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ELECTRON MICROSCOPE STUDY OF THE DISRUPTION OF RED-CELL MEMBRANES

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SUMMARY

This paper reports the results of an electron microscope study of the disruption of red-cell ghosts in distilled water and in strong salt solutions as observed in negative contrast preparations. Proteins released from the membrane by these treatments include a fibrous component, which appears to play some part in maintaining the structural integrity of the intact ghost. Preliminary estimates indicate that 5–10 % of nonhaemoglobin protein is released from the membrane by washing twice in distilled water, and a further 10–20 % is released if the pellet of distilled-water-washed vesicles is resuspended in strong NaBr solution.

INTRODUCTION

Two different techniques[1,2] applied recently to solubilize red-cell membranes have given evidence for an actin-like membrane protein component, in support of the earlier suggestion of OHNISHI[3] that muscle-type proteins form part of the membrane structure. In the first of these methods[1], red-cell ghosts are dialysed against 50 mM 2-mercaptoethanol with 0.3 mM ATP present, and, in the second[2] the red cells are haemolysed in a 0.1 % solution of the detergent Triton X-100, washed in 0.02 M ammonium acetate and then dialysed against deionized distilled water. The first method yields fibres with fine structure very similar to that of actin in electron micrographs. For protein solubilized by the second method, the suggested similarity to actin is based on amino acid composition and precipitation properties.

In the present work, red-cell ghosts have been prepared in 0.01 M phosphate buffer, then simply washed in distilled water and their disruption studied by electron microscopy. Their disruption in strong salt solutions has also been studied. For biochemical work and protein fractionation, it is desirable to achieve as complete a solubilization of membrane components as possible. For electron microscopy, on the other hand, the fact that only partial solubilization is achieved is no disadvantage but allows the early stages of mild disruption to be studied.

We have shown in a previous paper[4] that negative contrast examination can cause extensive modification of structure in red-cell ghosts if the ghosts are not prefixed, and we shall show later in this paper that, for ghosts prepared in Tris buffers,

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even osmium fixation is not sufficient to prevent disruption of structure at the negative contrast preparative step. It must be borne in mind, therefore, that in this work a final potentially disruptive step of negative contrast preparation is added to the break-up already achieved by washing, *etc.* This further disruption is not entirely a disadvantage for the aim of the work is, in fact, to disrupt the membrane in a controlled way so that its component parts can be seen.

METHODS

Red-cell ghosts were prepared from fresh human blood or 14-day-old bank blood by a modification of the method of DODGE *et al.*[5]. The red cells were washed 3 times in isotonic phosphate buffer (pH 7.0) and haemolised in 0.02 M phosphate buffer (pH 7.6) with a ratio of packed cell-to-haemolysate volume of 1:4. After two washes in 0.02 M phosphate buffer, the ghosts were then washed three times in 0.01 M phosphate buffer. After each washing and spinning, the dark lower layer of the pellet was discarded. Other preparations were made using 0.01 M Tris buffer for the final two washes.

Negative contrast examination was made in 2% sodium phosphotungstate (pH 7), 2% ammonium molybdate (pH 7) or 2% uranyl acetate unbuffered (pH 4.5). Prior fixation, where used, was for 15 min in osmium fixation solution of PALADE[6] at pH 7.4. Protein was assayed by the method of LOWRY *et al.*[7] using bovine serum albumin as a standard, organic phosphorous by the method of ALLAN[8], and hexose by the method of WINZLER[9].

For examination in thin sections, the ghosts were spun down to a pellet and fixed in the Palade osmium fixation solution. Sections were stained with uranyl acetate.

RESULTS

As a starting point for study of disruption, we can say that ghosts prepared from fresh blood in phosphate buffers, prefixed in osmium, withstand the stress of negative contrast preparation whether in sodium phosphotungstate or unbuffered uranyl acetate and give the expected appearance of sacs approx. 8μ in diameter, thrown into folds as they dry down on to the support film[4]. Ghosts prepared from 14-day-old bank blood are rather more fragile, but almost all appear as unbroken sacs when prepared in this way.

