ELECTRON MICROSCOPE REPLICAS FROM THE SURFACE OF A FRACTURE THROUGH FROZEN CELLS

G. H. HAGGIS, Ph.D.

From the Physiology Department, University of Edinburgh, Scotland

ABSTRACT

A technique is described, which is new in certain details, for fracturing frozen cells and taking replicas from the fracture surface. This technique has been developed as a cytological preparative technique complementary to those currently used in electron microscopy. The present paper describes results for human red cells, chosen for preliminary work to facilitate recognition of artifacts. The cells are suspended in 20 per cent glycerol, 0.9 per cent NaCl solution, allowed time to equilibrate, and rapidly frozen in a small chamber plunged into propane at −196°C. The chamber is kept at −196°C., and fractured *in vacuo*, in such a way that the fracture plane runs through the frozen suspension, and through the cells in its path. After fracture, the chamber is brought to −105°C., still *in vacuo*, to etch the fracture surface, and a platinum-carbon replica is deposited on the surface from a carbon arc. The replica is subsequently cleaned in concentrated alkaline solution and examined in the electron microscope. The outlines of the fractured cells can be recognised. There are indications that in areas of the replica which correspond to the interior of a cell, individual haemoglobin molecules can be seen, and in favourable cases the arrangement of some of the four molecular subunits.

The normal preparative technique used at the present time for the examination of cells and tissues in the electron microscope involves fixation with osmium tetroxide or potassium permanganate, and subsequent dehydration, embedding, and thin sectioning (1, 2). There can be little doubt that this preparative treatment gives a picture of the ultrastructure of cells which bears at least some relation to the ultrastructure *in vivo*, since correlation is found in many cases between structures seen in the electron microscope and the functional activity of cells (3–6), and between electron microscope, polarized light, and x-ray diffraction studies. However, if conditions during fixation and alcohol dehydration are compared with the conditions required to preserve cells in a state of normal activity, it must be admitted that this preparative treatment is drastic in the extreme. Osmium tetroxide destroys all enzyme activity, and brings about profound changes in the permeability of natural membranes (7). There is molecular rearrangement during fixation, and further rearrangement and loss of material during dehydration and embedding (8).

Linked with the problem of fixation for electron microscopy is the problem of obtaining sufficient contrast to show up molecular detail in very thin sections. Reducing the section thickness gives enhanced resolution, but reduced contrast. The normal preparative methods reveal little molecular detail for structures within cells, except in rather rare cases, such as that of ferritin (9), where the protein molecule has inherent electron contrast. For example, mammalian red cells are known to contain haemoglobin molecules in high concentrations, each molecule made up of four

subunits approximately 30 A in diameter, in near-tetrahedral arrangement (10). Since modern electron microscopes have an instrumental resolution better than 10 A, the haemoglobin subunits should be clearly resolvable, but nothing corresponding to haemoglobin molecules or their subunits can be seen in osmium- or permanganate-fixed red cells. Corresponding detail must be lost in other cells also.

Several attempts have been made to refine freeze-drying techniques to meet the critical requirements of electron microscopy (11, 12, 13). Recently Muller (14) and Sjöstrand and Baker (15) have introduced improvements in the embedding procedure: after rapid freezing to −160°C. or −185°C., the cells or tissues are brought up to a temperature between −40°C. and −70°C. for freeze-drying, and then embedded in methacrylate *in vacuo* at low temperature, polymerization of the methacrylate being brought about by ultraviolet light at −10°C. Bringing the cells up to −40°C., or even −70°C., during drying represents a potential hazard in the preservation of molecular arrangement, since migratory recrystallization and growth of ice crystals begins, during rewarming of frozen cells, once the temperature rises above about −130°C. (16, 17). In fact, passage through the temperature range −10° to −130°C. during either freezing or rewarming, in the absence of high concentrations of some protective agent like glycerol, can produce molecular changes more drastic than those produced by chemical fixation. High salt concentrations in the fluid regions between ice crystals may be expected to extract lipids from lipoprotein membranes (18) and to break up or modify any macromolecular structure held together by electrostatic forces. Although extensive, the molecular rearrangements produced by rapid freezing, or rewarming, should be different from those produced by chemical fixation. In pancreatic cells the "membranes" of mitochondria and endoplasmic reticulum show a similar appearance after both types of preparative treatment (3, 15), and it seems reasonable to conclude that these correspond to membranous structures present *in vivo*. Ribonucleoprotein particles are seen only in the chemically fixed material. There is however independent evidence for the existence of sedimentable ribonucleoprotein particles, at least in fragmented cell preparations (19), and lack of contrast may prevent their being seen in frozen-dried material.

