SCANNING ELECTRON MICROSCOPIC OBSERVATIONS
OF BIOLOGICAL SECTIONS CUT ON A
"VIBRATOME" AND INCUBATED
IN MEDIA FOR ATP-ASE AND
ALP-ASE ACTIVITY
(SEM APPEARANCE OF THE VIBRATOME SECTIONS)

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The suitability of non-frozen sections, cut with a Vibratome, for cytochemistry was examined with a scanning electron microscope.

Sections, 10 μ to 50 μ in thickness, were prepared from unfixed tissue or tissue fixed with perfusion of glutaraldehyde solution. They were incubated in Wachstein-Meisel medium for ATPase or in Mayahara-Ogawa medium for ALPase. The sections were dehydrated and dried under vacuum or with the critical point drying method.

The surface of sections prepared from fresh tissue which was fixed after sectioning often was coated with blood cells and extracellular fluid.

Judging from the SEM appearance, the Vibratome sections were comparatively uniform in thickness but had fine chatter marks on their surface while sections cut on a Tissue Sectioner were irregular in thickness but provided topographic areas where SEM examination was not impaired by knife marks.

Deposits of lead-phosphate were conspicuous on the surface of the incubated tissue from duodenum but not on the cut surface of the section. Irregularly distributed aggregates of precipitate were also a common finding on the surface of the tissue but not on the cut surface.

The advantage of using non-frozen sections for histochemistry at the electron microscopic level is well known to histochemists. Of the devices available for cutting non-frozen sections or slices, the Smith-Farquhar Tissue Sectioner (Sorvall TC-2) has been regarded as the best instrument in the laboratory. In addition to this instrument, "Vibratome" (Oxford. Model G) has been introduced within the past few years. This new machine claims versatility in sectioning fresh tissue and in producing sections of uniform thickness with fixed tissue (10). These two capabilities are apparently advantageous for cytochemistry at the electron microscopic level because the limited speed of penetration of incubation medium into the fresh tissue blocks, and the irregularity in penetration of medium into the fixed tissue sections, have resulted in inconsistent reactions observed in ultra-thin sections.

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The uniformity in thickness of the sections has been evaluated by the transmission electron microscopic appearance of resin embedded sections (2, 10).

This communication will describe the SEM appearance of the cut surface of sections from glutaraldehyde fixed tissue and from unfixed material cut on a Vibratome to compare with that of sections cut with the Tissue Sectioner (3-6, 8,9). It also describes the characteristics of sections incubated in Wachstein-Meisel medium for ATPase (11) and in Mayahara-Ogawa medium for ALPase (7).

MATERIALS AND METHODS

Rats and hens were perfused through the heart with balanced Ringer solution followed by perfusion with, and immersion in, 3% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) containing 4% sucrose (380-390 mosml). Sections 10 μ to 50 μ in thickness were cut on a Vibratome. The optimal adjustment of the Vibratome, judging from the SEM appearance of the section, was as follows:

- i) Angle of the knife: 15°C or less.
- ii) Speed of the advancement of the knife: intermediate to slow.
- iii) Magnitude of vibration of the blade: intensive to intermediate.
- iv) Type of razor blades: A half of a double edge razor blade was better than the single edge injector type blade (Schick) for soft tissue, especially when strong vibration was employed.

Small blocks of tissue were mounted on the metal pan (5 cm $\times$ 5 cm $\times$ 0.5 cm) with Loctite (Loctite Co., Newington, Conn.), Cyanoacrylate I.S.-12 (Intercontinental Chemical Co., Dublin, Ireland), Cyanobond RP (Sumitomo Chemical Co., Tokyo) or Aronalpha (Sankyo Co., Tokyo).

Fresh tissue sections were postfixed with buffered glutaraldehyde solution. Some were then incubated for 30 min at 20°C in either Wachstein-Meisel medium (11) for ATPase activity or Mayahara-Ogawa medium (7) for ALPase activity. Some were then postfixed in 2% osmic acid for one hour. The sections were thoroughly washed and dehydrated in a series of ethanol and propylene oxide. The dehydrated sections were dried under vacuum (Edwards freeze dryer Model EPD 2) or with a critical point drying method which employs isoamyl acetate-CO₂ exchange. With this method, the dehydrated sections were immersed in isoamyl acetate for 15 to 30 min and closed in a metal chamber. Liquid carbon dioxide was introduced into this chamber at a pressure of 50 to 70 kg/cm². The chamber was closed and warmed up to 25°C for 20 min, and then to 50°C for 10 min, during which the pressure gage indicated 60 to 70 kg/cm² and 70 to 90 kg/cm² respectively. The pressure was reduced to atmospheric within 60 min by gradually letting the gas out.

The dried sections were mounted on a copper stage with silver paste and coated with evaporated carbon and gold or gold-palladium (8 : 2) alloy. Electron micrographs were taken by a JEOL U-3 SEM at 25 KV with an original magnification of 50 to 10,000 $\times$.

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*** Designed by Dr. K. Tanaka, Dept. Anatomy, Tottori University and manufactured by Yonago Kiko, 2710 Yomi-cho, Yonago Tottori 683.