Negative contrast viewing of unfixed ghosts

Unfixed ghosts viewed in sodium phosphotungstate show extensive stromalytic forms[4,10] and sometimes circular profiles 300 Å in diameter (Fig. 1). The negative-contrast preparations are normally made at room temperature, but if unfixed ghosts are prepared in sodium phosphotungstate at 37° they show a quite different and interesting effect (Fig. 2). Scattered particles and clusters of particles are seen, with particle diameter of approx. 90 Å and centre-to-centre spacing of approx. 110 Å in the clusters. Sometimes these particles appear as rings with a dark centre.

Break-up of ghosts in distilled water or strong salt solutions

If ghosts prepared in phosphate buffers are washed twice in distilled water and the supernatant wash solutions are pooled and viewed by negative contrast,

a fibrous component with threads 20–40 Å in diameter can be seen lying among particles, perhaps globular proteins, heterogeneous in size (diameters 50–200 Å) (Fig. 3). Similar fibres are seen in ghosts breaking up in strong salt solutions (1.2 M NaCl). Hexose estimations indicated that no polysaccharide is freed from the membranes by these treatments, but some protein is released (see below). As a check

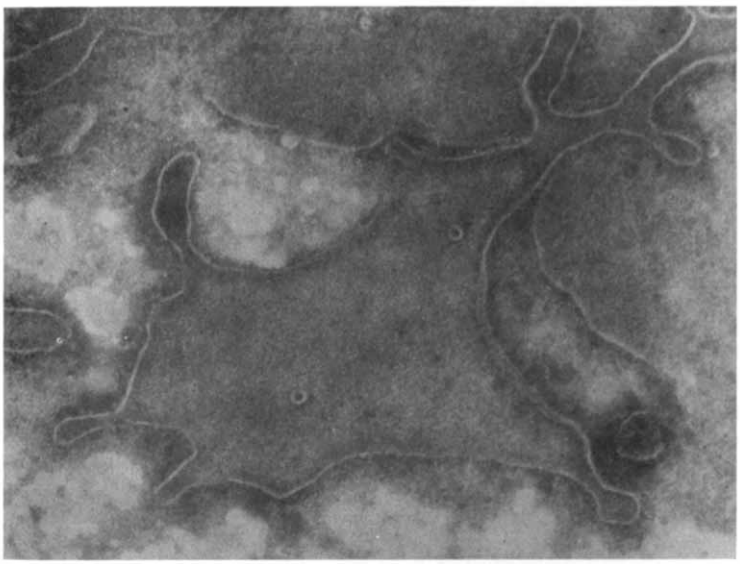

Fig. 1. Unfixed ghosts throwing out stromalytic forms during negative-contrast preparation (sodium phosphotungstate). Circular profiles are somtimes seen on the membrane approx. 300 Å in diameter. × 75000.

that the fibrous component was not fibrin or other protein spinning down with the ghosts during their preparation, the ghosts were sometimes spun up in $\rho = 1.20$ sucrose, then dialysed against 0.01 M phosphate buffer, prior to distilled-water washing. Glutaraldehyde fixation or addition of divalent ions to the wash solutions causes the fibres to aggregate into bundles (Fig. 4). The fibres sometimes appear beaded with a periodicity of 50–60 Å, but we have not observed any tendency for these fibres to twist in pairs to give the 'actin-like' form which MARCHESI AND PALADE[1,12] report for ghost fibrous protein released by mercaptoethanol or trypsin treatments[12].

