in these membranes and that the molecules are free to come closer together as the diglyceride coalesces into droplets. It is informative to apply this hypothesis to the vesicles of the sarcoplasmic reticulum, in which about 80% of the protein appears in negative stain as an irregular array of short rods of diameter 35 Å, projecting 50 Å from the surface (Greaser, Cassens, Hoeckstra & Briskey, 1969). It may be calculated that the observed loss of 70% of the phosphatidylcholine and half the area (Finean & Martonosi, 1965) would just bring these subunits into contact if they are embedded in the outer half of the lipid bilayer, as suggested by results of freeze-etching (Bertaud, Rayns & Simpson, 1970). Examination of freeze-etched specimens of influenza virus has also suggested a structure in which the projecting protein antigens are embedded in the outer half of the lipid bilayer (Bächi, Gerhard, Lindenmann & Mühlethaler, 1969).

A further striking example of the fluidity of membrane structure has appeared in a paper on the surface antigens of hybrid cells (Frye & Edidin, 1970). Cell lines of mouse and human origin were fused and the hybrids were treated with fluorescent antibodies (red for human and green for mouse antigens). At 10 min after fusion most hybrids showed distinct red and green halves, but within 40 min the colours were completely mixed. The process was not affected by inhibitors of protein synthesis or oxidative phosphorylation, but it ceased dramatically at temperatures below 15°C. It was calculated that this high rate of diffusion was consistent with the movement of molecules of diameter 200 Å in a medium of the viscosity of castor oil.

These arguments suggest that the organization of protein molecules in the plane of the lipid bilayer is governed relatively loosely by hydrophobic interactions. The organization in a perpendicular direction is likely to be dominated by more specific protein-protein interactions in an aqueous environment. A combination of electron microscopy with chemical or immunological labelling methods should enable one to decide where particular proteins are located. They may for example be on the inner or the outer surface of a vesicle, confined within the bilayer, or they may penetrate from one aqueous surface to the other. So far, negative staining has shown many examples of proteins that project from one surface or another of a membrane, while the ready cleavage of the bilayer along the central hydrophobic plane in freeze-fractured preparations suggests that proteins that pass through the membrane are relatively rare (da Silva & Branton, 1970).

Whatever the nature of the interactions between proteins on the membrane and proteins in the

membrane, it can be said that the observations discussed in this brief survey are inconsistent both with any major role for so called membrane structural proteins and with any network of proteins of the type visualized by Changeux (1969) as allosteric mediators of responses in excitable membranes. If there is any substance in this theory then excitable membranes must be built on rather different principles.

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The Proteins Released from Intact Erythrocyte 'Ghosts' at low Ionic Strength

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The methods currently in use for the preparation of haemoglobin-free mammalian erythrocyte 'ghosts' by different research groups vary considerably. This may explain to some extent the overall lack of agreement and co-ordination that exists in the field of erythrocyte membrane proteins. Even though haemoglobin-free erythrocyte 'ghosts' can reasonably be considered as a homogeneous membrane preparation, there can be little doubt that every research group is dealing with a slightly different product from which their protein extracts are made. The preparation method employed by the present author closely follows the procedure developed by Dodge, Mitchell & Hanahan

(1963). The incorporation of a single wash with tris-HCl buffer has been found to aid the release of haemoglobin from human erythrocyte 'ghosts', but phosphate buffer alone has been found successful for the preparation of bovine erythrocyte 'ghosts'. These 'ghosts' are intact as judged by phase-contrast and electron microscopy, though the bovine 'ghosts' do tend to show some stromalytic forms (Burger, Fujii & Hanahan, 1968).

