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A transmission electron microscope study of the effects of ion etching on cells

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SUMMARY

The effects of ion etching on blood cells have previously been studied by scanning electron microscopy. This present study by transmission electron microscopy was undertaken to evaluate the effects of the etching process on the cells. Critical point dried preparations were made, etched and subsequently processed and embedded in Araldite. Examination of thin sections of erythrocytes revealed disintegration of the plasma membrane; the residual membrane destruction products formed the tips of cones produced by long etching times. The effect of etching varied in erythrocytes in the same preparation. Nucleated cells showed a similar disintegration of the plasma membrane, but membranes of mitochondria, granules, vesicles and vacuoles did not exhibit effects of etching comparable to those of the plasma membranes. After treatment with a number of different fixatives, erythrocytes on carbon-coated copper grids were also etched and examined directly in a high voltage electron microscope at 1 MV. The effects were comparable to those seen in thin sections. To study the etch rates of biological materials, the resonant frequencies of quartz crystals were measured after application of thin films of albumen and cholesterol and again after these had been etched. The ratio of the frequency changes indicated that the etch rate of albumen was approximately 2·5 times that of cholesterol.

The results are discussed in the light of theories of the mechanisms involved in ion etching.

INTRODUCTION

Among the techniques developed for supplementing standard transmission and scanning electron microscopy in the study of cellular ultrastructure is that of ion etching (Lewis & Stuart, 1970; for bibliography see Spector *et al.*, 1974; Frisch *et al.*, 1974). Moreover, secondary ion emission microanalysis (ion probe analysis) has also been used to analyse the distribution of elements in biological cells and tissues (Galle, 1974). With the ion etching technique, the cells or tissues are subsequently coated with a thin layer of metal and examined in the scanning

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electron microscope. Three major difficulties have been encountered in the interpretation of etched cells.

(1) The cells are not observed directly but the metallic conductive coating is examined;

(2) Good resolution of cellular detail is not obtainable in the scanning electron microscope (SEM) at relatively high magnifications;

(3) It is frequently impossible to distinguish artefacts produced by the method from true cellular structures.

To overcome these problems etched cells have been processed for transmission electron microscopy (TEM). The results of a study by this procedure on erythrocytes and other blood cells are presented here. The findings provide an evaluation of ion etching applied to the study of cell structure. They are also relevant to the interpretation of results with secondary ion emission micro-analysis.

MATERIALS AND PREPARATIVE METHODS

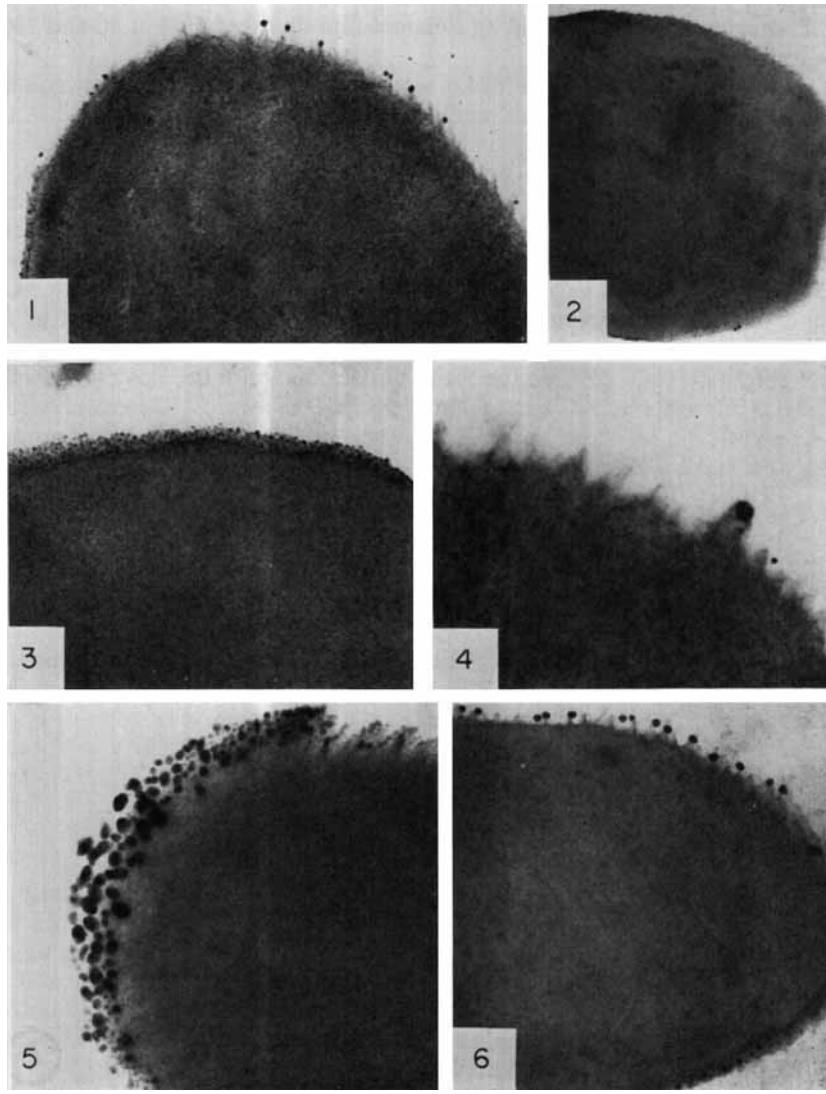
Venous blood from normal volunteers was used. The blood was drawn into a syringe with no anticoagulant, and then fixed directly in 2% glutaraldehyde in 0·1 M phosphate buffer, pH 7·3. In some cases, buffy coat concentrates were used. After a fixation period of at least 1 h at 20°C on a rotator the suspensions were centrifuged for 5 min at 3000 rev/min, the fixative removed and replaced by distilled water. This rinse was carried out three times. Drops of the cell suspensions were placed on glass coverslips, 10 mm diameter, on aluminium foil and on silver membranes (Flotronic). Some preparations were air-dried and others were processed by the critical point drying technique (CPD) (Anderson, 1951). Following fixation in glutaraldehyde and rinsing, cells were also osmicated in 1% osmium for 2 h, then rinsed again and drops of the suspensions were placed on carbon-coated copper grids and dried.

