sucrose, respectively. In phosphate-buffered saline it had no effect on the distribution of the intramembrane particles (Fig. 3a), while in lactate-buffered sucrose it was as effective as Acridine orange (Fig. 3b). Furthermore, just as Acridine orange, Rivanol dissolved in sucrose is capable of aggregating ribosomes of rabbit reticulocytes (Fig. 4a), while in phosphate-buffered saline the ribosome aggregation was absent (Fig. 4b). This means that Rivanol dissolved in phosphate-buffered saline cannot penetrate the plasma membrane. This phenomenon can be explained by the fact that Rivanol forms macromolecular acridine chloride complexes with sodium chloride [21] and becomes insoluble in the course of time. Although we used freshly prepared solutions showing no precipitation, higher molecular weight Rivanol-chloride complexes must have been formed which behaved as a polycation having no aggregating effect on intracellular structures. It is known that removal of negative charges of the outer red cell surface by neuraminidase or their reduction by high ionic strength promotes the low pH induced intramembrane particle aggregation in spectrin-depleted ghosts [10]. Similarly, in our experiments, blocking of these negative charges by acridine dyes may have contributed to the intramembrane particle aggregation observed, although by itself it cannot bring about aggregation.

In summing up, we believe that the intramembrane particle aggregation observed can primarily be explained by perturbation of the inner red cell surface by strongly cationic dyes. We suppose that in this perturbation both the spectrin meshwork and anionic pohospholipids may be involved. Like basic proteins and divalent cations in leaky ghosts, strongly cationic, penetrating acridine dyes may cause spectrin precipitation and concomitant intramembrane particle aggregation in intact erythrocytes. On the other hand, although very little is known about the effect of acridine dyes on the physicochemical properties of phospholipids, it is possible that these dyes may – like low pH and Ca^{2+} – cause phase separation of anionic phospholipids, thereby causing particle aggregation. It is also conceivable that acridines may

stack anionic phospholipids by directly cross-linking their polar headgroups and the particles are excluded from these domains. To what extent perturbation of spectrin and/or anionic phospholipids of the inner leaflet may contribute to this aggregation should be elucidated by further experiments. The aggregating effect of Acridine orange on spectrin-depleted [10,11] and on spectrin-free, stripped ghosts, as well as its effect on the in vitro precipitation of purified spectrin will be the subject of a separate communication.

The reversibility of aggregation may be explained by the fact that linkage of supravital dyes to different anionic structures is a concentration-dependent and partly reversible process. Since removal of the dyes from the extracellular space initiates a new equilibrium state, it should result in release of dye from both the outer and inner membrane surface. The agglutination of erythrocytes observed during incubation with Acridine orange or Rivanol disappeared after washing, indicating that the outer red cell surface regained its net negative charge. Regaining of negative charges of the sialic acid residues seems to be essential for the reversibility of intramembrane particle aggregation, because neuraminidase treatment prevented particle disaggregation after washing (Fig. 5). Although a certain amount of dye seems to be irreversibly retained in the erythrocyte membrane, it is not sufficient to maintain particle aggregation, probably because of electrostatic repulsion between the negative charges of the sialic acid residues regained after washing.

In spite of the marked intramembrane particle aggregation observed after incubation with Acridine orange, no ghost forms were encountered under our experimental conditions (Figs. 4a and 6). Here it should be mentioned that Wickramasinghe et al. [22] also found no ghost forms by thin-section electron microscopy when they incubated normal human erythrocycytes in 1% Brillantcresyl blue for 2 h. In our experiments, low concentration of this supravital dye caused no or only insignificant intramembrane particle aggregation, but at higher concentration the aggregation was more significant, although we also found no haemolysis.

All these data indicate that, in contrast to previous observations, significant clustering of intramembrane particles is certainly possible in non-haemolyzed red cells. Although the mobility of intramembrane particles is really limited in intact erythrocytes (the vesicles are practically intramembrane particle-free) this limitation is less severe than was previously thought.

What may be the significance of these experiments?

First, this method makes possible the investigation of the susceptibility of intramembrane particles to aggregation in human red cell diseases. Our aggregating procedure using Acridine orange might be a useful supplementary means in the investigation of different qualitative (hereditary elliptocytosis and pyropoikilocytosis) and quantitative (hereditary spherocytosis with spectrin deficiency) cytoskeletal disorders [23,24].