Following Hall's (20) and Meryman's (21) demonstration that it was possible to take replicas for electron microscopy from the surface of ice held at low temperature, Meryman and Kafig (16) explored the possibilities of a rather less conventional preparative treatment. They froze frog muscle by plunging it into liquid nitrogen, fractured the brittle frozen tissue *in vacuo*, and took replicas from the fracture surface by vacuum deposition of silicon monoxide. The surface was

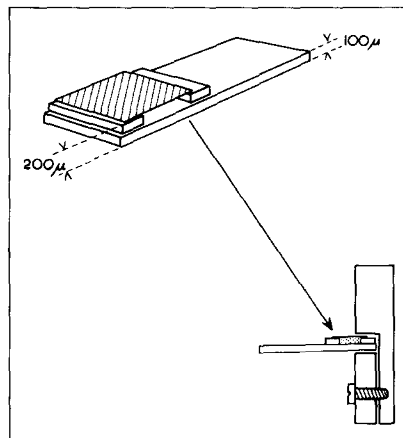


FIGURE 1

Freezing chamber and specimen holder.

found to be smooth, showing no structural detail. In order to show up structures embedded in the ice beneath this smooth surface, they tried focussing radiant heat from a filament onto the specimen *in vacuo* to bring about sublimation of water molecules and "etch" the surface. Replicas from the etched surface of the muscle fracture showed only the salt crystals which formed on removal of the surface water. At this point the technique seemed rather unpromising. However, Steere (22) showed that the arrangement of particles in virus crystals could be revealed in this way, both for crystals in aqueous suspension and for crystalline inclusions within cells.

The technique to be described in the present paper is similar to that of Meryman and Kafig with some modifications, and with Steere's arrangement of specimen and vapour trap in the vacuum chamber. The cells are frozen in 20 per cent glycerol-saline solution in a small chamber plunged into propane at −196°C. The chambers

are fractured *in vacuo* at $-196°C$. and allowed to warm, to etch the fracture surface, but they are not usually brought above $-105°C$. during etching, so that migratory recrystallization and other molecular rearrangement during warming is reduced to a minimum. The replica deposited on the etched surface is a mixture of platinum and carbon, which gives very high resolution. Human red cells were chosen for preliminary study, on the ground that if the individual haemoglobin molecules could be seen and clearly identified in these cells, then there would be every chance of applying the technique, with success, to more complex cells.

EXPERIMENTAL

Red Cells: A drop of fresh human blood, from a finger prick, is drawn into a small pipette and diluted about 1:10 in 0.9 per cent NaCl solution. The cells are centrifuged and the supernatant is withdrawn and replaced by a solution of 4 parts 0.9 per cent NaCl solution to 1 part of glycerol. The cells are stirred into this solution and left for 1 hour at room temperature, to allow time for the glycerol

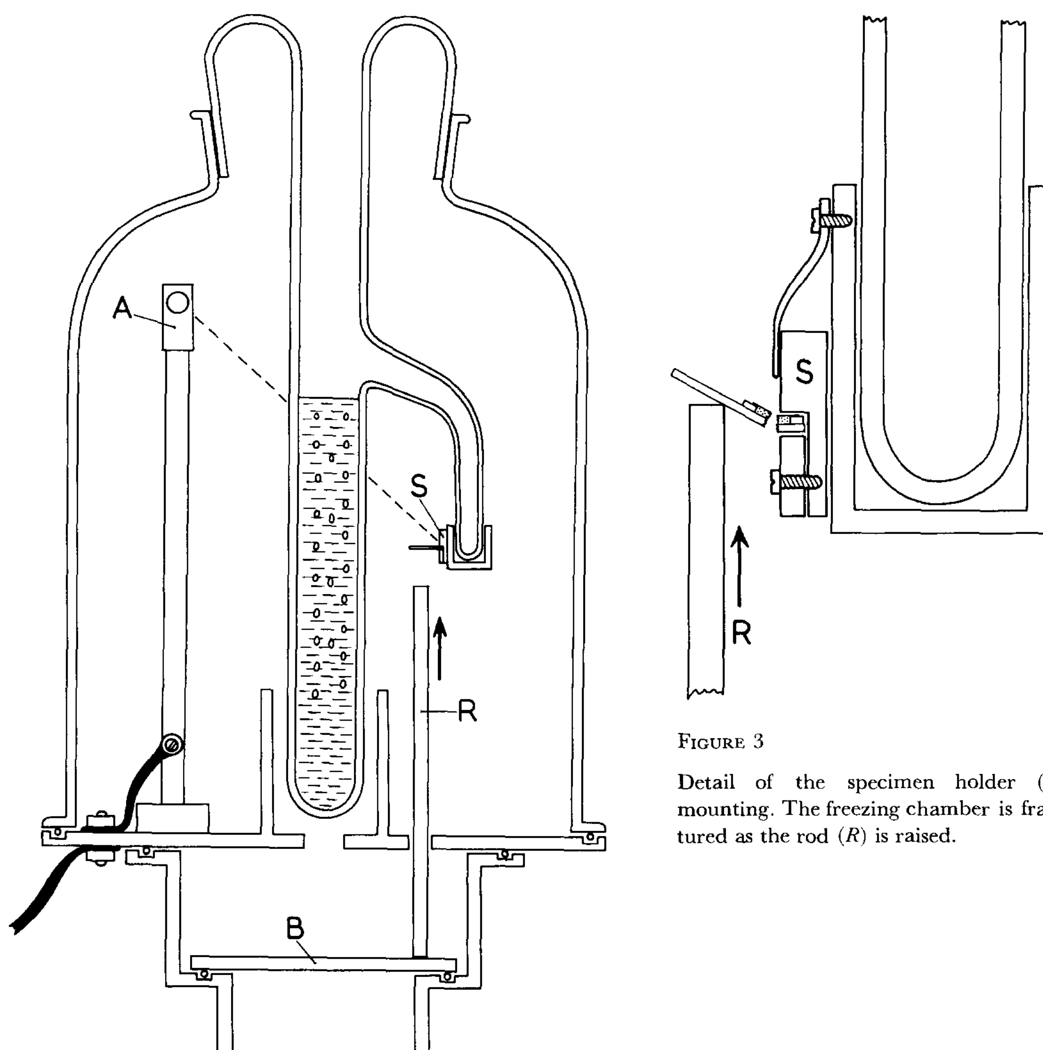


FIGURE 3

Detail of the specimen holder (*S*) mounting. The freezing chamber is fractured as the rod (*R*) is raised.