Cells were also fixed in Millonig's phosphate-buffered osmium tetroxide fixative; Luft's buffered permanganate fixative, and Dalton's chrome-osmium fixative (Glauert, 1967). The cells were then rinsed as described above and drops of the cell suspension were placed on carbon-coated copper grids and dried. Unfixed cells were also used; blood was diluted with 0·1 M phosphate buffer, pH 7·2, and the cells were dropped on carbon-coated copper grids and dried.

Etching was carried out in a radio frequency (rf) sputtering unit at 13·6 MHz in argon at pressures of $1–2 \times 10^{-2}$ Torr as measured by a Pirani gauge, and at power densities of 50–100 mW mm^{-2} for 3, 4, 6, 8, 9 and 10 min. Some preparations on aluminium foil, glass coverslips and silver membranes were then processed through absolute alcohol and propylene oxide and embedded in Araldite. The aluminium foil and the silver membranes were peeled off after the Araldite was cured. The glass coverslips were removed by thermal shock with liquid nitrogen. Thin sections were cut, stained with lead citrate and uranyl acetate, and then examined in an AEI 6B transmission electron microscope at 60 kV. Photographs were recorded on Ilford type EM 6 photographic plates at 1 sec exposure times.

Cells on carbon-coated copper grids from each type of fixative as well as unfixed cells were etched for 3 min. The grids were then divided into two sets, one was left unstained and one set was stained with lead citrate-uranyl acetate. The grids were examined in an AEI EM 7 at 1 MV. Photographs were recorded on Kodak 4489 EM film at 3 sec exposure times.

One preparation of each etching run was vacuum-coated with gold-palladium to a thickness of 35 nm and examined in the SEM (Cambridge S4) at angles of



Figs. 1–6. Erythrocytes etched for 4 min in argon.

Fig. 1. Note globules at the tips of some cones. × 54,000.

Fig. 2. Globules are not present on the tips of these cones. × 27,000.

Fig. 3. Fragmentation of the surface layer of the cells. × 54,000.

Fig. 4. Residual globule protecting cytoplasm beneath it. × 144,000.

Fig. 5. Edge of erythrocyte. The surface has disintegrated leaving globules of various sizes. × 90,000.

Fig. 6. Residual large globules on the upper surface of the erythrocyte; and fragmentation of its lower surface, not directly in the path of the impacting ions. × 54,000.

incidence of 0° and 45° of the primary electron beam to the surface of the specimen. Photographs were recorded on Polaroid film or Ilford FP4 at 40 and 100 s exposure times.

As controls, duplicate preparations of all etching runs were coated only and not etched. To check the effect of heat some specimens were placed upside down in the etching unit during the etching and subsequently coated; others were heated under vacuum or in air at 60°C, 90°C, 100°C and 120°C for periods of 10, 20, 60 and 120 min and then coated for comparison with etched and unetched specimens.

To measure the etch rates of biological materials solutions of 1% albumen and 1% cholesterol were used. A drop of each solution was placed on a 6 MHz quartz crystal of a quartz crystal microbalance and dried in air. The resonant frequencies of the crystals were measured to an accuracy of 10 Hz after application of the solutions. The crystals were then simultaneously etched on one electrode with control preparations of cells on the second electrode. After etching the resonant frequencies of the crystals were again measured. Between measurements the crystals were kept in a dessicator. The control preparations were coated and examined in the SEM.

RESULTS

Cells in thin section–erythrocytes

There was some variability in the degree of etching among red cells in the same preparation: in all the preparations which were examined, the red cell membrane was disintegrated by the impact of the ions and residual membrane destruction products, apparently more resistant, remained to form the tips of cones (Fig. 1). The surfaces of other erythrocytes showed no membrane remnants, and appeared smoother (Fig. 2). The superficial layers around the edges of many of the red cells had disintegrated into larger or smaller globules (Fig. 3). Occasionally adjacent cells showed a different etch effect; in one cell a relatively smooth removal of the surface material had occurred, in the other fragmentation was more evident. Sometimes the superficial layer of the cell had been removed leaving only an isolated globule (Fig. 4) indicating the depth of the etch. A striking feature was the variation in size of the 'globules'; in some cases they were very fine, in others quite coarse (Fig. 5). It is possible that cytoplasmic components, as well as membrane destruction products participated in the formation of the 'globules'. Disintegration of the cellular membrane was also seen along the lower edge, at a depth beyond that to which the ions had penetrated (Fig. 6).