On distilled-water washing, the ghosts break up into vesicles 0.1–1 μ in diameter. Ghosts break up in a similar way in 1.2 M NaCl. This disruption in distilled water or strong salt solutions can be clearly distinguished in electron micrographs from the break up of unfixed ghosts in negative-contrast viewing. In the latter case, provided there are only a few ghosts per grid square, the area occupied by fragments and vesicles from one ghost is separated by large clear spaces from other areas where other ghosts have fragmented. Fragmentation can then be assumed to be taking place during the drying of the negative stain. For fixed distilled-water vesicles on the other hand, well-preserved flattened sacs 0.1–1 μ in diameter are randomly distributed over the grid square.

A curious effect is quite often seen during the disruption of ghosts from 14-day-old bank blood in distilled water or sometimes for these ghosts in the 0.01 M

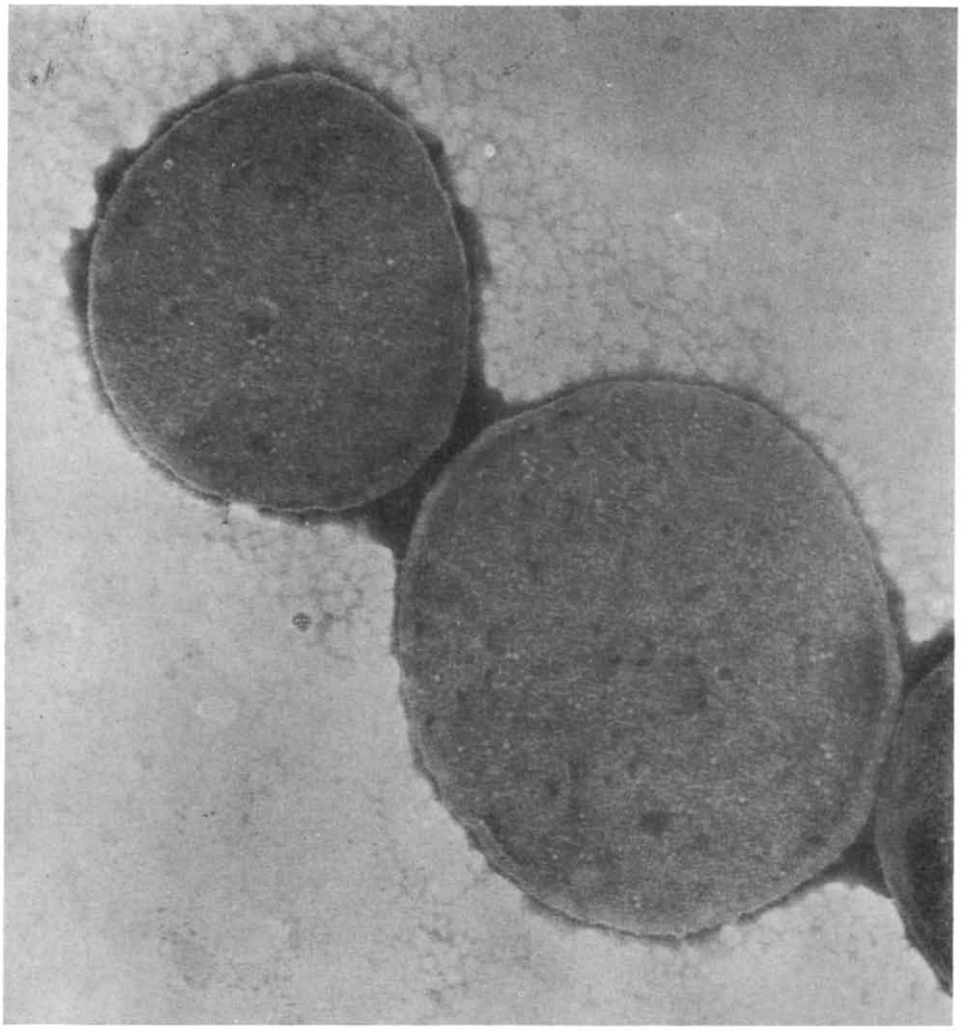

Fig. 2. Unfixed ghosts prepared in sodium phosphotungstate at 37°. The ghosts break up. (Vesicles in this micrograph are approx. 1 μ in diameter.) Particles are sometimes seen in clusters with centre-to-centre distance of approx. 110 Å. × 74000.