RESULTS

i) Comparison of the surface of the sections cut on a Vibratome with those cut on a Tissue Sectioner (Figs. 1-3).

As indicated by the SEM appearance of the sections of hen oviduct, the "Vibratome" was better than the "Tissue Sectioner" for producing sections of uniform thickness. On the other hand, the cut surfaces of the Vibratome sections were marred by chatter marks caused by the vibration of the knife. These marks appeared, more or less, constant but should not affect the interpretation of an ultrathin section, which is actually a cross or oblique section of the Vibratome section. The Tissue Sectioner produces sections which are somewhat irregular in thickness but their surfaces have topographic areas which are unimpaired with knife marks, thus are better for SEM preparation. The differences between the two instruments are illustrated in Fig. 3.

The versatility of the Vibratome in cutting thin sections was superior to that of the Tissue Sectioner but this difference did not affect the surface features of the sections observed with the SEM.

ii) Optimal adjustment of the Vibratome and preparation of specimen for SEM.

For soft tissue such as the cerebrum, liver, duodenum and oviduct, the chatter marks on the surface of the sections as judged from their SEM appearance, could

FIG. 1. A cross section of the albumen-secreting region (magnum) of the hen oviduct cut on a Vibratome, dried under vacuum, and coated with evaporated carbon and gold-palladium alloy. $\times 400$.

FIG. 2. A section of the albumen-secreting region of the hen oviduct cut on a Tissue Sectioner otherwise treated the same as the section in Fig. 1. $\times 400$.

FIG. 3. A diagrammatic illustration of the different feature of cross sections cut on the Vibratome and the Tissue Sectioner. Note the uniformity in thickness and the tiny chatter marks on the surface of the Vibratome section. Considerable irregularity in thickness but topographical flatness of the surface are characteristic of the tissue cut with the Tissue Sectioner.

be decreased by adjustment of the Vibratome knife. In addition to the conditions described in "Materials and Methods", differences in preparation also had an effect on the SEM appearance of the sections.

a) Versatility of the critical point drying method: (Figs. 4 and 5)

This method (1) is claimed to prevent deformation of the SEM appearance of blood cells and to preserve the cilia standing on cell surfaces. The certain effect of this method was also evident on sections cut either with a Vibratome or a Tissue Sectioner. Figs. 4 and 5 illustrate the SEM appearance of the surface of cerebral cortex section dried under vacuum and with the critical point drying method.

b) Differences in thickness of the Vibratome sections: (Figs. 6 and 7)

Figs. 6 and 7 are sections of different thickness of the rat liver, $10\ \mu$ and $50\ \mu$ in thickness respectively. As previously noted in (i) the differences in the SEM appearance between the two sections were minimal, if any.

iii) Unfixed sections cut on a Vibratome. (Figs. 8–11)

It is more difficult to cut fresh tissue sections with a Vibratome than was anticipated. The degree of difficulty is dependent upon the differences between tissues. The central nervous system was one of the more suitable tissues for cutting fresh sections as mentioned by Ago and Ogawa (2). The liver (Figs. 8, 9, and 10) was comparatively difficult to cut, and $20\ \mu$ in thickness was the minimum that could be achieved with this instrument. The SEM appearance of the cut surface of the liver was contaminated with blood cells indicating the necessity for perfusion with a balanced salt solution prior to sectioning (Fig. 8). Furthermore, the surface

was often coated with extracellular fluid. Quick postfixation of the surface and drying under vacuum could not preserve the fine structure of the fresh sections (Fig. 9). Attempts at digestion with enzymes and light ultra-sonication to remove masking substances from the surface of the sections, have not been satisfactory. Freeze-drying of the section (Fig. 10) was occasionally encouraging in this respect but its usefulness for electron microscopic cytochemistry requires further evaluation. Other type of unfixed tissues, such as rat intestine (Fig. 11), provides smooth cut surfaces but no cellular structures could be identified in these areas.

iv) SEM appearance of Vibratome sections incubated in the substrate media (Figs. 12–15).

The resolving power of the SEM, approximately 100 to 200 Å for the type used in this investigation (JEOL U-3), is ten times less than that of the TEM. Observation of the reaction products of enzymatic activity with the SEM is also hampered by the necessity for metallic coating of the specimen. The present investigation was intended to evaluate the type of the deposits of the surface of the intestinal villi and the appearance of the cut surface after incubation in a solution containing lead.

FIG. 4. A section of the rat cerebral cortex perfused with buffered glutaraldehyde, cut on a Vibratome, dried under vacuum, and coated with carbon and gold. N: nucleus. $\times 2200$.

FIG. 5. A Vibratome section of the same tissue as in Fig. 4, dried with the critical point drying method. N: nucleus. $\times 3000$.

FIG. 6. A comparatively thin section ($10\ \mu$) of rat liver perfused with buffered glutaraldehyde solution and dried with the critical point drying method. $\times 330$.

FIG. 7. A thicker section ($50\ \mu$) of the same tissue as in Fig. 6. The difference due to the variation in thickness was not evident in the SEM appearance. $\times 330$.