In more heavily etched cells (6–10 min) residual membrane destruction products were only rarely found (Fig. 7). In most cases no membrane was

Fig. 7. The cellular membrane can be seen at edge on the left. × 135,000.

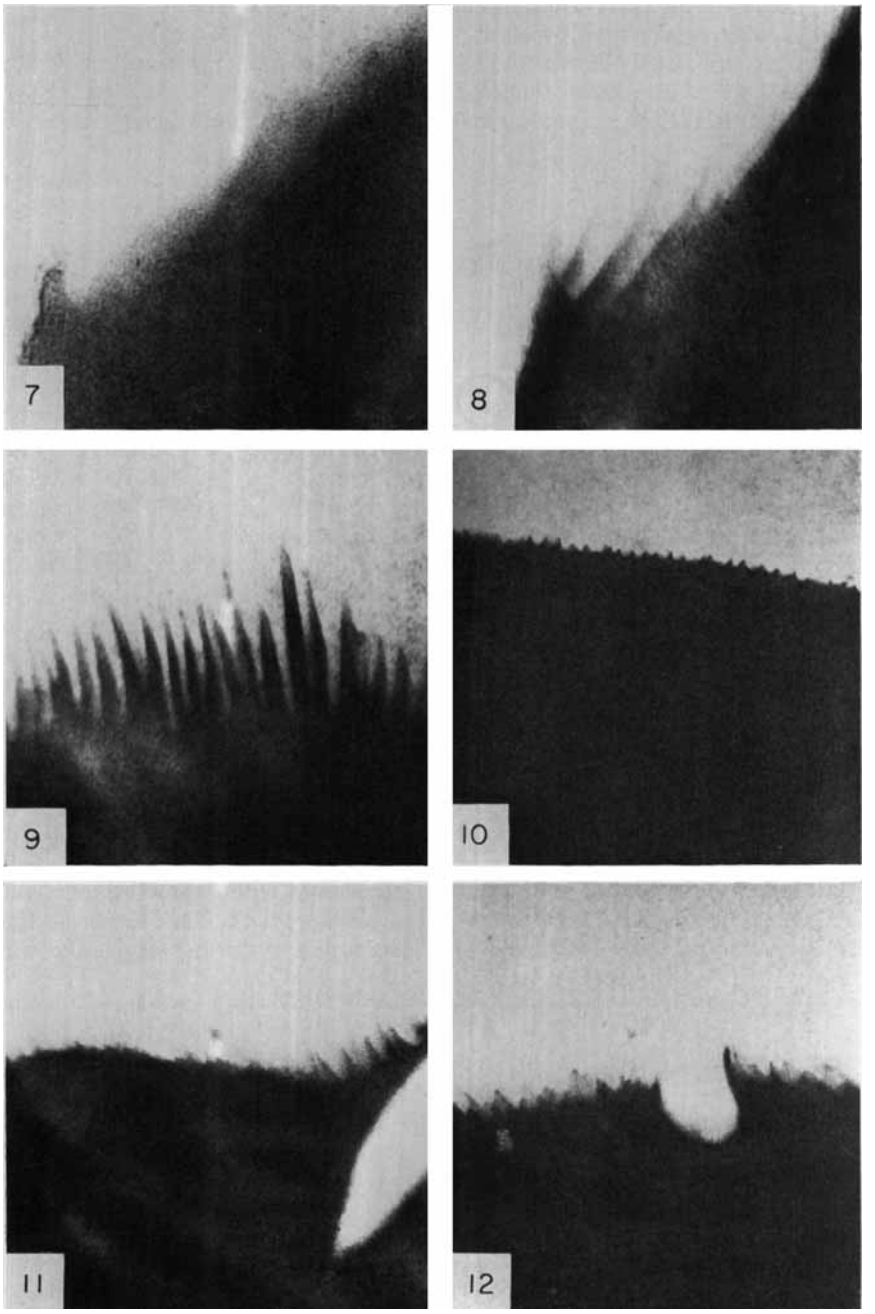
Fig. 8. Prominent cone formation at the edge of the cell. × 81,000.

Fig. 9. Erythrocyte surface showing tapered cones of varying height. × 81,000.

Fig. 10. Erythrocyte showing very short unevenly spaced stub-like projections. × 54,000.

Fig. 11. Erythrocyte exhibiting both short and long cones. × 54,000.

Fig. 12. The limiting membrane of the vacuole is evident. × 81,000.



Figs. 7–12. Erythrocytes etched for 6 min in argon.

identifiable (Fig. 8). Again, there was considerable variation in the surface pattern. In some cells long cones which were of variable width and height, separated by deep clefts, were found (Fig. 9). In other erythrocytes the etched surfaces were represented by short, stublike, unevenly-spaced projections (Fig. 10). There was also a difference in etching across their diameter in some erythrocytes, and a smooth erosion occurred side by side with cone formation (Fig. 11). Where the red cells contained vesicles or vacuoles, these were clearly visible after etching (Fig. 12).

