Summary. Using a simple glow discharge apparatus with a low vacuum, biological specimens were bombarded by ions of various energies and directions. Three kinds of human cells fixed and dried previously were etched by this method and were, after metal coating, observed by scanning electron microscopy.

The etched surface of erythrocytes was sponge-like, possibly suggesting an etch-resistant reticular structure in the cell. In the etched leukocytes the nucleus and cytoplasmic granules could be exposed. The internal structures of the spermatozoa and their abnormal forms could be revealed after etching as the acrosome, postnuclear cap and some other organelles showed their surfaces.

These results may support the usefulness of the application of ion etching to soft biological materials.

The impact of ions of high energy causes ejection of atoms from the surface of the material and the latter becomes etched. The rate of the atom ejection depends upon the mass and velocity, i.e., energy, of the ions on one hand, and upon the mass of the atoms to be ejected and the energy combining them to the substance of the material on the other hand. The images after etching thus may reflect the intracellular distribution of the physically and chemically "etch-resistant" structures.

The etching effects are usually examined with the scanning electron microscope (SEM). The recent advent of a field emission type SEM which gives secondary emission images of a resolution (30 Å) higher than the previous, thermal emission type SEM's (100 Å), seems to accelerate the studies of surface etching.

Attempts to apply the ideas of ion etching to soft biological materials have been made for several years in British laboratories. Lewis and his associates (1968, 1970) suggested a visualization of the interior structures of red blood cells. Human erythrocytes fixed in glutaradehyde were etched with ions of argon, hydrogen, oxygen or aqueous vapor in a radio-frequency ion source (13.5 mHz) with vacuum of $10^{-3}$-$10^{-6}$ Torr. By this method Lewis and his associates demonstrated a radial arrangement of ridges in the erythrocyte and considered it to be an intrinsic structure supporting the concave disc shape of the cell.

Recently Hodges and his associates (1972) applied the same method to some cultured animal cells and chicken erythrocytes and found cony projections and pores on the etched cell surfaces which seemed essentially identical among different cells. These structures were thus judged by the authors not to reflect any of the intrinsic structures or substance distributions in the cell.

Using the epithelium of the bovine urinary bladder and a kind of resin, polytetrafluoro-ethylene, as model material, Fulker and his associates (1973) demonstrated
that characteristic low angle cones are produced both in biological and non-biological materials after they are ion-bombarded in a uniform direction.

The cones are formed under inclusions and contaminations which are more etch-resistant than other parts, and do not indicate an intrinsic structure or substance distribution in the material. Fulkér and his associates then succeeded in demonstrating the intrinsic structures of the bladder epithelium, e.g., reticular ridges apparently corresponding to the tonofibrils in the cells, by rotating and tilting the materials during the ion etching.

In the present study we made a simple device to utilize the ion impact of glow discharge. Ions of various energies and directions produced in this device were applied to blood cells and spermatozoa. We believe that the SEM images obtained by this method indicate the usefulness of ion-etching in “digging out” the internal structures of cells.

Instrument

The instrument used in the present study is diagrammatically shown in Figure 1. By an alternating current discharge (50Hz) occurring between two aluminum plates in a chamber of low vacuum ($1 \times 10^{-1} - 5 \times 10^{-2}$ Torr), nitrogen, oxygen and some other ions in the air are moved in lower velocities and in considerably variable directions. The electrode voltage is about 700V, current about 2mA. The specimen put on one of the plates is thus mildly irradiated by the ions of different kinds, energies and directions.

Specimen Preparation

Red blood cells

Venous blood from normal humans was dropped in glutaraldehyde (1% in a 0.1 M phosphate buffer, pH 7.4) to be fixed for 2–3 hrs and the cells were rinsed with water. Either after postfixation in 1% $\text{Os}_2\text{O}_4$ or without this procedure, the cells were dehydrated in acetone to be air-dried on glass slides (TOKUNAGA et al., 1969). Some specimens were emulsified in amyl acetate and dropped on glass.

White blood cells

Venous blood, citrated, from a patient with chronic granulocytic leukemia was centrifuged and the buffy coat containing leukocytes was fixed in the same 1% glutaraldehyde, postfixed in 1% $\text{Os}_2\text{O}_4$ and air-dried through amyl acetate. A light microscope examination of the smears of the same blood indicated that numerous neutrophil, eosinophil and basophil leukocytes were contained.
Fig. 2. Human erythrocytes etched for 2 hrs with the present method, showing sponge-like structure of the cells. A cell on the right hand shows a "scaling-off" image in its concavity. $\times 13,000$

Fig. 3. An erythrocyte showing the porous etched surface. $\times 13,000$
Spermatozoa

Ejaculates of normal and sterile men in their 20's were, after liquification, dropped in the same glutaraldehyde fixative as described above (Fujita et al., 1970) and critical point dried on glass slides.

The specimens were put in the discharge apparatus and exposed to ions for 1–3 hrs.

The specimens were then coated with gold-palladium on a stage with a rotating and tilting movement in a vacuum evaporator.

Observation was made partly with a field emission SEM (Hitachi HFS-2) and partly with an ordinary type SEM (Hitachi HSM-2). Accelerating voltage of 20–25 kV was used.

Results

Red blood cells

The red blood cells etched by the present method retained the shape of biconcave disc but the entire surface appeared sponge-like with numerous pores (Fig. 2). In the concavity the pores were larger in size and number than in the thickened periphery of the disc (Fig. 3). The peripheral part thus was supposed to be more etch-resistant than the central portion. In a few cells a superficial layer of the cell was partly stripped off (Fig. 2), suggesting an etch-resistant surface layer of the cell.