FIGURE 2 General view of the arrangement of components in the vacuum chamber showing the vapour trap partly filled with liquid nitrogen, with the specimen mounted on a side-arm. *S*, specimen holder; *A*, carbon arc; *B*, vacuum pump baffle plate; *R*, fracture rod rising with baffle plate.

FIGURE 4

Thermocouple traces for freezing and thawing of 20 per cent glycerol-saline solution under the conditions described in the text. The upper horizontal line is at 0°C., the lower line at −200°C. Marker repeat time 20 msec.

to penetrate into the cells (23). The cells are then centrifuged again and freezing chambers are filled with the concentrated suspension of red cells in 20 per cent glycerol-saline.

Freezing Chambers: The freezing chambers are made from coverglass (100 μ thick) and platinum foil (8 μ thick). Microscope coverglasses (No. 0) are cut into strips 2 mm. × 1 cm. Small pieces of the same glass, 2 mm. × 1 mm., are cemented to these strips as spacers (Durofix, Rawplug Company, England), and platinum foil is cemented above the spacers (see Fig. 1). Chambers made in this way can be filled by allowing the cell suspension to flow in from one side from a small pipette, the layer of cell suspension being 100 μ thick between an 8 μ thickness of platinum and a 100 μ thickness of glass. The inner glass surfaces of the chamber are covered with a thin layer of lucite (Perspex, I.C.I., England) cast from dilute solution in chloroform, to prevent the pH change and loss of protein from the cell surface which would occur in this small volume, if the cell suspension were in contact with glass (24).

Freezing, Fracture, and Etching: The chambers are frozen immediately after filling by plunging them into propane at −196°C., suitable precautions being taken to minimise dangerous condensation of atmospheric oxygen on the propane surface (25). If removed from fluid at −196°C., these small chambers warm up very quickly, and it is important at this stage to keep them below −130°C. to prevent migratory recrystallization and ice crystal growth in the frozen cell suspension (16). The chambers are therefore transferred to liquid nitrogen in a small cup filled with liquid propane at −196°C. and mounted under liquid nitrogen in a copper specimen holder, like a miniature vice (see Fig. 1). Several chambers may be filled and frozen, then transferred together to the liquid nitrogen. One chamber is mounted in the specimen holder and the rest stored in liquid nitrogen until required. The specimen holder has a much greater thermal capacity than the chambers, and may now be transferred without its temperature rising above −130°C. to a mounting in the vacuum chamber, previously cooled to −196°C. with liquid nitrogen. The vacuum chamber is shown in Fig. 2, with details of the specimen holder mounting in Fig. 3. The specimen is mounted on a side-arm of the vapour trap, which acts both as a water trap during later etching, and as an oil trap between pump and vacuum chamber. After the specimen holder has been mounted on the vapour trap side-arm, the vacuum chamber is put down onto the base plate, and evacuated to a pressure around 10^{-5} mm. Hg.

The oil-diffusion pump baffle plate (see Fig. 2) is controlled from outside the vacuum. When the baffle is opened during pumping down, at approximately 10^{-3} mm. Hg, the movement of the baffle plate raises a rod, as indicated in Figs. 2 and 3, which fractures the chamber at the point where it emerges from the specimen holder. (A clean fracture through the cell suspension is facilitated by lightly cutting the foil along the fracture line with a razor blade, under liquid nitrogen, after the chamber has been mounted in the specimen holder, but before transfer to the vacuum chamber. Manipulations under

Key to Abbreviations

RBC, red blood cell *EXT*, extracellular *CB*, cell boundary

FIGURE 5

Area of replica showing a fracture through the main part of one red cell and part of another in the lower right corner of the picture. Folds in the replica formed during thawing or subsequent handling can be seen, running across the waist and the upper part of the main cell. Part of the replica, in the upper left corner of the picture, has become detached along the cell boundary and lost. The area outlined is enlarged in Fig. 6. Surface brought to −110°C. during etching. ×50,000. Scale marker 1 μ.

RBC
RBC
EXT
5

liquid nitrogen are carried out in a cardboard box, half filled with the liquid.)

Initially the main part of the vapour trap and the side-arm are completely filled with liquid nitrogen, but after fracture of the chamber the liquid nitrogen in the side-arm is allowed to evaporate away, while the main part of the trap is kept topped up to just below the side-arm junction (as indicated in Fig. 2). As the specimen temperature rises above about −130°C., water molecules begin to sublime from the fracture surface. The mean specimen temperature is measured with a thermocouple, and at a chosen temperature between −120°C. and −90°C. this etching is stopped, by again filling the side-arm of the vapour trap with liquid nitrogen. The higher the chosen temperature, the deeper will be the etch.

