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Authors

Glaeser, Robert M.

Hayes, Thomas

Mel, Howard

et al.

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Division of Medical Physics and Donner Laboratory
Lawrence Radiation Laboratory
University of California, Berkeley, California

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According to the commonly accepted model of Davson and Danielli, the surface of red blood corpuscles (RBC) consists of two parallel monolayers of lipid molecules, coated on either side with protein. The historical development of this model has been reviewed by Davson [4]. Electron micrographs of sectioned human RBC, fixed and stained with KMnO_4 , can be interpreted in a manner consistent with the lipid bilayer model [18]. Still, in recent years, various authors have pointed out what seem to be serious deficiencies in this model. Haydon and Taylor have stated that the Davson-Danielli protein-lipid-protein membrane would be a thermodynamically unfavorable structure [8]. Oncley has argued along much the same lines, and has suggested that one might more profitably think in terms of lipoglycoprotein subunits, with a basic structure perhaps not too unlike that of the serum lipoproteins [16].

It would seem relevant, then, to draw attention to information which can be obtained with the electron microscope when one looks at the RBC surface "face on" rather than in section. Hillier and Hoffman in 1953 [13] and Hoffman in 1956 [14] treated human RBC ghosts with phosphotungstic acid, air-dried them, shadowed them with metal in the usual way, and then took such face-on pictures. Their pictures show a pebbly or granular surface

appearance (the characteristic bump diameter being about 200 Å for human RBC ghosts), which they interpreted in terms of plaques lying over the RBC surface. Ponder [17] has suggested that insufficient experimental control existed with regard to the production of artifacts, but later work by others employed a carbon replica technique to demonstrate that the surfaces of OsO₄-fixed, air-dried RBC [2], and of frozen dried RBC [7], possessed the same type of pebbly or granular surface texture.

We wish to report work which supports the interpretation that the pebbly or granular surface appearance is not an artifact, but is directly related to the structure of the RBC surface as it exists when the cell is suspended in buffered NaCl at a physiological ionic strength. We further report some filamentous structures which we have seen only in special unshadowed preparations of the RBC surface. Although the relationship of these latter structures to the cell surface is still under experimental investigation, we feel that their reproducibility in many experiments, and their potential significance for understanding cell membrane structure, justifies some discussion at this time.

Methods

Blood was obtained from the vena cava of ether-anesthetized rats and washed twice in standard buffer (0.145 M NaCl, 3×10^{-4} M NaHCO₃). The final pellet was resuspended in standard buffer to obtain a 10 percent vol/vol cell suspension. One volume of this cell suspension was added to a mixed solution composed of (a) two vol 0.143 M sodium barbital, 0.143 M sodium acetate, (b) two vol 0.10 M HCl, and (c) five vol of 2 percent OsO₄ in standard buffer. After 1 min the cells were centrifuged for 2 min at approximately 500 g. The supernatant was discarded, and the fixed cells were washed twice in 100 vol standard buffer and again twice in 100 vol distilled

water. Even after suspension in distilled water, the OsO_4 -fixed cells were intact and full of hemoglobin. A final suspension (0.1 to 1.0 percent fixed cells in distilled water) was sprayed onto the prechilled surface of freshly cleaved mica (mounted on an ordinary microscope slide), and water was removed by the freeze-drying technique. Carbon was then evaporated onto the slide at an angle of 90 degrees to the surface of the slide in order to deposit a uniform film (Fig. 1a). This carbon film with cells attached was floated off the glass slide (Fig. 1b) onto 0.145 M NaOH, and the organic material was digested for 2 hours or more at room temperature (Fig. 1c). Some of the undigested cell material remained attached to the carbon film. The carbon film was then transferred to a surface of distilled water to remove excess NaOH (by dilution), after which fragments of the carbon were picked up from underneath with a stainless steel electron microscope specimen grid. Some of the preparations were shadowed from the top (Fig. 1d); nichrome wire was used for shadowing. The electron microscope used was the Hitachi model HU-11. All photographs are printed in such a way that regions of high density (high electron scattering efficiency) appear bright.

In the following we distinguish between a carbon replica, which should in principle be completely free of any of the original substrate, and "replicon," a term here coined to denote a replica with some organic material still attached.

In the technique of replicon preparation sketched above, it occasionally happens that during digestion or subsequent washing the part of the replicon covering the cell falls down and is supported by the edges of the hole in the carbon film where the cell originally stood. More frequently the replicon falls to one side of the hole (Fig. 1c). This is of little importance if one

works with shadowed replicons, for then only true surface features stand out. If, however, one wishes to look at unshadowed replicons, it is quite important to observe those areas where the replicon has only one layer of carbon. In this way one can be certain that no salt or debris has been trapped between two layers of carbon.