There was no apparent difference in the appearance of etched red cells in the TEM when they had been processed from preparations air-dried on glass coverslips or on aluminium foil, compared to cells which had been critical point dried on silver membranes. Cells in preparations which were etched upside down were indistinguishable from unetched specimens which were only coated. Likewise, heated cells showed no discernible effects when they were subsequently examined in the SEM.

Nucleated cells

Only critical point dried nucleated cells were used for study in the TEM after etching. Light etching resulted in a fragmentation of the plasma membrane (Figs. 13, 14). In all cases globules were observed (Fig. 14), some of which formed the tips of cones (Fig. 15), irregularly distributed over the cellular surface (Figs. 15 and 16). In many instances intact membrane was seen along the sides of folds or projections (Fig. 15). Even after heavy etching, the cones were shorter, blunter and more widely spaced than those of the majority of erythrocytes. When cellular processes were long and thin, a membrane residue or destruction product at the top of the process frequently remained, though none could be seen on the cellular surface surrounding it (Fig. 17). Where the side of a cell lay normal to the impact of the ions the surface layer was removed, while the other side, not in in the direct pathway of the ions, showed disintegration of the plasma membrane (Fig. 18). The globular residues were mostly small, though occasionally larger ones were seen (Fig. 19). Sometimes these did not form the tips of cones, but appeared to lie directly on the etched surface (Fig. 20). In some cells, etching produced cone formations on the cellular projections with a smoother removal of surface material in the adjacent areas (Fig. 21). Where the cell had few projections, the etching produced cones, irregularly spaced, and of varying height and width

Fig. 13. Disintegration of the plasma membrane, but the etch has not yet penetrated into the cytoplasm. × 81,000.

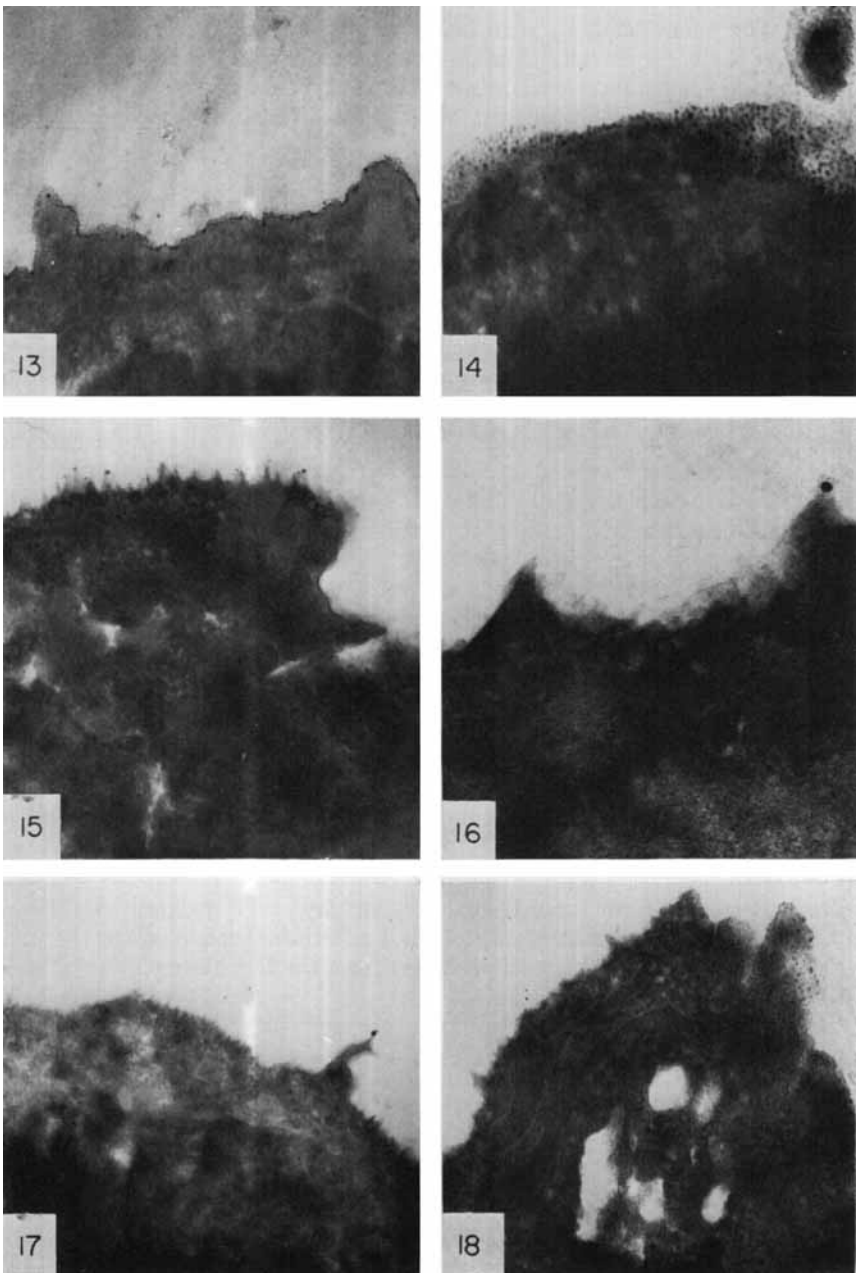
Fig. 14. Uneven fragmentation of the membrane with separation of the globules at right and left. × 81,000.