Replica Deposition and Electron Microscopy: With the fracture surface again at −196°C. a carbon arc is fired to deposit a replica on the etched surface. The line from arc to specimen is at 45° to the average fracture plane, and the arc-to-specimen distance is 9 cm. The arc is fired off quickly, so that the temperature of the fracture surface remains below −130°C. during replica deposition. The arc carbons (Link WLI, Morgan Crucible Company, London, England) are turned down to fine points and round these is wound a 5 mm. length of 36 S.W.G. pure platinum wire. This gives a high contrast non-granular replica of the type described by Bradley (26). After the replica has been deposited, the specimen holder is removed from the vacuum and allowed to warm up to room temperature. The replica at this point lies on the surface of the glycerol solution in the trough that remains after fracture of the freezing chamber. It may be floated off onto a shallow dish of water, to which a drop of detergent has been added to minimise fragmentation of the replica (27). Pellets of NaOH are dropped into the dish, and the replica is left floating on concentrated NaOH for some hours to digest away all adhering cellular material. Finally the replica is picked up on a carbon backing film on an electron microscope grid and thoroughly washed in distilled water. The electron microscope, a Metropolitan-Vickers E.M.6, was used at an electron magnification of 20,000 to 50,000, with a 50 μ objective aperture. Astigmatism was corrected on a hole in the backing film shortly before taking the pictures.

Cell Survival: The survival of cells after rapid freezing and thawing in the small chambers may be studied by replacing the platinum foil with a sheet of lucite, cast from chloroform, about 10 μ thick. For these experiments the ends of the chambers are sealed with Vaseline grease immediately after filling. Rapid thawing is achieved by plunging the chambers into isopentane at room temperature in a special clip, which keepts them below −130°C. until the moment of impact with the isopentane surface. The cells in these chambers may be observed under a light microscope before and after freezing and thawing. Cooling and warming rates were determined with a thermocouple and a cathode ray oscilloscope with internal beam-intensity-modulation time marker. The thermocouple was made from wires of copper and constantan 25 μ in diameter.

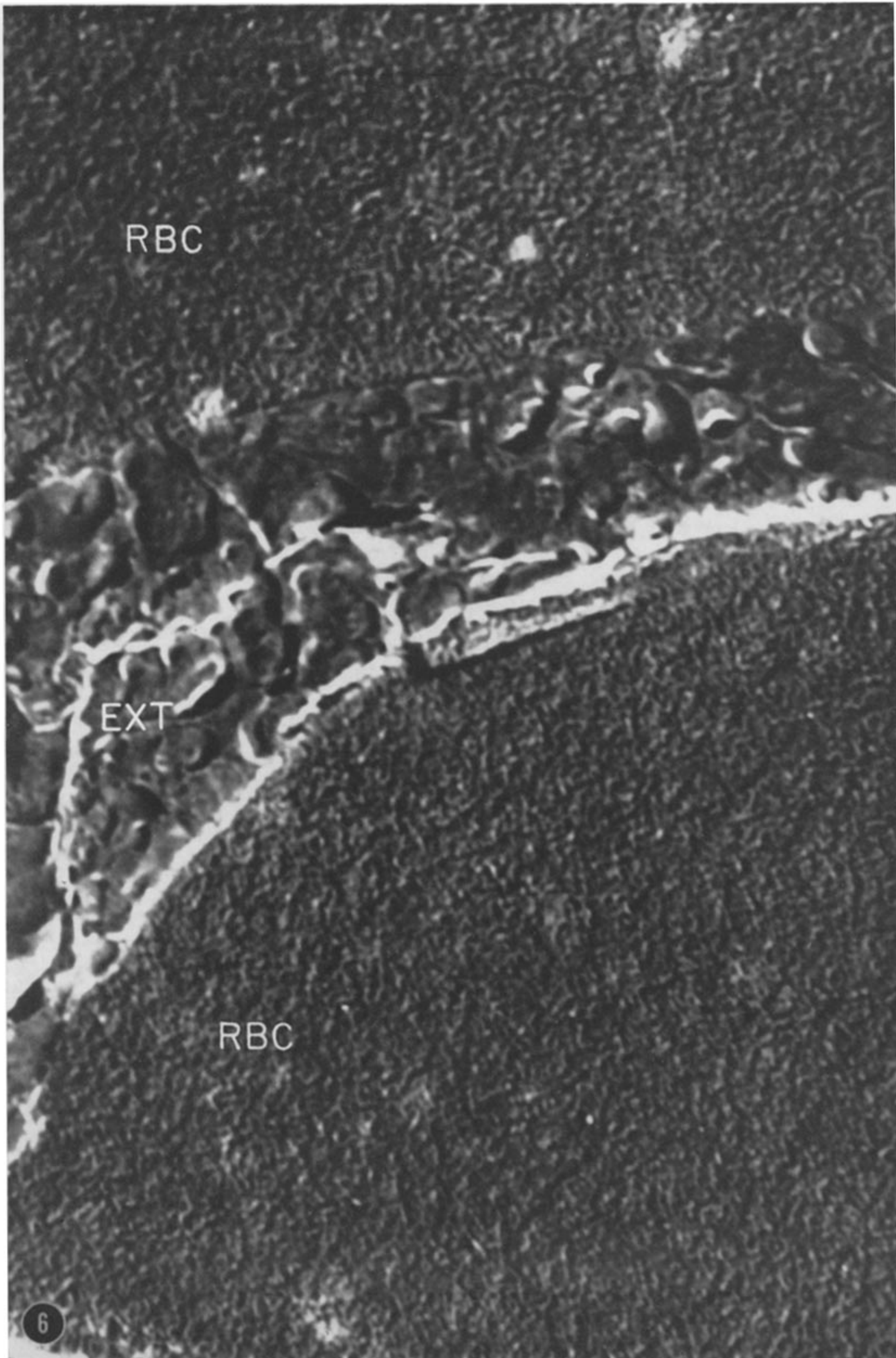
RESULTS AND DISCUSSION

When the freezing chambers are plunged into liquid propane at −196°C. their temperature falls from room temperature to below −130°C. within 150 msec. (Fig. 4). In experiments to test survival of cells after freezing and thawing, thawing is completed within 400 msec. These rates are similar to those recorded by Taylor (28) for freezing and thawing of tissues under comparable conditions.