Results

Previous work in our laboratory has shown that the electrophoretic mobility of rat RBC, fixed with OsO_4 as above, is identical, in the pH range from 1.5 to 9.5, to the mobility of unfixed RBC [6]. This means that the type of charged groups and net charge density per unit area are the same after OsO_4 fixation as before. It has also been shown that neither exposure to distilled water nor complete dehydration in ethanol has any apparent effect upon the charge properties of OsO_4 -fixed RBC [6]. Stability of this configuration can be further seen by the fact that the fixed cells do not hemolyze even in hot water. We have used the working hypothesis that, although providing mechanical stability, fixation with OsO_4 has not altered the charge-carrying parts of the surface. Evaporated carbon should give a faithful replica of that surface, and subsequent NaOH digestion should do nothing to the unreactive carbon film itself but should react differentially with the organic material adhering to the carbon film.

Metal shadowing will, of course, show the surface contours of the replicon, and will tend to obscure the underlying material. Figures 2 and 3 are pictures taken of shadowed specimens, and they show the granular or pebbly appearance that has already been reported by others. This same pebbly appearance has also been observed in this laboratory in frozen-dried fragments of RBC ghosts [5]. In the experiments presented here the typical bump diameter is about 400 Å. Whether the discrepancy between this value and the one quoted by Hillier and Hoffman (200 Å) would be attributed to the

method of preparation or to the type of animal from which blood was obtained is a question that remains to be answered.

When replicons are examined in the electron microscope without additional shadowing, one may see some of the structures that derive from undigested cellular material attached to the carbon: the carbon merely acts as a rigid frame supporting these structures. The appearance of the undigested material varies somewhat from cell to cell on a given replicon; the most satisfactory pictures are obtained when all but a few hundred angstroms of thickness of the cellular material are digested away. Such a picture is reproduced in Fig. 4. One may still observe some bumps, but most of the picture is composed of filamentous or strand-like structures with frequent bends or loops. Occasionally a single strand may be followed uninterrupted for more than one micron in length and many of the strands appear to generally follow the surface contour of the cell; others cross obliquely to this direction. The diameter of the strands is variable; in regions where digestion was apparently less complete, there are strands 300 Å in diameter, but in many places there are uninterrupted uniform strand segments of about 200 Å diameter. This may be clearly seen in a more magnified segment from another replicon in Fig. 5. A suggestion of fine structure within the strands themselves is indicated by a continuous electron dense line bisecting most of the 200-Å strand regions. We believe this thin line not to be a diffraction artifact, since it is not affected in a through-focus series. In some places (e.g., "X" on Fig. 5) the line deviates from the center, indicating a possible twist in the strand.

If strands are underlying structural elements in the membrane, then the pebbly appearance of shadowed replicons (as in Figs. 2 and 3) might be due to looped configurations of the filamentous structures observed in

Figs. 4 and 5. It appears that looping of the filaments would in principle be feasible because of the excessive, tortuous length present. In fact, close examination of the bumps present in the portions of Fig. 3 in the areas not covered by metal (e. g., "Y" in Fig. 3) suggests their possible identification with filamentous structures. The appearance of the undigested material varies somewhat from cell to cell on a given preparation. It is even more strongly dependent upon the length of hydrolysis time.

Discussion

This work has confirmed for rat RBC the pebbly appearance of the red cell membrane surface as previously reported for human, mouse, and other RBC by Hillier and Hoffman [13], Hoffman [14], Coman and Anderson [2], and Haggis [7]. In addition, it has provided evidence that the bumps on the surface of the red cell may be associated with a filamentous structure and that the bumps could represent the tops of loops of these filaments.

In 1952 Moskowitz and Calvin, discussing the results of their work on the components and structure of the human red cell membrane, stated: "The picture of the red cell membrane that is suggested by this work is as follows: Long, fibrillar material, called elinin, is arranged in a manner such that the fibrils lie side-by-side and parallel to the surface of the cell. These fibrils are joined together by the ether-extractable lipids to form the framework of the membrane, which is referred to as stromin" [15]. It is of interest to consider the possibility that the filamentous material observed in our replicons is elinin. Further work on the physical properties of elinin by Dandliker, Moskowitz, Zimm, and Calvin produced the following data: Electron micrographs of elinin showed the presence of rods ranging in length from about 2,500 to 10,000 Å, with the narrowest rods about 125 Å

in width [3]. The rods of elinin are held together by ether-extractible lipids. Elinin has a relatively high particle weight, about 4×10^7 , and is a lipid-carbohydrate-protein complex. It would seem quite likely to us that the filamentous structures we observe in our replicons are in fact composed of elinin.