phosphate buffer (negative-contrast viewing was in this case supplemented by thin sectioning). The ghost membranes can apparently take up the cup shape that is induced in whole red cells by hypotonic solutions[13] and the edges of the cup can then perhaps fuse. By this, or some other mechanism, double-walled ghosts 5–6 μ in diameter are formed (*i. e.*, diameter = 8 $\mu/\sqrt{2}$) (Fig. 5). The double-walled ghosts then break up into smaller double-walled vesicles in distilled-water washing (Fig. 6).

Ghosts prepared in Tris buffer

In micrographs of thin sections, ghosts prepared in Tris buffers show a normal membrane profile[14]. However, in negative-contrast viewing there is a marked dif-

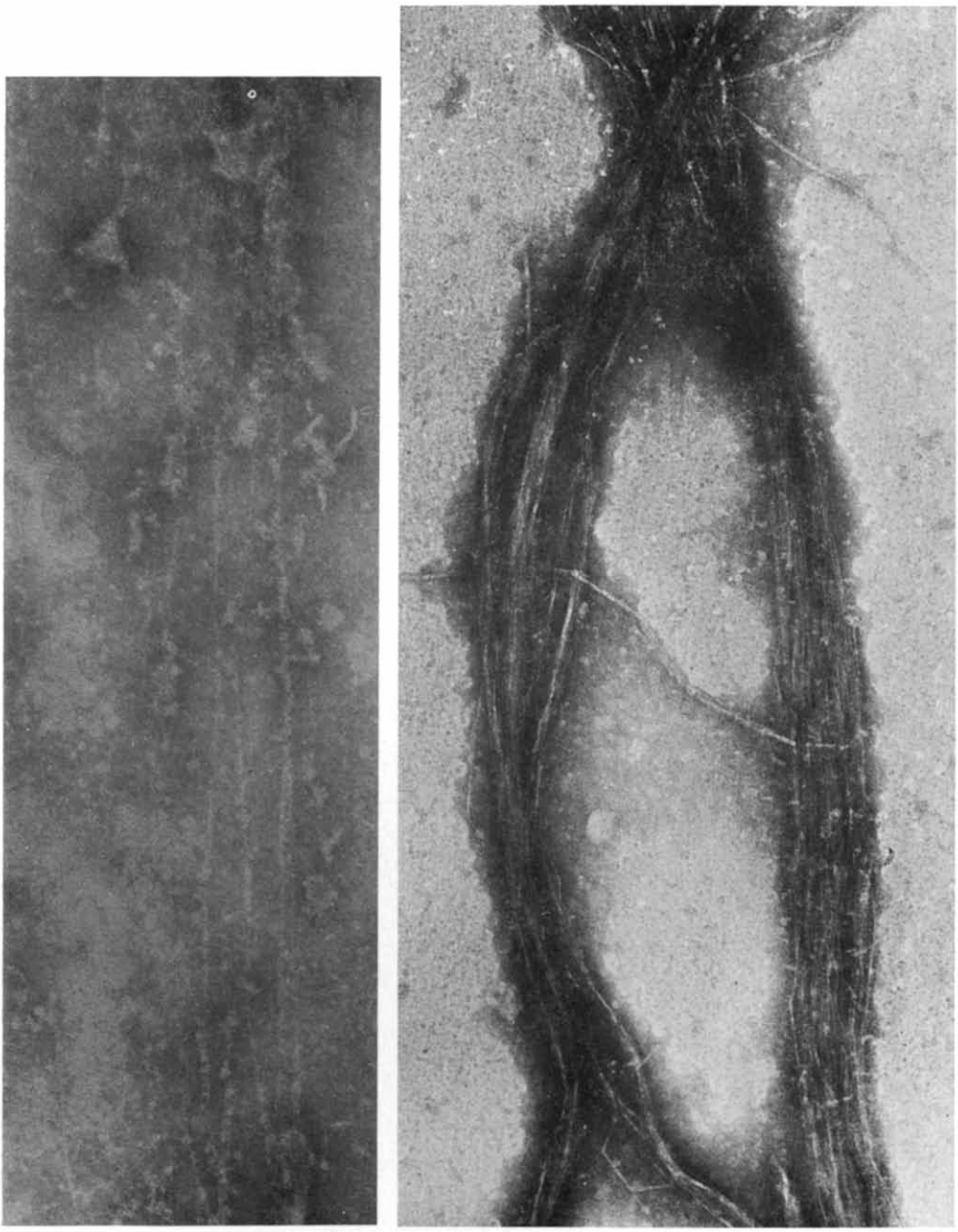

Fig. 3. Ghosts prepared from 14-day-old bank blood, spun up in $\rho = 1.21$ sucrose to remove any possible serum protein contamination, then dialysed against 0.01 M phosphate buffer and subsequently washed twice in distilled water. Supernatant of these two washes pooled, concentrated and viewed in this micrograph (sodium phosphotungstate). Fibrous proteins, apparently still attached in places to membrane fragments, lie among heterogeneous particles 50–200 Å in diameter × 120000.

Fig. 4. Supernatant fraction, after distilled water washing of ghosts and further standing for several days in 1.5 M NaCl at 4°. Fixed in 0.5% glutaraldehyde then viewed in sodium phosphotungstate. Similar fibre bundles are seen in the supernatant of distilled-water washing, without the further salt treatment, or if $MgCl_2$ is added to this supernatant fraction at 10 mM, without glutaraldehyde fixation. × 100000.