It was estimated, by direct observation of the chambers under a light microscope before and after freezing and thawing, that over 70 per cent of the red cells in 20 per cent glycerol-saline retained a normal, smooth, biconcave appearance after this treatment. The remainder presumably undergo haemolysis at some point during either freezing or thawing. In 10 per cent glycerol-saline 10 to 40 per cent survive, and in 5 per cent glycerol-saline only a few cells are seen, after freezing and thawing under these conditions. The replicas described in this paper were all taken from fractures through frozen suspensions of cells in 20 per cent glycerol-saline. In general, the fact that a high proportion of cells survive freezing and thawing under given conditions does not prove that this treatment has produced no change in the cell ultrastructure, since some regions of the cell may be irreversibly altered in a way which is not detected by the criteria chosen to test survival. One can only claim that the molecular movement and rearrangement that takes place during freezing under these conditions must be to a large extent reversible. After freezing, in the present technique, the only molecular movement prior to replica deposition is that taking place during re-

FIGURE 6

Enlargement of outlined area of Fig. 5. × 150,000.


RBC
EXT
RBC
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warming, between $-130°C.$ and $-105°C.$ Although there will be rotation and local movement of water and glycerol molecules over this temperature range, and, of course, sublimation of water molecules from the fracture surface, it is possible that protein molecules and larger structures will remain unchanged in form and position, provided the surface etch is sufficiently shallow.

For human red cells equilibrated in 20 per cent glycerol-saline, the haemoglobin concentration is about 30 per cent (29) and the glycerol concentration, inside the cell, 10 to 15 per cent. At this protein concentration the average distance between the centres of haemoglobin molecules (75 A) is not very much greater than their molecular diameter (60 A) (29). After rapid freezing, if intracellular ice crystals remain smaller than about 20 A, the haemoglobin molecules will remain uniformly distributed through the cell at the same average separation. If larger intracellular ice crystals form during freezing, or during rewarming to $-105°C.$, local concentrations of both protein and glycerol may be higher than average, with the spaces between haemoglobin molecules less than 15 to 20 A. The inflexion in the thermocouple cooling curve (Fig. 4) shows that considerable crystallization of ice has taken place during cooling (30) but does not, of course, show whether or not this is entirely extracellular.

In examination of the replicas the first point which may be noted is that where the advancing edge of the fracture hits a cell membrane, it is apparently quite undeflected and passes straight through the cell. The etched fracture surface shows a marked contrast between areas of the replica which correspond to the inside and outside of a cell (Figs. 5 and 6). This difference between different areas of the replica, which is found consistently in replicas taken in this way, shows that the intracellular appearance is not just an artifact arising from granulation of the platinum-carbon deposit. The intracellular fracture shows in fact the type of appearance which might perhaps have been expected, in view of the close spacing of the haemoglobin molecules in the cell and the high concentration of glycerol. As water is removed from the surface, the haemoglobin molecules presumably dry down onto one another amongst the glycerol. In Figs. 5 and 6 individual haemoglobin molecules cannot be distinguished.