FIG. 8. A thick section ($50\ \mu$) prepared from the fresh rat liver. The section was postfixated with glutaraldehyde and dried with the critical point drying method. The cut surface is contaminated with blood cells (arrows) and coated with extracellular fluid. $\times 330$.

FIG. 9. A fresh section of rat liver, cut on a Vibratome, dried under vacuum and quickly fixed in buffered glutaraldehyde. Any cellular structures but some indications of cell boundary (arrows) are not identifiable. $\times 3300$.

FIG. 10. A fresh section of rat liver, freeze-dried, and coated with carbon and gold. C: a central vein. $\times 2200$.

FIG. 11. A fresh section of rat duodenum. Note the uniformity in thickness. The cut surface is clear and smooth. No cellular structures are identified in this area. $\times 120$.

FIG. 12. A Vibratome section of rat duodenum incubated for 30 min at 20°C for the ALPase reaction. The surface of the microvilli are coated with precipitates. $\times 1000$.

FIG. 13. A Vibratome section of rat duodenum incubated for 30 min at 20°C for the ATPase reaction and postfixated with osmic acid. The distribution of lead salt on the luminal surface of the intestine was the same as that in Fig. 12. $\times 440$.

FIG. 14. A control section of rat duodenum boiled for 5 min prior to incubation for the ATPase reaction. Only few precipitates (arrows) are found on the surface of the villi. $\times 600$.

FIG. 15. A control section of rat duodenum incubated in substrate-free medium. Some precipitate still remain on the luminal surface but the number of precipitates is significantly reduced. $\times 150$.

Precipitation of lead-phosphate on the surface of the duodenum was exactly the same in both the ALPase reaction (Fig. 12) and the ATPase reaction (Fig. 13). In addition to the precipitation of the metallic salt, some irregular aggregates of precipitate were distributed over the surface of the microvilli. The significance of these deposits is unknown but it should be mentioned that boiled sections (Fig. 14) and the sections incubated in substrate-free medium (ATP sodium salt or β -glycerophosphate was omitted) had only minimal deposits of lead salt on their surfaces (Fig. 15).

DISCUSSION

The differences observed with the SEM between the sections cut on the Vibratome and those cut with the Tissue Sectioner are logical considering the different principles employed in the mechanisms of these two instruments. The principle of sectioning with the Vibratome is based on low frequency vibrations imparted to the knife along its longitudinal axis in the plane of the section, while the Tissue Sectioner employs a chopping mechanism. The differences in the surface features therefore are comparable to the differences between the surfaces of freeze-etched and freeze-fractured tissue.

The usefulness of the Tissue Sectioner for investigating intracellular structures with the SEM has been reported by Makita and Sandborn (3, 4), Makita and Kiwaki (5, 6) and Sandborn and Makita (8, 9). The Vibratome has no advantage over the Tissue Sectioner for this purpose since the Vibratome sections are, more or less, marred with chatter marks caused by the vibrations of the knife.

As stated in the instruction booklet for the Vibratome, the setting of amplitude, speed, and blade angle for each type of tissue is determined initially by "trial and error". Ago and Ogawa (2) described a method for adjustment of this instrument. The present findings however suggest that a slightly greater magnitude and speed of the knife are optimal to minimize chatter marks on the surface of the sections. The use of a double edge razor blade allows an increase in the amplitude of vibration without fracturing delicate tissue, including fresh tissue.

It is claimed that sections of fresh tissue of acceptable thickness can easily be cut with the Vibratome. In our experience it is much easier than with the Tissue Sectioner but it nevertheless remains difficult. The lower limit of thickness for fresh tissue sections appeared to be 10 μ at best. Smith (10) indicated that sections of less than 20 μ in thickness cut with the Vibratome were of superior quality. This difference was not noticeable in the SEM appearance of the Vibratome sections. In attempting to cut thinner fresh sections, the following procedures provided occasional encouraging results:

- i) Embedding the tissue in 6–7% agar on the rough surface of the metal pan and while cold (approximately 4°C) quickly processing the tissue block.
- ii) Mounting fresh tissue blocks on the metal pan with an excess of adhesive reagent for embedding. The effect of the chemicals on the enzyme activity has not been evaluated.
- iii) Embedding a piece of the fresh tissue in a large block of fixed tissue. The vibration of the blade should be minimized since the buried tissue is likely to

be dug out from the surrounding fixed tissue. This is much more difficult than in cryostat sectioning.

iv) For some visceral tissues, holes punched by a piece of thin but solid shipper were made and the shipper which contains the fresh tissue was mounted and cut with an injector type single razor blade with an intensive magnitude of vibration.

The SEM appearance of the incubated sections represents only the profile of the reaction products. The principle of electron microscopic cytochemistry is to examine the cross sections of the Vibratome preparations. The histochemistry of tissue examined at the light microscopic level, however, is essentially the same as that seen in the SEM appearances. It has been noted that the precipitate observed may include lead itself, lead-substrate complex and some others, so it is probable that not all the deposits encountered here are lead-phosphate as expected for the final product of the reaction. However these precipitates are considerably decreased in number when the sections are incubated in substrate-free media or boiled for several min prior to incubation. The details of these results will be described elsewhere.

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