ference between ghosts prepared in Tris and phosphate buffers, particularly for ghosts prepared from 14-day-old bank blood. The Tris ghosts are more fragile, and osmium fixation is no longer adequate to protect the membrane from the stress of negative-contrast viewing in sodium phosphotungstate (Fig. 7). If the membranes were breaking up into the lace-work of Fig. 7 in the Tris washing or during osmium fixation (rather than as a result of the negative-contrast preparation) the effect would be detected in thin-section micrographs, particularly as the lace-work effect is often accompanied by the formation of stromalytic forms. It seems that ghost membranes remain morphologically intact in Tris buffers but are more fragile than ghosts in phosphate buffers. They are also much more permeable to ferritin[15].

Estimation of the amount of protein released by distilled-water washing and by strong salt solutions

In general, for any method used to break up membranes or release membrane proteins, chemical estimation of the amount of material solubilized is rather arbitrary and depends on the conditions of centrifugation used to spin down the fragmented membrane material. Proteins and lipids are considered to be 'solubilized' if they do not sediment, for example, at 35000 $\times g$ for 45 min[16] or 78000 $\times g$ for

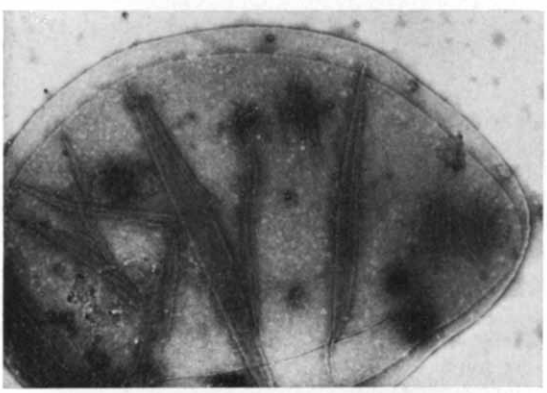

Fig. 5. Double-walled ghost (diameter 5–6 μ) in 0.01 M phosphate buffer in a preparation from 14-day-old bank blood. Osmium fixed then viewed in sodium phosphotungstate. × 10000.