Fig. 15. Irregularly spaced cones, some with globules at their tips, are present on the cellular surface; relatively intact plasma membrane is seen within the fold at right. × 36,000.

Fig. 16. The cones are relatively widely spaced but have broad bases; few globules remain. × 76,500.

Fig. 17. Very slight cone formation is present on this cellular surface and only the tip of the projection shows a globule. × 27,000.

Fig. 18. The surface of this cell is eroded on the left and shows membrane fragmentation on the right. × 81,000.



Figs. 13–18. Nucleated cells etched for 4 min in argon.

(Fig. 22). When heavy etching resulted in removal of the top layer of the cell, the underlying organelles were exposed, for example, intracellular granules (as in Fig. 22), and mitochondria (Fig. 23) whose surfaces appeared rather evenly eroded. The same occurred with sheets of endoplasmic reticulum (Fig. 24). Etching of these intracellular membranes did not produce globules analogous to those formed from the plasma membranes, and possibly also from superficial cytoplasmic components, during the initial phases of etching.

Erythrocytes in HVM

The effects of etching on erythrocytes after fixation by different fixatives are illustrated in Figs. 25–32. Disintegration of the superficial cellular layer and cone formation were evident after all procedures. There was a striking heterogeneity in globular shape and size. 'Globules' were produced in the red cells etched after fixation by glutaraldehyde (Figs. 25 and 26), chrome osmium (Fig. 27) and osmium (Fig. 28). 'Globules' were less prominent in unfixed etched cells, but were observed after staining with lead citrate-uranyl acetate (Fig. 29); likewise potassium permanganate fixed etched cells also did not show globules before staining (Fig. 30), but they were made visible by the stain. Except for these two instances, staining served only to enhance features that were readily apparent in the unstained etched cells. As seen in the thin sections, etch effects varied in erythrocytes in the same preparation. Similarly also, relatively larger disintegration products were often observed over the outer convexity of the cell (Fig. 26). The etch 'pattern' over the central area of the erythrocytes appeared sharper and more clearly defined in glutaraldehyde-fixed etched cells than in erythrocytes prepared by the other fixatives or etched unfixed (cf. Figs. 31 and 32).

Measurements of the quartz crystal resonant frequencies

The results are shown graphically in Fig. 33. Comparison of the gradients of the curves shows that the etch rate of the albumen was approximately 2·5 times that of the cholesterol. Cells etched together with the crystals showed cones.

DISCUSSION

Previous studies from these laboratories and by other workers have demonstrated the effects of ion etching on biological material as seen in coated specimens in the scanning electron microscope. Apart from the few observations by Baker

Fig. 19. Residual globules at the tip of the cone to the left, while on the right an agglomerated globule spans the tips of several cones. × 108,000.

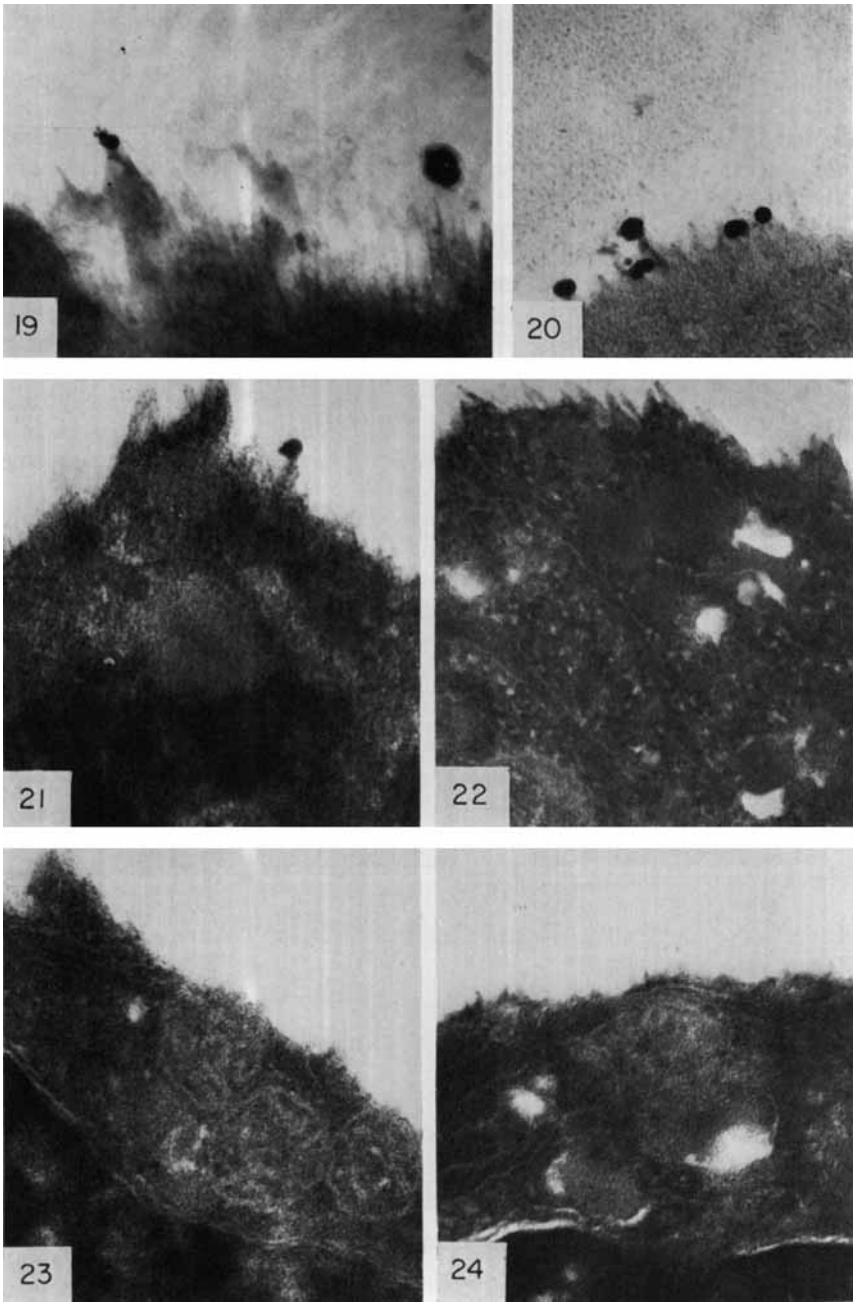
Fig. 20. Globules at different levels, some lying directly on the etched surface. × 90,000.