The visualization of the strands in the cell surface depends on two important parts of our preparative technique. First, the red cells are fixed in OsO_4 . The action of OsO_4 is twofold: as a fixative and as an electron stain. In our study the staining properties are of less importance than the fixative effects, that is, the property of osmium tetroxide to make rigid the spatial arrangement of the biological system. It has been shown in our laboratory that OsO_4 can fix the structure of the serum lipoproteins, chylomicrons, and triglyceride particles [9, 10, 11, 12]. Osmium tetroxide fixative can thus preserve lipid structures much as other fixatives preserve the protein framework. Treatment with KMnO_4 , on the other hand, does not preserve these lipid structures. It would thus seem that OsO_4 fixation of the red cell membrane could preserve the lipid junctions between the individual units of elinin and thereby allow the filamentous structure to be visualized. The second critical point in the preparative technique is the digestion of organic material by NaOH . This digestion must proceed to a point where most of the cell contents have been removed, but it must not be so drastic as to remove the "elinin" itself.

We now feel there is substantial reason to believe that the pebbly or granular surface structure revealed in the shadowed replicons is not an artifact. Nevertheless, there remains some considerable question regarding the relationship to the actual membrane structure of the organic material that remains stuck to the carbon. If this material were of the natural

membrane, the membrane thickness would be from 400 Å to 1,000 Å. There is some precedent to think that the RBC membrane is as thick as 1,000 Å [17], but these values are far out of line with the electron microscopic value of 75 Å obtained from sectioned KMnO_4 -treated RBC [18]. It may be relevant to this point to reiterate that surface charge characteristics of KMnO_4 -fixed RBC are not the same as for normal RBC [6].

Other recent work by Baker has demonstrated filamentous structures, 200 Å to 1 μ in diameter and a few microns long, emanating from the surface of hemolyzed RBC [18]. It is tempting to think that these structures are related to the ones we report in this paper; they may represent the unraveling of single or multiple strands of the same material we see attached to the carbon film after treatment with NaOH. The filaments seen by Baker are, however, unique to specimens air-dried in the presence of buffered phosphotungstic acid (PTA). They are not seen, for example, if the PTA is washed off prior to air-drying, or if the ghosts are dried in the absence of PTA.

The eventual reconciliation of what the electron microscope tells us about the cell membrane from sections with what it tells us by the use of replicons and "face on" surface techniques still remains a task for the future. One obviously needs more information about the effects of surface fixing and staining agents on the molecular structure of membranes. Current work is being directed towards testing for the conservation of other membrane properties (than surface charge), such as membrane capacitance and resistivity, as well as the use of newer fixation reagents and the extension of these studies to other cell surfaces. Experiments are also in progress to gain further information about the thickness of the RBC membrane as seen

by sectioning techniques, for this will bear directly upon the interpretation of the filaments seen in unshadowed replicons.

Summary

A carbon "replicon" technique is used to study the membrane of OsO_4 -fixed rat erythrocytes. When metal-shadowed, the replicons show pebbly or granular structure at the level of 400 to 500 Å. Examination of unshadowed replicons reveals organic material, attached to the carbon, in what seems to be a repeatedly looped, filamentous structure. The possible relationship of these filaments to elinin is discussed.

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We thank Professor Oncley for giving us a copy of his manuscript prior to publication.

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Figure Legends

Fig. 1. Steps in the preparation of "replicon" specimens for electron microscopy: (a) carbon deposition over OsO_4 -fixed, lyophilized RBC; (b) stripping of carbon and RBC from mica; (c) digestion: (i) shows that the fragment is sometimes supported by the edges of the hole where the RBC previously was, (ii) shows that more frequently the fragment falls to one side of the hole, and (iii) indicates the differential digestion of organic material by the NaOH solution; (d) shadowing of the replicon from the top.

Figs. 2 and 3. Metal-shadowed carbon replicons of OsO_4 -fixed RBC. The typical bump diameter is about 400 Å, though many larger bumps are present in Fig. 2. It is suggested that these may represent the fusion of the more regularly sized 400-Å bumps.

Figs. 4 and 5. Unshadowed carbon replicons. A small amount of organic material still remains after prolonged exposure to 0.145 M NaOH. This material is organized as bumps having a uniform diameter of 400 Å, and as a filamentous "yarn" which is often seen as a strand 200 Å, to 300 Å thick bisected by a thin bright line (presumably a region of high osmium concentration).

MUB-6057

Fig. 1

ZN-5221

Fig. 2

ZN-5220

Fig. 3

ZN-5222

Fig. 4

ZN-5219

Fig. 5

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