In a series of publications the present author has developed a method for the release of several proteins from haemoglobin-free erythrocyte 'ghosts' by causing membrane fragmentation to take place as a result of exhaustive dialysis against distilled water followed by freeze-thawing (Harris, 1968, 1969*a*, 1971). Two of these proteins have been characterized by gel electrophoresis, ultracentrifugation and electron microscopy. A molecule with a quaternary conformation resembling a hollow cylinder, approx. 170 Å long, 125 Å external diameter and 40 Å internal diameter, with a sedimentation coefficient of 22.5S, has been isolated from both human and bovine erythrocyte 'ghosts'. Another component has a quaternary conformation resembling a torus or ring, approx. 130 Å external diameter, 60 Å internal diameter and 40 Å thick, and has a sedimentation coefficient of 9.0S. This second protein, which photographic rotation suggests has ten subunits (Harris, 1969*b*), has only been isolated from human erythrocyte 'ghosts'. At the moment it is not possible to say whether or not the single-torus protein is a dissociated form of the hollow-cylinder protein, though the electron-microscopic results may indicate this. Purified preparations of each protein are very stable at pH 8.0. As yet no function can be ascribed to these proteins.

The main method used for the purification of the above-mentioned proteins has been sucrose-density-gradient centrifugation, which avoided the problem of protein aggregation that was generally encountered when the protein extracts were applied to Bio-Gel or Sepharose columns. Samples were taken directly from the sucrose gradients for polyacrylamide-gel electrophoresis at pH 8.0 in the absence of dissociating agents. After dialysis samples were studied electron microscopically by negative staining.

The electron-microscopic images given by the single-torus and hollow-cylinder proteins are meaningful with respect to protein symmetry. Even though the electron-microscopic image of a protein is a two-dimensional representation of a three-dimensional structure, it has been shown that for the proteins under discussion it is possible to interpret these images in three-dimensional terms (Harris, 1969*b*). For proteins that do not have such a geometrical quaternary conformation this may

not be possible, since the number of orientations of the protein on the carbon backing film might then be very large, resulting in a large number of slightly different electron images. The single-torus protein exhibits simple cyclic symmetry, whereas the hollow-cylinder protein appears to have a rather complex dihedral and cyclic symmetry. In the electron microscope the hollow-cylinder protein oriented on its side is seen to be composed of four stacked rings. Careful examination of this image suggests that the inner pair of rings are fused more closely than each of the inner rings is to its adjacent outer ring. This might indicate that all four rings are not of identical composition or that steric hindrance is playing a significant role. The inner pair of rings might be identical and also the outer pair. As no aggregates of two, three, five or more protein rings are found, this might support the above suggestion. The binding affinities between the subunits or adjacent protein rings must determine the possible number of rings that can stack together. The inner rings may possess an affinity for each other on one side and an affinity for the outer ring on the opposite side. The outer rings may have no affinity for each other but possess on one side an affinity for the surface of the inner-ring dimer. Proposals such as this are purely speculative, but are thought to be reasonable in terms of the molecular symmetry observed in the electron microscope.

Recent studies have suggested that the hollow-cylinder and single-torus proteins may be located on the inner surface of the erythrocyte membrane. As mentioned above, bovine 'ghosts' prepared in phosphate buffer tend to give rise to stromalytic forms. Inside some of the stromalytic projections the hollow-cylinder protein has clearly been seen by negative staining with ammonium molybdate. In the case of the hollow-cylinder protein situated on a large area of membrane (Harris & Agutter, 1970) it is not possible to say for certain whether the molecule is located on the inner or the outer surface of the collapsed membrane. The interpretation applicable to the electron image of the stromalytic tube is more precise and there can be little doubt that the molecules are located inside the membrane projections. The assumption is implied that the inner surface of the stromalytic projection is continuous with the inner surface of the normal membrane, and, though likely, this cannot be accepted as fact. A further result that is indicative of the single-torus and hollow-cylinder proteins being loosely bound to the inner surface of the membrane is that small membrane lesions, probably produced at the time of preparation of the negatively stained specimens, often have large numbers of these molecules located on the adjacent carbon backing film. It is reasoned that the trauma that produced

the membrane lesion permitted the escape of the molecules.