Fig. 21. Cones are more evident in the cellular projection than lateral to it. The globule appears larger than the cone forming its base, probably due to undercutting of the cone. × 90,000.

Fig. 22. Note penetration of etch into the granule, at right. It appears more eroded than the surrounding cytoplasm. × 54,000.

Fig. 23. Note fairly even erosion of cytoplasm and mitochondria. × 54,000.

Fig. 24. Erosion of endoplasmic reticulum. × 54,000.



Figs. 19–24. Nucleated cells etched for 6 min in argon.

(1969) made on thin sections of etched metal-coated erythrocytes, no detailed analysis of the surface structures of etched cells as seen in thin sections in the transmission electron microscope or of whole etched cells as seen in the high voltage microscope, has previously been published. It is clear that such a study is a prerequisite to understanding the effect of the etching process on the cells, and particularly if the method of analysis by secondary ion emission is to be applied to the investigation of the topographical distribution of elements within cellular membranes and cells. Because of the complexity of biological tissue, it is essential to obviate the physical factors which contribute to the etch effect before attempting to assess the biological implications of etching.

When plasmas are generated by radio-frequency discharges in oxygen, hydrogen, argon or other appropriate gases, they contain reactive atoms, ions and excited molecular species (Thomas & Hollahan, 1974). Many theories have been proposed to explain how target material is released by the impacting ions; these have included localized thermal sublimation, formation by trapped ions of gas bubbles which then erupt, and the development of volatile compounds, as well as *physical* sputtering, that is, the release of target atoms and clusters by direct momentum transfer or indirectly by the disturbance created by the ion in its passage in the target, resulting in energy being released at the surface by target atom recoil (Holland & Priestland, 1972).

In investigation of inorganic materials, the development of surface topographies such as cones, pits, hillocks and hummocks is generally explained by variations of the sputtering yield across the specimen surface due to initial compositional, crystallographic or topographical irregularities, i.e. more resistant surface inclusions (Fulker *et al.*, 1973; Barber *et al.*, 1973).

Another factor (of particular importance in the investigation of biological material) which may affect the speed of erosion is chemical bonding (Galle, 1974), for when a specimen is bombarded by ions, the intensity of secondary ion emission—and hence the amount of material removed from the surface—depends not only on the concentration of that particular element, but also on the strength of its chemical bonding to other elements in the sample. Both the sputtering yield for

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Fig. 25. Glutaraldehyde-fixed osmicated erythrocyte, unstained, top of outer edge. × 180,000.

Fig. 26. Glutaraldehyde-fixed osmicated erythrocyte, unstained, edge of outer convexity. × 180,000.

Fig. 27. Edge of erythrocyte fixed in chrome osmium prior to etching, unstained. Note variability in size of the globules. × 180,000.