Secondly, the intense intramembrane particle aggregation observed at 5 mM Acridine orange concentration (Fig. 1c) may allow investigation of the topographical relationship between intramembrane particles and different cell surface receptors by freeze-etching in non-haemolyzed erythrocytes. So far, this has been carried out in ghosts only, where the intramembrane particles were clustered by trypsin treatment and low-pH incubation [25–28]. This long and rather cumbersome method may be substituted by a rapid one in the following way. Incubation of red cells with ferritin-conjugated ligand followed by washing, then incubation in 5–10 mM Acridine orange for 30 min, fixation in the presence of the dye, washing in distilled water, freeze-etching. Since this method requires only some tenths of millilitres of blood, it might especially be useful for the investigation of red cells in such species where collection of blood for ghost preparation is difficult or impossible.

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References

1 Weinstein, R.S. (1974) in The Red Blood Cell (Surgenor, D.M., ed), pp. 213–268, Academic Press, New York
2 Steck, T.L. (1974) J. Cell Biol. 62, 1–19
3 Nicolson, G.L. (1976) Biochim. Biophys. Acta 457, 57–108

4 Lux, S.E. (1979) Semin. Hematol. 16, 21–51
5 Lux, S.E. (1979) Nature 281, 426–429
6 Speth, V., Wallach, D.F.H., Weidekamm, E. and Knüfermann, H. (1972) Biochim. Biophys. Acta 255, 386–394
7 Pinto da Silva, P. (1972) J. Cell Biol. 53, 777–787
8 Nicolson, G.L. (1973) J. Cell Biol. 57, 373–387
9 Nicolson, G.L. and Painter, R.G. (1973) J. Cell Biol. 59, 395–406
10 Elgsaeter, A. and Branton, D. (1974) J. Cell Biol. 63, 1018–1030
11 Elgsaeter, A., Shotton, D.M. and Branton, D. (1976) Biochim. Biophys. Acta 426, 101–122
12 Gerritsen, W.J., Verkleij, A.J. and Van Deenen, L.L.M. (1979) Biochim. Biophys. Acta 555, 26–41
13 Verkleij, A.J., Zwaal, R.F.A., Roelofsen, B., Confurius, P., Kastelijn, D. and van Deenen, L.L.M. (1973) Biochim. Biophys. Acta 323, 178–193
14 Wise, G.E., Shienvold, F.L. and Rubin, R.W. (1978) J. Cell Sci. 30, 63–76
15 Hui, S.W., Stewart, C.M., Carpenter, M.P. and Stewart, T. (1980) J. Cell Biol. 85, 283–291
16 Lelkes, G., Lelkes, G. and Hollán, S.R. (1980) Eur. J. Cell Biol. 22, 212 (abstr.)
17 Lelkes, G., Lelkes, Gy., Breuer, J., Pick, J. and Hollán, S.R. (1981) Haematologia 14, 446 (abstr.)
18 Kosenow, W. (1952) Acta Haematol. 7, 360–368
19 Bessis, M. and Breton-Gorius, J. (1964) Nouv. Rev. Fr. Hémat. 4, 77–94
20 Steinbuch, M. (1972) Vox Sang. 23, 92–106
21 Steinbuch, M. (1980) in Methods of Plasma Protein Fractination (Curling, J.M., ed.), pp. 34–56, Academic Press, New York
22 Wickramasinghe, S.N., Hughes, M., Higgs, D.R. and Weatherall, D.J. (1981) Clin. Lab. Haematol. 3, 51–60
23 Cohen, C.M. and Branton, D. (1981) Trends Biol. Sci. 6 (10) 25–27
24 Agre, P., Orringer, E.P. and Bennett, V. (1982) N. Engl. J. Med. 306, 1155–1161
25 Tillack, T.W., Scott, R.E. and Marchesi, V. (1972) J. Exp. Med. 135, 1209–1227
26 Pinto da Silva, P. and Nicolson, G.L. (1974) Biochim. Biophys. Acta 363, 311–319
27 Triche, T.J., Tillack, T.W. and Kornfeld, S. (1975) Biochim. Biophys. Acta 394, 540–549
28 Kuettner, C.A., Staehelin, L.A. and Gordon, J.A. (1976) Biochim. Biophys. Acta 448, 114–120