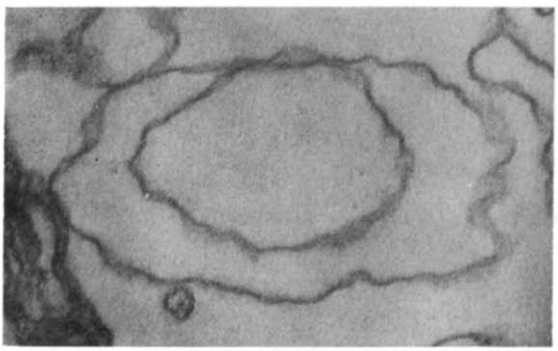

Fig. 6. Thin-section micrograph of double-walled ghost vesicle (diameter approx. 7000 Å) seen after distilled-water washing of ghosts from 14-day-old bank blood. × 75000.

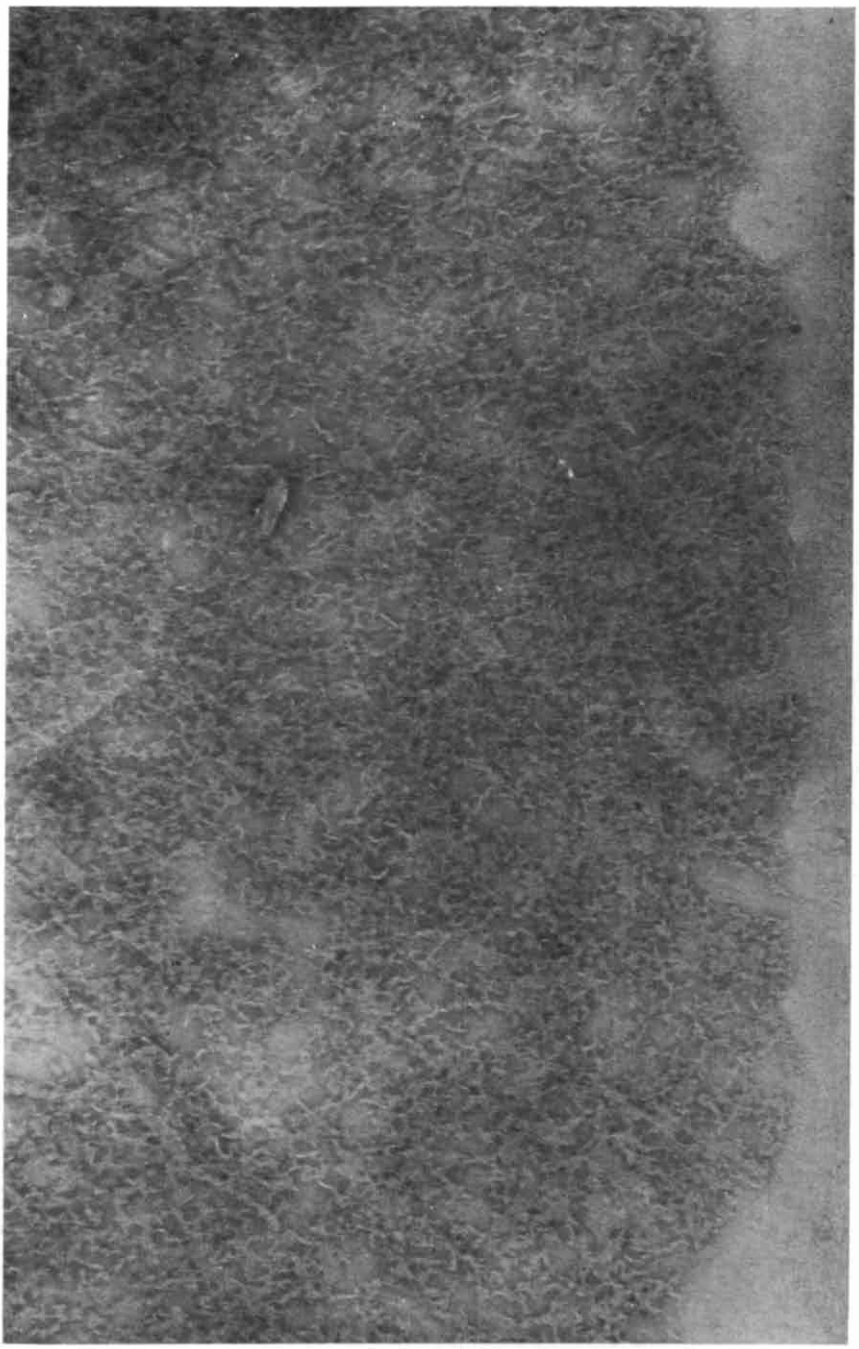

Fig. 7. Ghosts prepared with the final two washes in 0.01 M Tris buffer. Osmium fixation, then sodium phosphotungstate. Without the Tris washes the ghost membrane would appear structureless, but well preserved, after this treatment. After the Tris washes, osmium fixation no longer prevents the membrane breaking up into a lace-work, presumably during drying in sodium phosphotungstate. × 67 500.

2 h (ref. 1). In the present work, after two washes in distilled water (packed cell-to-wash volume ratio, 1:20) with the ghost vesicles spun down in each case at 45000 × *g* for 30 min, a certain amount of lipid was present in the pooled supernatant fraction. Electron microscope examination showed that small vesicles approx. 1000 Å in diameter were present in this fraction. Chemical estimations for the pooled wash supernatant solutions and for the pellet after distilled-water washing are given in Table I. This table also includes determinations made on the skin formed by adding NaBr to a resuspended pellet of distilled-water-washed ghost vesicles to bring the final density to $\rho = 1.21$ (approx. 2.5 M NaBr) and spinning for 15 h at 130000 × *g*. This gives an estimate of the amount of further protein released from the membranes by strong salt solutions. The bulk solutions after the NaBr spin, like the distilled-water-wash solutions, contains small vesicles of approx. 1000 Å in diameter.

