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Release of a macromolecular protein component from human erythrocyte ghosts

The method to be outlined below depends upon the fragmentation of *intact* erythrocyte ghosts. (The term *intact* refers to the electron microscopic appearance, see below.) This fragmentation is done by the removal of the buffer in which they are suspended by dialysis against distilled water, or alternatively by the addition of sodium dodecyl sulphate to 0.1 mg/ml. Most previous attempts to disrupt or solubilize erythrocyte ghosts have employed more drastic treatments than these. Thus, MADDY[1], and REGA *et al.*[2], used a two-phase *n*-butanol–water system to solubilize over 90% of the ghost protein, as did MORGAN AND HANAHAN[3], by the use of an ultrasonicated single-phase 10% *n*-butanol–water system. A sodium dodecyl sulphate treatment (50 mg/ml) was used by BAKERMAN AND WASEMILLER[4], and a pyridine treatment (33%, by vol.) by BLUMENFELD[5]. A dialysis system using β-mercaptoethanol and ATP was developed by MARCHESI AND STEERS[6], which solubilized a protein component that could be polymerized to give actin-like fibrils, visible in the electron microscope by negative contrast staining.

Haemoglobin-free erythrocyte ghosts are prepared as follows from fresh or 14-day-old human whole blood, in acid citrate dextrose, by a modification of the method of DODGE, MITCHELL AND HANAHAN[7]. The cells are first washed 3 times with isotonic phosphate buffer (pH 7.0), the buffy coat being removed at each wash. Haemolysis is then performed by the addition of approx. 5 vol. of 0.01 M phosphate or Tris–HCl buffer (pH 7.4). The ghosts are then washed in this buffer until haemoglobin free. Occasionally it is found that the phosphate-washed ghosts remain pink. Two further washings with the Tris–HCl buffer will release the bound haemoglobin. Ghosts of creamy white colouration were usually obtained, but a slight haemoglobin contamination does not appear to alter the extraction.

At this stage *intact* ghosts can be seen in the electron microscope (A.E.I., E.M.6B) using the negative contrast staining technique. Using uranyl acetate (pH 4.5) as the negative stain it is not necessary to fix the ghosts, but with sodium phosphotungstate (pH 7.0) it is essential to fix the ghosts with osmic acid or glutaraldehyde prior to negative contrast staining. When preparing the ghosts from 14-day-old blood it is desirable not to use the Tris–HCl buffer, otherwise fragmentation of the ghosts occurs.

To release the material under discussion, the intact ghosts in 0.01 M phosphate or Tris–HCl buffer (pH 7.4) are dialysed 1–4 days against glass-distilled water at 4°. To prevent bacterial growth during the longer dialyses, the antibiotic tetracycline hydrochloride (approx. 10 μg/ml) was used, and removed by distilled water dialysis for 12 h, before the electron microscope study. Samples taken for viewing in the electron microscope show that the ghosts have undergone fragmentation, together with the release of a considerable amount of fine material. This material can be seen all over the background in Fig. 1, two types of profile being revealed. The first type consists of ring or torus forms, having an external diameter of approx. 110 Å and an inner diameter of approx. 50 Å. The other profile to be seen has a rectangular shape, with dimensions of approx. 110 Å and 180 Å. These rectangles appear to repre-

sent a stacked aggregate of four single-ring structures; thus, they are in fact cylindrical. In Fig. 1 all the single-ring structures are lying in a horizontal plane on the carbon backing film and the cylinders on their sides. In areas of deeper negative stain, the single rings can also be found lying in a vertical plane and in this position they show up as pairs of dots approx. 50 Å apart (see Fig. 2). Likewise, the cylindrical structures are occasionally seen lying vertically in the negative stain, showing up as clear rings with a dense central hole (see Figs. 3 and 4). (Theoretically both the single rings and cylinders could also lie in any position between the vertical and horizontal, in a deep pool of stain.) To summarize the main features of Figs. 1–4, two particles can be seen, a single ring structure and a cylindrical four-ring structure. Each structure has been described lying in a vertical and a horizontal plane, thus resulting in four profiles.