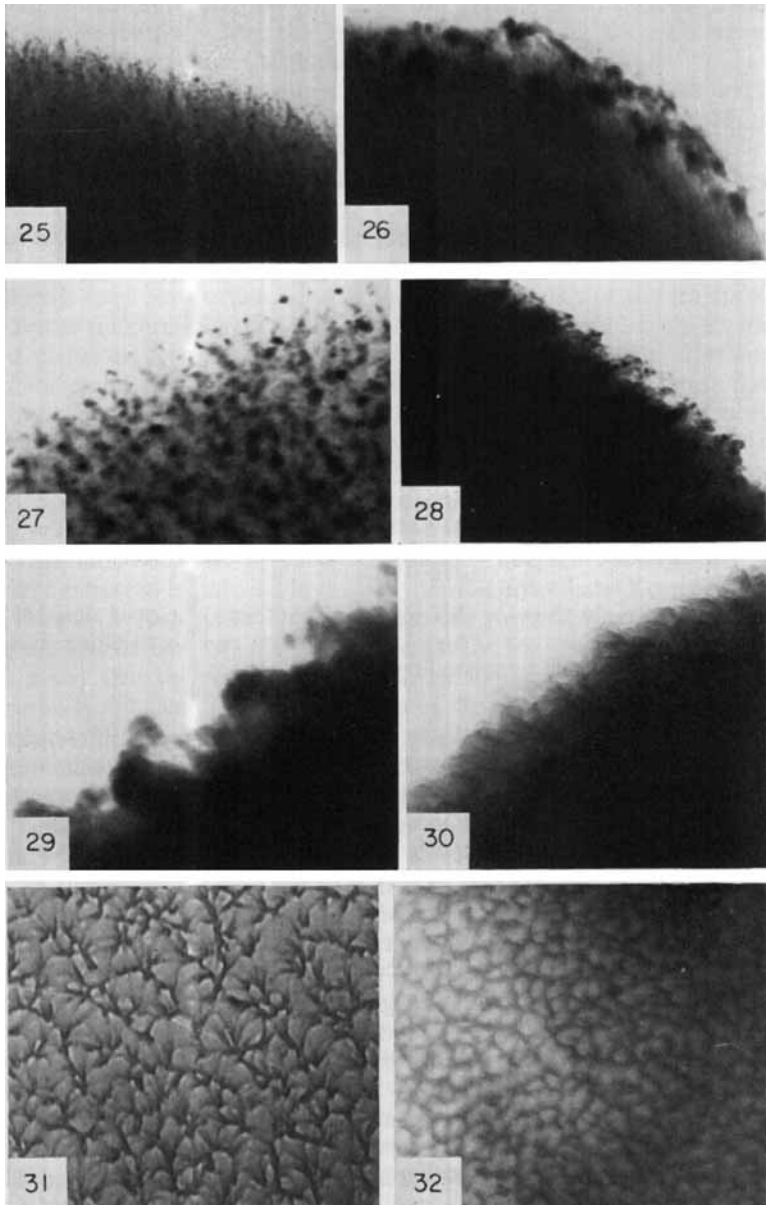
Fig. 28. Edge of erythrocyte fixed in osmium only, unstained. × 180,000.

Fig. 29. Unfixed erythrocyte, stained with lead-citrate-uranyl-acetate after etching. × 180,000.

Fig. 30. Potassium permanganate fixed erythrocyte, unstained, no globules evident. × 180,000.

Fig. 31. Glutaraldehyde-fixed erythrocyte; unstained 'face-on' view over central area. Note spikes, irregularly spaced. × 90,000.

Fig. 32. Osmium-fixed erythrocyte, unstained, 'face-on' view over central area, etch pattern representative of that seen in erythrocytes not fixed in glutaraldehyde. × 90,000.



Figs. 25–32. Erythrocytes etched for 3 min in argon, unprocessed, as seen in HVM.

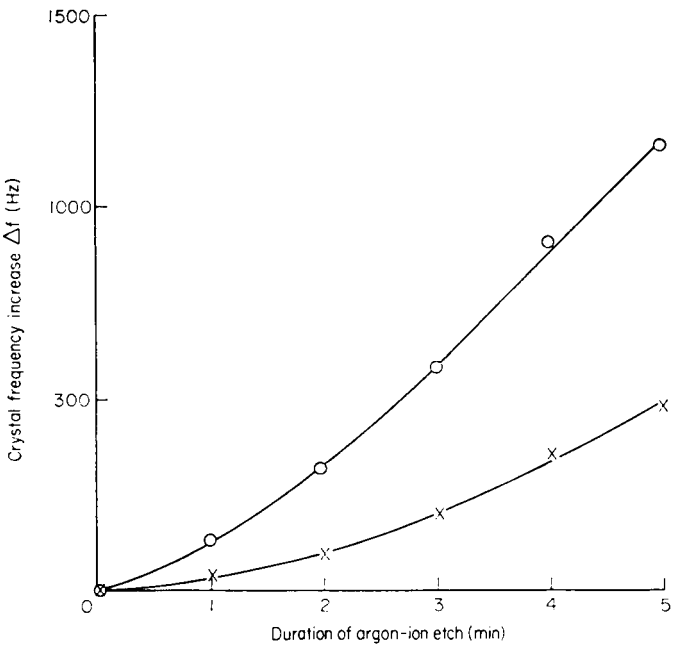


Fig. 33. Graphs showing changes in resonant frequencies of albumen- (○–○) and cholesterol- (×–×) coated quartz crystals resulting from removal of material by argon ion etching.