Prior to chemical determination, the pooled supernatant solutions of distilled-water washing were concentrated by packing in dialysis bags in Aquacide 2 (Calbiochem. Co.) for 15 h at 4°. The haemoglobin content of the concentrated wash solutions (5–20 % of the protein present) was estimated from absorption measurements at 412 mμ and subtracted from the total protein present to give the protein value in Table I. All solutions set aside for chemical determinations were dialysed for 3 days at 4° against distilled water to remove inorganic phosphate.

To accurately determine the amount of protein freed from the membrane by these procedures, fractionation of the supernatant solutions of the distilled-water

TABLE I

PROTEIN AND PHOSPHOLIPID ESTIMATIONS

Values given per mg of phospholipid of ghosts in buffer.

Treatment of ghosts previously prepared in 0.01 M phosphate buffer	*Protein (mg) (not including haemoglobin)*	*Phospholipid (mg) (organic phosphate × 25)*
Distilled-water washing		
Ghost vesicle pellet after two washings in distilled water		
Fresh blood	1.47	0.87
14-day-old bank blood	1.37	0.89
	1.46	0.93
	1.37	0.96
Pooled supernatant of the two distilled-water washings		
Fresh blood	0.43	0.13
14-day-old bank blood	0.35	0.11
	0.22	0.07
	0.17	0.04
Further spinning in $\rho = 1.21$ NaBr		
Skin formed when pellet of above is spun up in $\rho = 1.21$ NaBr	0.87	0.76
(14-day-old bank blood)	0.88	0.86
Bulk solution and pellet of this $\rho = 1.21$ NaBr spin after removal of skin	0.38	0.13
	0.43	0.06