Very often the two outer rings of the cylindrical structures (lying horizontally) show up more clearly than the central pair. This may be a staining artifact, but may indicate that the inner two rings have a different composition to the outer rings. One can thus speculate why no groups of two rings are observed in the electron microscope. When the cylindrical structures break down, the central pair of rings

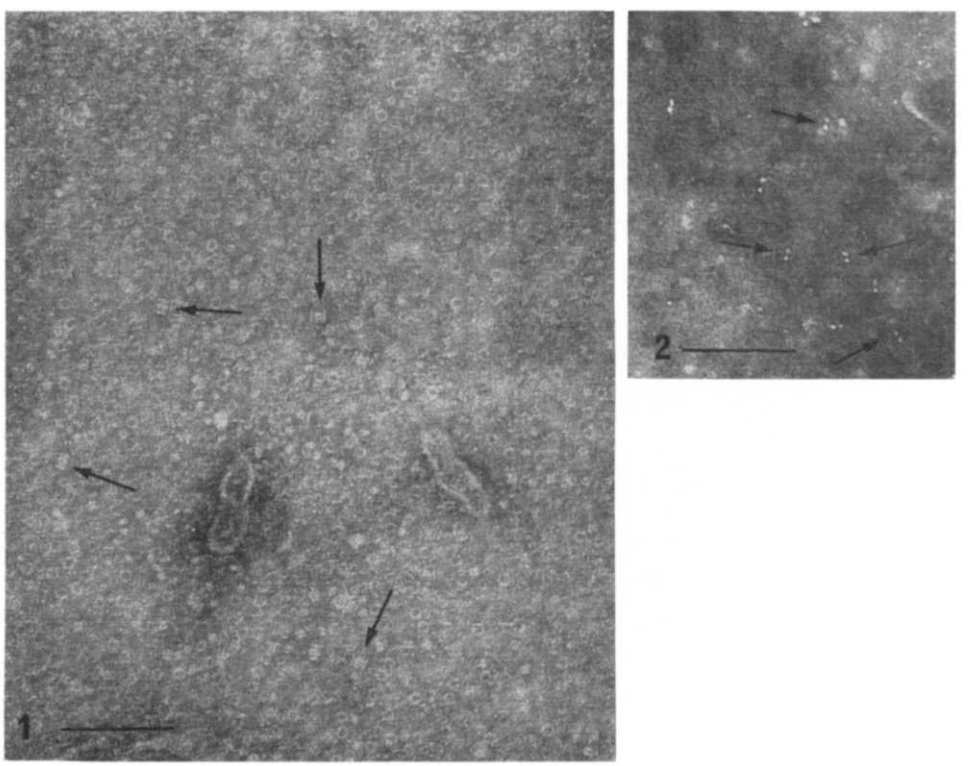


Fig. 1. Human erythrocyte ghosts after distilled water dialysis at 4° for 4 days. Negatively stained with 2% sodium phosphotungstate (pH 7.0). The line marker indicates 1000 Å. Arrows indicate the cylindrical structures. Membrane fragments and many single-ring structures are also visible.

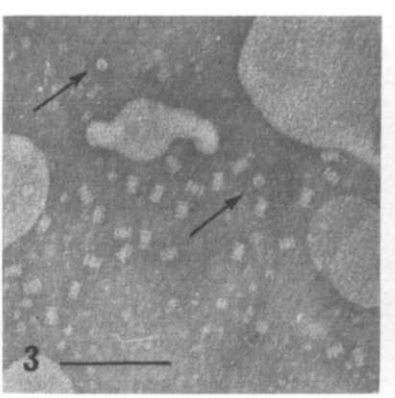
Fig. 2. Human erythrocyte ghosts after distilled water dialysis at 4° for 4 days. Negatively stained with 2% sodium phosphotungstate (pH 7.0). The line marker indicates 1000 Å. In this deep pool of negative stain, the single-ring structures are lying in a vertical plane, being revealed as paired white dots (arrowed).

may be destroyed, leaving intact only the outer rings which do not tend to pair together. The fact that no groups of three, five, or more rings are observed is also evidence supporting the hypothesis that the centre pair of rings in the cylindrical four-ring structures are different from the outer rings. Nevertheless, all four rings in the cylindrical structures may be identical, this aggregate of four and the single-ring structure being the stable configurations.

Many of the single-ring structures in Fig. 1 give an indication of being composed of subunits. It is proposed to apply the photographic rotation technique for contrast enhancement of MARKHAM, FREY AND HILLS[8] to this structure.

The alternative procedure for releasing the structures described above from *intact* ghosts, is to cause fragmentation by the addition of sodium dodecyl sulphate to 0.1 mg/ml. When this is done with ghosts from fresh blood only the cylindrical structures are found by electron microscopy (see Fig. 3). However, when ghosts from 14-day-old blood are treated in this manner both single-ring and the cylindrical four-ring structures are found. This, together with the observations from the water dialysis treatment, suggests that when the ghosts fragment, the cylindrical structures are released, and that these tend to break down to give the single-ring structures and possibly other material 'invisible' in the electron microscope. It must also be stressed that an aggregation of the more fundamental subunit composing the ring structures could occur after removal from the ghosts.