the specimen atoms and the secondary ion yield vary greatly for different elements (Andersen, 1969; Benninghoven, 1971). Chemical reactivity between impacting ions and target atoms must also be taken into account. Some of the mechanisms which are operative in the process of ion etching of inorganic materials have already been shown to be applicable to biological targets also. For example, a reactive gas, e.g. oxygen, has been used in the surface etching or complete 'ashing' of organic targets by the formation of volatile reaction products (Thomas, & Hollahan, 1974). Previous studies from these laboratories have shown that even with short etching times, up to 4 min, erosion of cells was greater with oxygen, water vapour or air, than it was when argon was used. With this latter gas chemical reactivity was minimal, if it occurred at all. Thus, the lack of uniformity of the etch between erythrocytes in the same preparation, or even across the diameter of a single red cell, points to differences in surface conditions. Erythrocytes in a sample of venous blood are representative of all cell ages from reticulocyte to senescent red cells and the cellular composition varies with age (Pennel, 1974). With increasing age there are decreases in the total lipid, cholesterol and phospholipid content of the cell, and young cells have different etch patterns from older ones (Lewis & Stuart, 1970). Data on the relative etch rates of biological material have not yet been established, but the results obtained with the quartz crystal resonant frequency measurement clearly indicate that protein and lipid etch at different rates. Earlier work on red cell membranes by Baker (1969) has shown that the appearance of lightly etched membranes is consistent with the membrane mosaic hypothesis. As pointed out by Baker, the number of plaques in red cell membranes, as measured by Hillier & Hoffman (1953), corresponds to the number of holes seen in erythrocyte membranes after light etching. In addition, studies

made on erythrocyte membranes in which the proteins had been aggregated by translational motion prior to fixation showed that these aggregates etched more rapidly than their surroundings (Frisch *et al.*, 1974). Taken together these data support the asssumption that the 'globules' produced by the etch are mainly lipid disintegration products of the surface layer of the cell.

The effects of ion etching on erythrocytes as seen in thin sections were substantially similar to those observed on the surface of whole, unprocessed, glutaraldehyde-fixed red cells as observed in the high voltage transmission mode. The fact that 'globules' were not prominent in unfixed cells and cells fixed by potassium permanganate indicates that the chemical changes brought about by fixation, (see, for example, Maunsbach, 1966; Korn, 1966; Hopwood, 1972; Kretzer, 1973), do effect the subsequent etch pattern. Recent studies on the freeze-etching of fixed cells have shown that fixation may alter the path of the fracture-plane during freeze fracture (Nermut & Ward, 1974). After glutaraldehyde fixation the fracture plane occurred between the membrane leaflets, while after osmium fixation the number of cross-fractured cells was increased, due presumably to interactions between the lipid molecules, as much of the protein is extracted. Glutaraldehyde does not cross link the lipids, which might account for the prominence of globules after glutaraldehyde fixation. The similarity between glutaraldehyde-fixed etched erythrocytes as seen in processed cells in thin section and the unprocessed cells, as seen in HVM, deserves comment. Normally, following fixation in glutaraldehyde, lipids are unchanged and are subsequently almost completely extracted by ethanol in the dehydration procedure (Korn & Weisman, 1966; Morgan & Huber, 1967). It appears that the globules produced by ion etching are surface components altered chemically, for instance by cross linking, in such a way that they are less soluble in ethanol and their extraction during dehydration is reduced. The disintegration of the superficial cellular layer could well be due to breaking of chemical bonds and removal of material by sputtering. The variation in size of the 'globular' destruction products could be brought about by two mechanisms; firstly, uneven surface composition which is influenced by or even due to fixation, and, secondly, recondensation of sputtered material. Membrane break-up in cellular areas, not directly in the path of the impacting ions, could be due to reflected primary or secondary ions or to other particles in the radio frequency system, such as electrons or excited energetic neutrals. Free electrons particularly are accelerated to high velocities in the oscillating electromagnetic field. The occurrence of large globules on the outer convexity of the red cell fits well with earlier observations on the location of cholesterol in the red cell membrane by Murphy (1965), who demonstrated by autoradiography that in erythrocytes the tritiated cholesterol was concentrated around the periphery of the biconcave discs. Cholesterol constitutes approximately 26% of the total red cell lipids (Nelson, 1967); in the mature red cell, all the cellular lipid resides in the membrane (Van Deemen & de Gier, 1974).

Analysis of the features of etched nucleated cells is complicated by the irregular cellular contours due to numerous processes and projections. In this situation, there will be a certain protective effect of the processes on some areas of the cellular surface on the one hand; on the other, there will be a simultaneous release of both membraneous and cytoplasmic components after the initial phase of etching. Where large 'blobs' were found on the surface of etched cells, these were probably due to undercutting of the cones during etching and not to melting effects because the blobs appeared first, and sharp cones were left after the blobs had been removed by further etching. It was remarkable that while the membranes of red cell vacuoles appeared to contain more etch-resistant areas, the outer

membranes of mitochondria, granules and sheets of endoplasmic reticulum did not clearly exhibit such properties. This indicated a difference in their composition at the molecular level. Such a distinction between plasma and intracellular membranes has, indeed, been shown biochemically (Van Deeman & de Gier, 1974); a particularly relevant example is the high level of cholesterol in plasma membranes, as compared to that of intracellular membranes.

CONCLUSION

The present study has demonstrated that the initial effect of etching depends on the composition of the superficial cellular structures and on their surface topography. Aggregates produced by the disintegration of the superficial cellular layer form the tips of cones propagated by longer etching. On the basis of the quartz crystal microbalance resonance measurements, we deduce that these aggregates (or globules) are predominantly lipid-derived.

ACKNOWLEDGMENTS

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