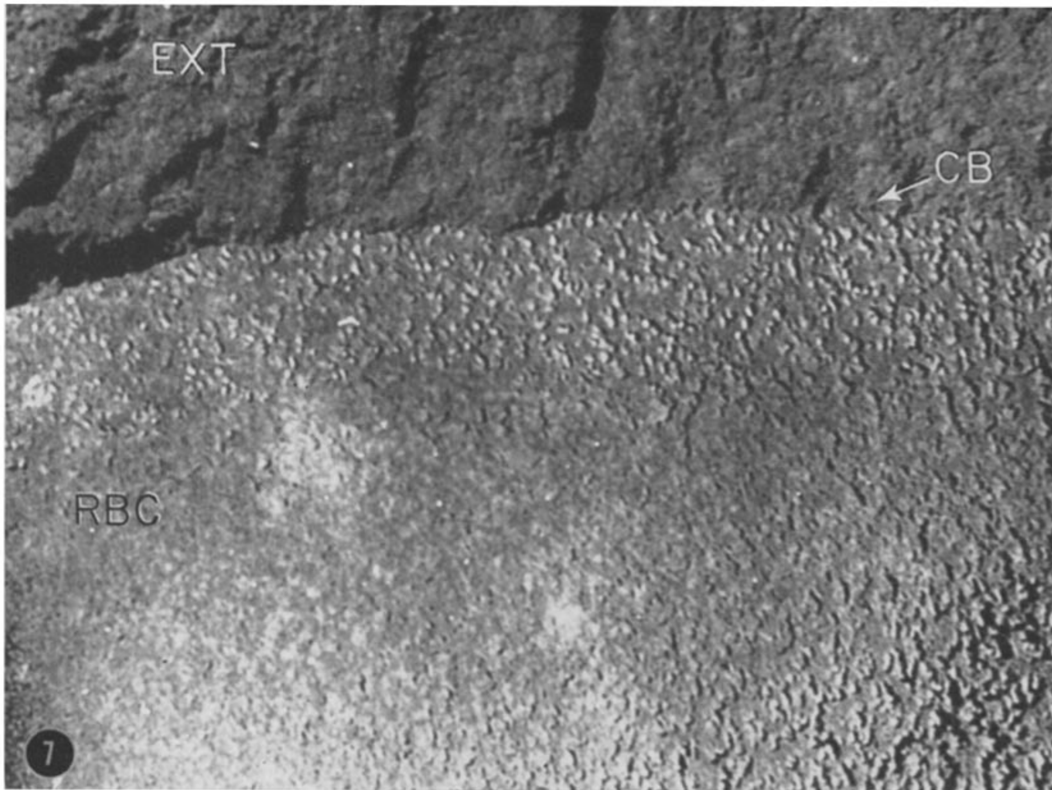
The appearance of the intracellular fracture surface is somewhat variable, partly as a result of local variation in the angle of the fracture plane. Although often as in Figs. 5 and 6, it is also often as shown in Figs. 7 and 8. Small "bumps" under 100 A in size can be seen against a relatively structureless background, and in some cases (Fig. 8) a larger different type of "bump" ranging in size from 100 A to 600 A. So far as is known there are no formed bodies ranging in size from 100 A to 600 A present at high concentration in human red cells *in vivo*. The larger "bumps" seen in Figs. 8 and 9 are therefore probably ice or glycerol-hydrate crystals. The specimen is mounted in such a way that the shadowing direction is also the direction of advance of the freezing boundary through the specimen during cooling (see Fig. 2). It is thus possible to tell from the direction of the shadows that these intracellular crystals are mainly formed on the side of the cells nearest to the foil wall of the chamber. This suggests that they form during freezing rather than during etching.

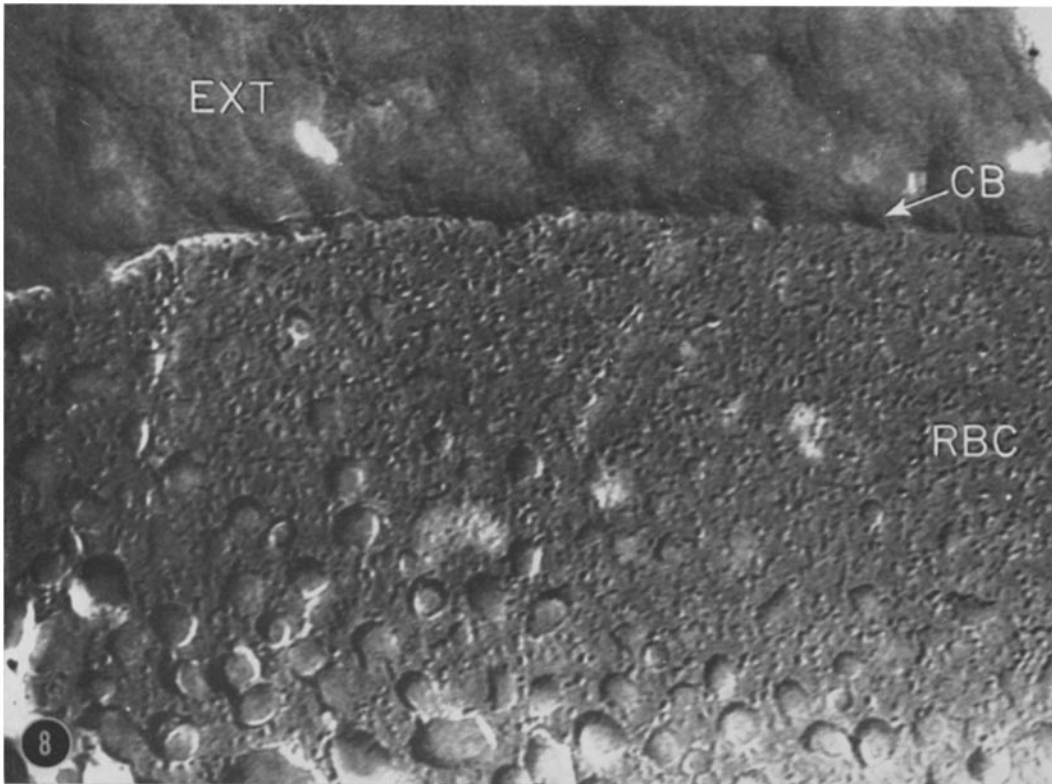
Differential expansion between the interior and exterior of the cell during warming and thawing, and handling of the replica subsequent to thawing, apparently lead to frequent breaks and folds in the replica at the cell boundary (Fig. 5). Where there are no signs of artifacts of this kind (Figs. 7 and 8) there is an abrupt transition from the appearance typical of the inside of the cell to the appearance typical of the extracellular fracture, without sign of any structure at the interface. Either there is no true membrane at the cell boundary or in these preparations the cell membrane is obscured by glycerol.