Thus, no suggestion can yet be put forward with conviction as to the possible location of the structures described in the *intact* ghost. If cylindrical structures of the dimensions quoted above are located on or in the outer surface of the *intact* ghost membranes, one might expect to see them by negative contrast staining, but this is not the case. On the other hand, if they are located on or in the inner surface, they would be unlikely to be observed in the electron microscope. It is possible that the



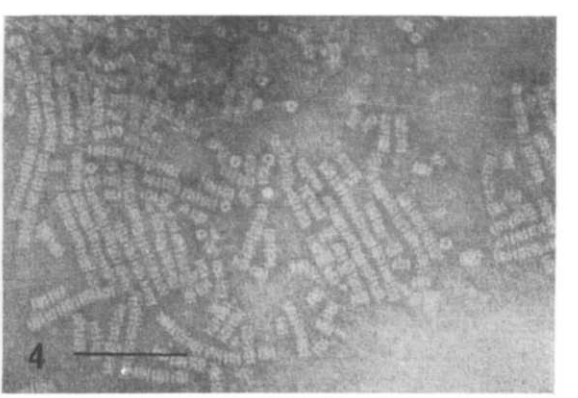


Fig. 3. Human erythrocyte ghosts from fresh blood after treatment with sodium dodecyl sulphate (0.1 mg/ml). Negatively stained with 2% sodium phosphotungstate (pH 7.0). The line marker indicates 1000 Å. Membrane fragments and cylindrical (four-ring) structures are visible. Arrows indicate cylinders lying in a vertical plane.

Fig. 4. Ox erythrocyte ghosts after distilled water dialysis at 4° for 2 days. Negatively stained with 2% sodium phosphotungstate (pH 7.0). The line marker indicates 1000 Å. Many cylindrical (four-ring) structures are visible, lying individually and in long columns, being arranged in this latter array by the forces of drying. (Membrane fragments are also present, but not included in this print.)

structures presented do not represent a membrane component and that they are intracellular structures that have been bound by the membrane during haemolysis and the subsequent washings.

Work is in progress regarding the purification and characterisation of this extractable material. Preliminary results indicate it is of protein composition and may represent up to 5 % of the protein of the *intact* ghost. Two preparations of ox erythrocyte ghosts have revealed a very similar release of material when fragmented by either of the treatments described (see Fig. 4). The significance of the results presented cannot be fully discussed at the present stage of the investigation. But it would appear that, as the material is so readily released not only by sodium dodecyl sulphate but by the mild treatment of water dialysis, it is unlikely that an artifact is being created.

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1 A. H. MADDY, *Biochim. Biophys. Acta*, 117 (1966) 193.
2 A. F. REGA, R. I. WEED, C. F. REED, G. G. BERG AND A. ROTHSTEIN, *Biochim. Biophys. Acta*, 147 (1967) 297.
3 E. MORGAN AND D. J. HANAHAN, *Biochemistry*, 5 (1966) 1050.
4 S. BAKERMAN AND G. WASEMILLER, *Biochemistry*, 6 (1967) 1100.
5 O. O. BLUMENFELD, *Biochem. Biophys. Res. Commun.*, 30 (1968) 200.
6 V. T. MARCHESI AND E. STEERS, JR., *Science*, 159 (1968) 203.
7 J. T. DODGE, C. MITCHELL AND D. J. HANAHAN, *Arch. Biochem. Biophys.*, 100 (1963) 119.
8 R. MARKHAM, S. FREY AND G. J. HILLS, *Virology*, 20 (1963) 88.

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The activation by leucine of ouabain-sensitive ATPase of HeLa cells

Cell membrane ATPase which is Mg^{2+} dependent and (Na^+-K^+) activated is believed to be the carrier enzyme for the active transport of Na^+ and K^+ at cell surface (*cf.* ref. 1). It has been suggested that this kind of ATPase contributes to amino acid transport in ascites tumor cells[2] and L cells[3] on the basis of the findings that glycine uptake in tumor cells was greatly decreased by ouabain, as in the case of cation transport, and that certain Na^+ concentrations in the medium are capable of accelerating the inward transport of α-aminoisobutyric acid in L cells. The authors have noted that the penetration of glycine and leucine into HeLa cells is greatly inhibited by 10^{-5} M ouabain, while on the other hand, HeLa cells have been fractionated into specifically (Na^+-K^+)-activated and also ouabain-sensitive ATPase. In the present experiments, two ATPase preparations from HeLa cells were examined for activation of the enzyme activity with amino acids.

HeLa cells were cultivated in the glass flask as monolayers with medium consisting of 90 % (v/v) Hanks balanced salt solution, 10 % (v/v) inactivated bovine serum and 0.4 % (w/v) lactalbumin hydrolysate (NBC).