![Fig. 4. A leukocyte before etching. × 20,000](image_url)
White blood cells

The SEM images of human leukocytes after the present fixing and drying method (Fig. 4) have been published elsewhere (Hattori, 1972).

The result of ion-etching in white cells was less uniform than in red cells. In some cells the superficial cytoplasm remained like a labyrinth, but in many other cells it was clearly removed and numerous granules were exposed. In some cells the granules were large (0.3–0.7 μ). They were mostly round but irregularly shaped and elongated (mitochondria?) ones were intermingled. These cells were thought to be either eosinophils or basophils (Fig. 5). Granules of smaller size (0.1–0.3 μ) corresponding to those of neutrophils were exposed less successfully. In the cell, for instance, shown in Figure 6, reticular remnants of cytoplasm overlap this type of granules.

In many cells large masses of substance corresponding to the nucleus were revealed. The cell in Figure 5, for instance, reveals a structure suggesting a bilobulated nucleus which makes it probable that this cell is an eosinophil. The surface of this structure is provided with many indentations which presumably correspond to nuclear pores.

Fig. 5. A probable eosinophil after 3 hrs etching. A bilobular nucleus and large cytoplasmic granules are shown. ×20,000
Fig. 6. A probable neutrophil. Those granules as indicated by arrows may correspond to the cytoplasmic granules exposed by ion-etching. × 20,000

Fig. 7.
Spermatozoa

The surface views of normal and abnormal human spermatozoa have been published elsewhere (Fujita et al., 1970).

After ion etching the plasma membrane and the underlying cytoplasm usually are removed from the spermatozoa. The acrosome and postnuclear cap are exposed and the groove between them appears much clearer than in natural spermatozoa (Fig. 7). The acrosome shows a roughly granular surface, whereas the postnuclear cap a more finely granular one. It is likely that these images do not represent the natural surfaces on the structures but their slightly etched phases (Fig. 7).

In some atypical spermatozoa ion-etching may expose the deformity of the acrosome and postnuclear cap. In Figure 8, for instance, a spermatozoon from a sterile male is shown whose postnuclear cap reveals its unique shape as its anterior end projects laterally. The globular masses attached to the posterior portion of the head

Fig. 7. Head of a normal spermatozoon etched for 2 hrs with the present method. The surfaces of the acrosome and postnuclear cap apparently are exposed. $\times 26,000$

Fig. 8. Head of an abnormal spermatozoon from a sterile patient. Note the unusual shape of the postnuclear cap and the cytoplasmic remnant, possibly mitochondria (C). $\times 26,000$
Fig. 9. Head and middle piece of a normal spermatozoon etched for 2 hrs. The partly torn middle piece shows a spiral structure probably corresponding to the mitochondrial sheath. ×26,000

Fig. 10.
presumably represent mitochondria which had been included in a cytoplasmic droplet before etching.

Most spermatozoa were torn at the proximal or distal end of the middle piece.

The middle piece appeared thinner than in natural spermatozoa probably due to the loss of superficial cytoplasm. A spiral configuration most likely corresponding to the mitochondria is often recognized (Fig. 9, 10).

In the main piece of the tail fine circular fibers ensheathing it were sometimes recognized (Fig. 10).

Discussion

As pointed out by FULKER and his associates (1973), etching with ions of high velocity and uniform direction causes the appearance of low angle cones in biological and non-biological materials.

In a preliminary stage of this study we etched red blood cells with argon ions in a higher vacuum ($10^{-5}-10^{-6}$ Torr) and with a higher electrode voltage (7 kV). Only cone-like and plate-like structures corresponding to the direction of ion bombardment could be produced by this method.

The figures of radiating plates demonstrated by LEWIS and his associates (1968, 1970) in their ion-etched red blood cells are suspected to be a modification of cone figures.

In the present method of mild ion-etching with low vacuum ($10^{-1}-10^{-2}$ Torr) and low electrode voltage (700 V) the ions run rather irregular courses and an etching effect similar to that obtained by FULKER (1973) by rotation of specimens is attained. Thus, the surface of red blood cells etched by this method looks quite natural. It is, however, unknown whether such a cavernous structure as shown in this study is really hidden in the red blood cells, either as a dense structure of support or as a heterogenous distribution of materials. In this respect, the etched images of leukocytes shown in this paper may provide more reliable evidence for the present method of etching, as the structures shown are those known to occur in the cells.

In the leukocytes we believe that the large, eosinophil and basophil and the small neutrophil granules could be digged out, though some other bodies such as mitochondria are probably intermingled. Cytoplasmic debris seems to remain on the granules, causing their granular surface appearance. Chromatin which, at least after OsO4 treatment, seems to be more resistant than other parts to ion impact, remains in the cell. The part of the nucleus lacking chromatin under the nuclear pores is etched rather deeply. The holes thus made may be too sparse for nuclear pores. We suppose that only a part of the nuclear pores are distinctly etched.

In human spermatozoa our result gives further evidence that some cell organelles are etch-resistant and can be exposed by ion etching. We could thus demonstrate the acrosome, postnuclear cap, mitochondria and circular filaments of the tail. This method thus may help in the morphological analysis of anomalous spermatozoa whose interior organelles are often difficult to detect by ordinary SEM observation.

Though the present paper demonstrates the etched structures of only a few cell kinds by only limited methods, it may serve to indicate the usefulness of ion-etching for cell research and stimulate study in this field using a variety of materials and techniques.

Fig. 10. A part of the tail of a normal spermatozoon. Mitochondrial sheath of spiral arrangement in the middle piece (upper right) is obvious. Ring fibers in the main piece of the tail may be visible on the lower left (arrow). ×52,000
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