Fig. 9 shows at high magnification a small part of the *intracellular* area of a replica similar to those of Figs. 7 and 8. The small "bumps" are seen to be of very variable shape and form, occasionally showing an appearance suggestive of a "shad-

FIGURES 7 AND 8

Further areas of replica showing interior of cells (lower part of figures), cell boundary, and extracellular region (upper part of figures). In Fig. 8 ice or glycerol-hydrate crystals can be seen, ranging in size from 150 A to 600 A. Surface brought to $-105°C.$ during etching. $\times$ 150,000.


EXT
CB
RBC
7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8

owed" haemoglobin molecule made up of four subunits in near-tetrahedral arrangement (10). (All the plates are printed as "shadowed" electron micrographs, light areas in the plates corresponding to thicker areas of replica.) It seems possible that an individual haemoglobin molecule might occasionally stand out in this way against a variable background of glycerol and protein. Since such molecules would protrude from the background to a varying extent in different orientations, the apparent size of the subunits would be expected to vary somewhat, and the dimensions of the "molecules" indicated by arrows in Fig. 9 are consistent with this interpretation. One cannot be certain however that these effects are not produced by granulation of the deposit, or by granulation of glycerol or salt on the etched surface.

Granulation of the replica is sometimes quite marked, particularly if the replica is very thin or if the platinum:carbon ratio is too high. However, under these circumstances the whole area of the replica is granular and the effect is clearly recognisable. If the effects shown in Fig. 9 are not produced by haemoglobin molecules, a granulation of glycerol and salt on the etched surface seems a more probable explanation than local granulation of the replica.

The study of frozen haemoglobin solutions is not so helpful in interpreting these intracellular effects as might be supposed. Replicas from haemoglobin solutions frozen without glycerol show a granular appearance somewhat similar to the intracellular appearance of Fig. 6. The molecules are apparently crowded together by ice crystals during freezing, and individual molecules cannot be distinguished. Replicas from haemoglobin solutions with added glycerol show an appearance similar to that of the intracellular areas of Figs. 7, 8, and 9. In replicas from both the intracellular areas and frozen haemoglobin-glycerol solutions doublets are fairly frequently seen (about 60 A by 30 A) suggestive of haemoglobin molecules partly protruding from the underlying glycerol-protein layer. Replicas from frozen glycerol-saline solutions are relatively structureless (compare the extracellular areas of Figs. 7 and 8). However, areas of the glycerol-saline surface do sometimes show salt and glycerol in ridges and rows of granules, not so very different in appearance from those of the etched surface of the frozen haemoglobin-glycerol solutions. It must be allowed that the small "bumps" of Figs. 7 and 8 might be granules of salt or glycerol, presumably seen much more frequently in intracellular areas than in extracellular areas as a result of some difference in the freezing conditions in the two cases.

Figs. 10 and 11 show at high magnification the *intracellular* appearance after deeper etching (specimen brought up to −95°C. rather than −110°C. or −105°C.). The appearance of the surface is now quite different. No granulation suggestive of "shadowed" haemoglobin molecules is seen, but doublets and triads of dark dots, surrounded by deposit, are seen quite frequently. Their appearance suggests that some haemoglobin molecules now protrude so far from the underlying substratum that the molecules become completely surrounded by deposit, and that the deposit penetrates to some extent between the subunits. The protein itself would be of lower electron opacity than the deposit, but is probably in any case digested away during the cleaning of the replica in concentrated NaOH (the subunits thus showing up in these prints as dark dots). There is no question, in this case, of these effects being produced by granulation of the deposit, and no comparable effects have been seen in replicas from frozen glycerol-saline solutions. It seems rather probable, therefore, that these effects in the deeply etched surface represent a visualization of the haemoglobin subunit structure.

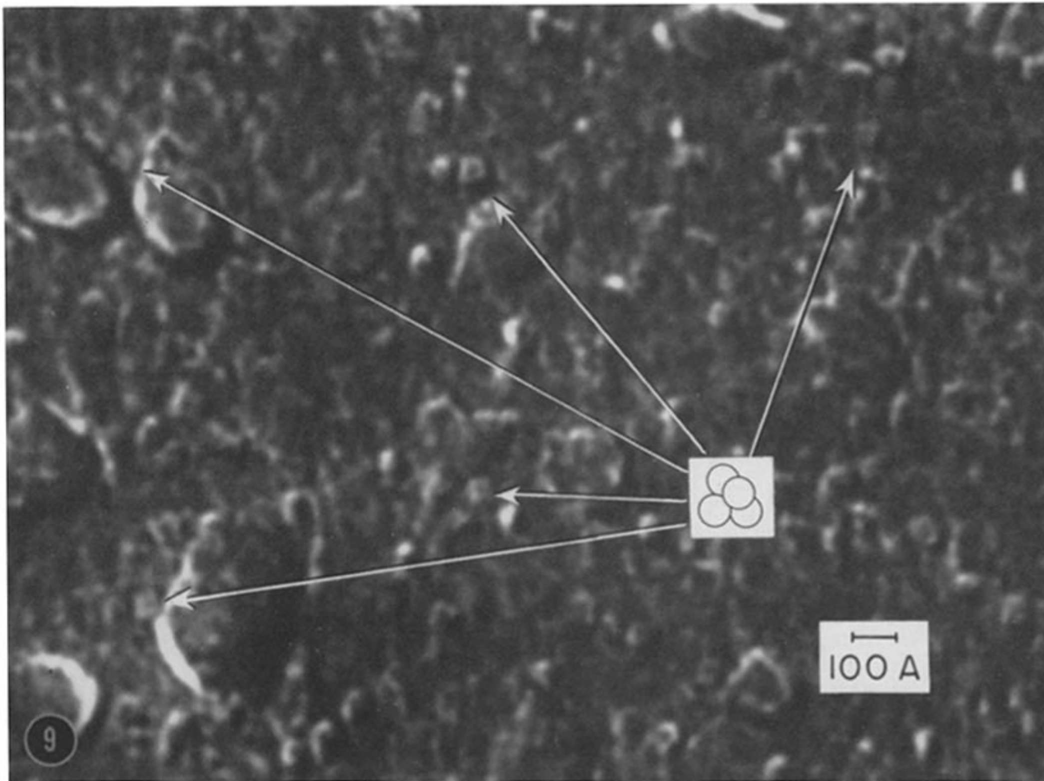
The aim of this work has been to investigate the