washing and the bulk solution of the NaBr spinning would be necessary, into protein and lipoprotein subfractions. A rough estimate can be made from the measurements of Table I if it is assumed that the small vesicles in the supernatant solutions after distilled-water washing have the same protein/phospholipid ratio as the larger vesicles which spin down into a pellet, and that the small vesicles in the bulk solution after the NaBr spin have the same protein to phospholipid ratio as the larger vesicles which form a surface skin. Treated in this way the results of Table I give an estimated 5–10% for the ghost protein released by distilled-water washing. The fibrous component forms only a part of this 5–10% (Fig. 3). Comparison of results for 14-day-old bank blood with those given in Table I for fresh blood and with earlier results by others[16] shows that the cells stored 14 days lose 10–15 % of their protein during ghost preparation. (The 14-day-old blood ghosts are also more fragile as noted above.) The amount of protein released by distilled-water washing is, however, much the same for both fresh and 14-day-old cells. The results of Table I give an estimate of 10–20 % (expressed as percentage of the protein of ghosts in 0.01 M phosphate buffer) for the protein released by the further treatment of suspension in strong salt solutions (2.5 M NaBr).

DISCUSSION AND CONCLUSIONS

Distilled-water washing and strong salt solutions may be expected to release proteins bound to the membrane by predominantly ionic bonds, while protein bound to lipid by predominantly hydrophobic bonding will remain with the main lipoprotein fabric of the membrane in these mild treatments. 15–30% of human red-cell-membrane protein appears to be held by predominantly ionic bonds, and this includes a fibrous protein essential for maintaining the stability of the 8 μ sac of the red-cell ghost, since its removal causes the ghost to break up into smaller vesicles. It is perhaps removal of this fibrous protein by distilled-water washing that allows the glycoproteins of the membrane to be solubilized by butanol in the treatment of MADDY[17]. It is not certain that the fibrous protein released by distilled-water washing (less than 10 % of the whole membrane protein) is identical with actin-like material yielded by haemolysis in Triton X-100, followed by distilled-water washing[2] or with fibrous protein released by mercaptoethanol[1] or trypsin[12] treatments. In fact, there seems to be a difference in the the fine structure of the distilled-water fibres, described in this paper, and the fibres released by mercaptoethanol and trypsin treatments, described by MARCHESI AND STEERS[1].

The membrane particles approx. 90 Å in diameter and approx. 110 Å centre-to-centre, seen in negative contrast preparations of the red-cell ghosts made at 37° (Fig. 2) sometimes appear to be arranged in limited areas of hexagonal array suggestive of the more extensive areas of hexagonally packed particles reported by BENEDETTI AND EMMELOT[18] for liver-cell membranes. BENEDETTI AND EMMELOT suggested originally that their particles might be globular micelles arising from a phase transition of the membrane lipid or lipoprotein from lamellar to micellar form at temperatures around 37°. For liver cells it is now considered more probable that they represent a surface component of the membrane, present at cell contact areas[19]. For the red cell also, the particles seen in 37° preparations might arise through a micellar phase transition but more probably represent a surface component, perhaps

a multi-enzyme complex. Mild treatment of red-cell ghosts yields, in solution, a hollow protein cylinder, 180 Å long and 110 Å in diameter, apparently made up of four rings[11] with each ring made up of ten subunits. The particles seen in the negative-contrast micrographs at 37° sometimes appear to have a dark centre and could be these cylinders seen end-on. It is not clear, however, if there are structures of this form and size in or on the membrane, why they are not seen in normal negative-contrast preparations at room temperature. A further interesting question is whether the negative-contrast particles at 37° correspond to the surface particles seen on the red-cell membrane in freeze-etch preparations[20].

The circular profiles seen in Fig. 1 remain puzzling. They could be holes in the membrane formed by artifactual rearrangement of the membrane components or as a result of breaking-off of a tubular stromalytic form.

In the disruption of Tris ghosts by negative-contrast preparation (Fig. 7), breaks appear to be forming at random in a continuous sheet. There is no indication of globular or micellar membrane substructure. It is not clear what type of structural membrane change makes ghosts prepared in Tris buffers more fragile and more permeable to ferritin than those prepared in phosphate buffers, but this might be a result of removal of divalent ions from the membrane by complexing with Tris during preparation of the ghosts.

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