The tentative conclusions drawn from the above observations are supported by the immunological approach of C. Howe, who has shown that antibodies prepared against the single-torus protein do not agglutinate intact erythrocyte (personal communication). Also, Hoozeveen, Juliano, Coleman & Rothstein (1970), using intact human erythrocytes and erythrocyte 'ghosts' that had been labelled with a fluorescent stilbene compound, have shown that on extraction at low ionic strength all the fluorescence remained with the residual membrane fragments. Both these observations suggest that the proteins that are released when the intact erythrocyte 'ghost' undergoes fragmentation at low ionic strength come from the inner surface of the membrane.

The possibility remains that the proteins under discussion are predominantly adsorbed cytoplasmic contaminants. Hoozeveen *et al.* (1970) present a calculation that argues very much against this being the case. The present author considers that, as the proteins have bound to the 'ghosts' through the extensive washings during which the haemoglobin is released, it is unlikely that other cytoplasmic proteins would remain bound. Even if in the intact erythrocyte an equilibrium exists between membrane-bound and cytoplasmic protein, it is reasonable to consider the proteins to be membrane-associated, though not membrane 'structural' proteins.

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The Proteins of Erythrocyte Membranes: Where are They?

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Among the proteins present in human erythrocytes there are two major components that are located, at least in part, on the external surface of the cell. These have been located by their ability to react with a non-penetrable reactive reagent, formylmethionyl sulphone methyl phosphate, of

extremely high specific radioactivity. These two components have apparent molecular weights, determined by sodium dodecyl sulphate-gel analysis, of about 105 000 (a) and 90 000 (b). Each is present in approx. 4×10^6 copies/cell; together they might cover at least 25% of the cell surface, and probably more. Component (a) appears devoid of sialic acid and carbohydrate. Component (b) bears about 50% of the cell-surface carbohydrate and about 70% of the cell-surface sialic acid. Its apparent molecular weight of 90 000 is therefore likely to be misleading, since a large fraction of its mass (perhaps half) is carbohydrate. Components (a) and (b) are now being 'fingerprinted'.

The reagent also labels phosphatidylserine and phosphatidylethanolamine. Only slight labelling of these lipids can be detected on the external surface of erythrocytes although they are heavily labelled when 'ghosts' are exposed to the reagent. This suggests either (1) that these phospholipids are predominantly built into the inner surface of the cell or (2) that they are present equally on both inner and outer surfaces, but are less available [being possibly screened by proteins (a) and (b)] on the outer surface than on the inner surface. It is not possible, as yet, to distinguish between these two alternatives.

Separation and Partial Characterization of Some Human Erythrocyte Membrane Protein Components

By M. J. A. TANNER and D. OWENS. (*Department of Biochemistry, University of Bristol, Bristol BS8 1TD, U.K.*)

Extraction of human erythrocyte 'ghosts' with EDTA solutions containing 2-mercaptoethanol (Marchesi, Steers, Marchesi & Tillack, 1970) followed by solutions containing 1 M-sodium chloride results in the solubilization of many of the bands seen on polyacrylamide-gel electrophoresis of intact 'ghosts' in sodium dodecyl sulphate and 8 M-urea. The insoluble residue remaining contains 47% of the protein, 58% of the total phosphate, 57% of the cholesterol, 75% of the hexose and 50% of the sialic acid present in the intact membrane, and gives only a diffuse band over the molecular-weight range 65 000–40 000 and a low-molecular-weight 'mini-protein' band (Laico, Ruoslahti, Papermaster & Dreyer, 1970) on sodium dodecyl sulphate-gel electrophoresis. Tryptic-peptide 'maps' of erythrocyte 'ghosts' show a small number (17–20) of intensely staining peptide spots against a high background of a large number of peptides staining with low intensity. 'Ghosts' extracted as above (salt-extracted 'ghosts') yielded a tryptic-peptide