FIGURE 9

Area from cell interior. In this area five of the small "bumps" have an appearance which suggests "shadowed" haemoglobin molecules (*cf.* inset, in which four subunits are drawn in near-tetrahedral array). Ice or glycerol-hydrate crystals can be seen between 100 A and 400 A in size. Surface brought to −105°C. during etching. × 600,000.

FIGURES 10 AND 11

Areas from cell interior showing haemoglobin molecules by "negative contrast." Surface brought to −95°C. during etching. × 800,000. (*Cf.* insets, in which four subunits are drawn in near-tetrahedral array.)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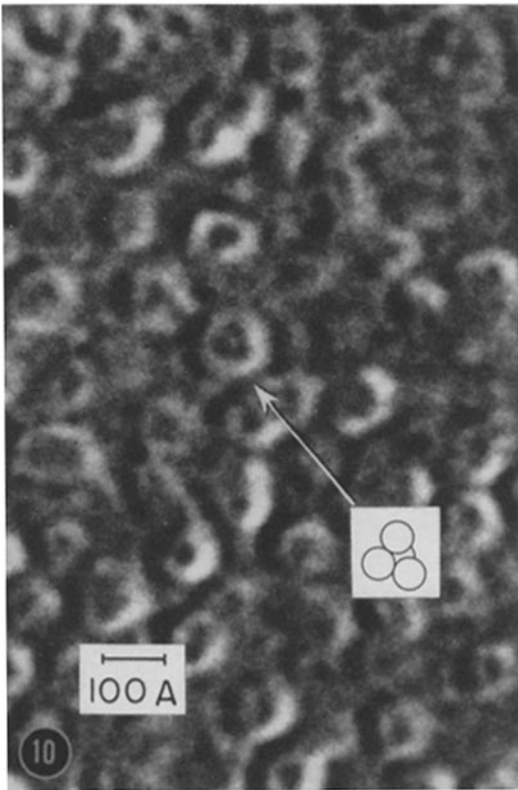
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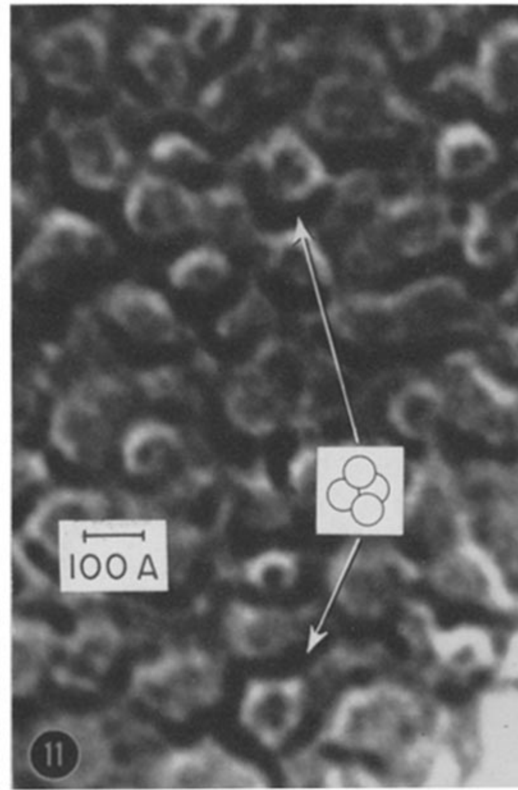
100 A

possibility of studying molecular structures *within* cells by the fracture replica technique. In a preliminary study of replicas from frozen glycerol-saline and protein solutions it was soon found that conditions in the frozen block were highly variable depending on local freezing rates and local salt and glycerol concentrations. In the study of intracellular freezing there is no substitute for the cells themselves, and mammalian red cells were chosen as the simplest material for study. Freezing rate, initial glycerol concentration, and etch temperature were varied until conditions were found which appear to be useful in showing up molecular detail, with freezing conditions compatible with a high percentage of survival on thawing, and hence with most of the cell membranes presumably intact in the frozen block.

The usefulness of the technique applied to other cells or tissues remains to be demonstrated, but cells which could be studied most profitably would seem to be those, like muscle or pancreas, in which certain structures (*e.g.* actomyosin filaments or ribosomes) are present in large numbers. Although mostly obscured by glycerol in the etched surface, an occasional filament or ribosome should stand out to reveal some structural detail. In view of the known "protective" effect of glycerol during freezing and thawing (23), any structural detail revealed in this way should correspond quite closely to that prevailing *in vivo*.

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