Scanning Microscopy

Volume 7 | Number 1 Article 12

12-14-1992

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Scanning Microscopy, Vol. 7, No. 1, 1993 (Pages 107-114)
Scanning Microscopy International, Chicago (AMF O'Hare), IL 60666 USA

0891-7035/93$5.00+.00

THE USE OF SONIC FREQUENCIES AS A CLEANING AGENT OF SPECIMENS TO BE OBSERVED BY SCANNING ELECTRON MICROSCOPY

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(Received for publication July 15, 1992, and in revised form December 14, 1992)

Abstract

The presence of mucus and/or cellular debris can obscure the fine morphology of the gastrointestinal or respiratory luminal surface, when observed by scanning electron microscopy. With the intent of obtaining a good cleaning of the mucosal surface without altering the ultrafine morphology of epithelial cells, a new model of sonicator/ultrasonicator is presented.

The instrument is supplied with a control system for wave frequency, amplitude and form, and permits a precise regulation of the wave energy. With this instrument it is possible to produce a "cleaning effect" by using any kind of frequency (either sonic or ultrasonic) and/or amplitude and/or waveform and/or liquid.

We report the application of sonic frequencies through water as a fluid for immersion to obtain a gentle and slow removing of mucus and in order to explore the possibility to clean hydrated tissues.

With the employment of sonic frequencies (from 5 to 15 kHz modulated by 200 Hz) and water as the immersion fluid, we were able to generate a gentle wave energy which effected an optimal removal of the mucus, with the consequent exposure of a well preserved epithelial surface of rat trachea and small intestine.

Key Words: Scanning electron microscopy, ultrastructure, specimen preparation, tissue exposure, ultrasonication, sonic frequencies, epithelium, intestine, trachea, rat.

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Introduction

Conventional scanning electron microscopy techniques do not permit a satisfactory view of the gastrointestinal or respiratory mucosal surface, because of the presence of mucus and/or cellular debris. In fact, at high magnification, these materials can obscure the fine surface morphology of epithelial cells.

Several studies proposed different cleaning procedures, besides washing, such as ultrasonication (Takagi *et al.*, 1974), brushing (Zalewsky and Moody, 1979), the use of enzymes followed by mechanical agitation (Wood and Dubois, 1981) and rubbing of the mucosal surface with gloved fingers (Al-Tikriti *et al.*, 1986). These methods may have side effects which mainly result in a surface damage of the luminal aspect of epithelial cells detectable at high resolution. The mechanical procedures such, as digital rubbing, may induce flattening of microvilli and/or epithelium removal as well as alteration of the cellular microtopographic relationships. In addition, this technique allows only occasional good cleaning because it cannot be standardized. Chemical digestions using enzymes, though specific, are not always reliable, and are used on unfixed, fresh samples. Treatment performed with ultrasonic instruments commonly known as "tank cleaners" elicited a better selective microdissection of biological tissues (Highison and Low, 1982; Low and McClugage, 1984; Low, 1989).

The use of "tank cleaners" should not leave some physical characteristic of the sonic-ultrasonics waves out of consideration (Apfel, 1981). The frequency of the waves (i.e., the amount of kHz employed) has been usually considered crucial for obtaining an acceptable surface cleaning without significant cellular damage. However, many other factors, such as the amplitude, the morphology of the wave, the temperature and the liquid used, either at sonic or ultrasonic level, affect the quality of dissection. With the conventional "tank cleaners" having a standard ultrasonic frequency (20-80 kHz) and amplitude, it is necessary to increase or decrease the time of application (within short limits) to vary the microdissection power of these instruments. Furthermore, acetone is the liquid considered useful for the best microdissection (Low, 1989).

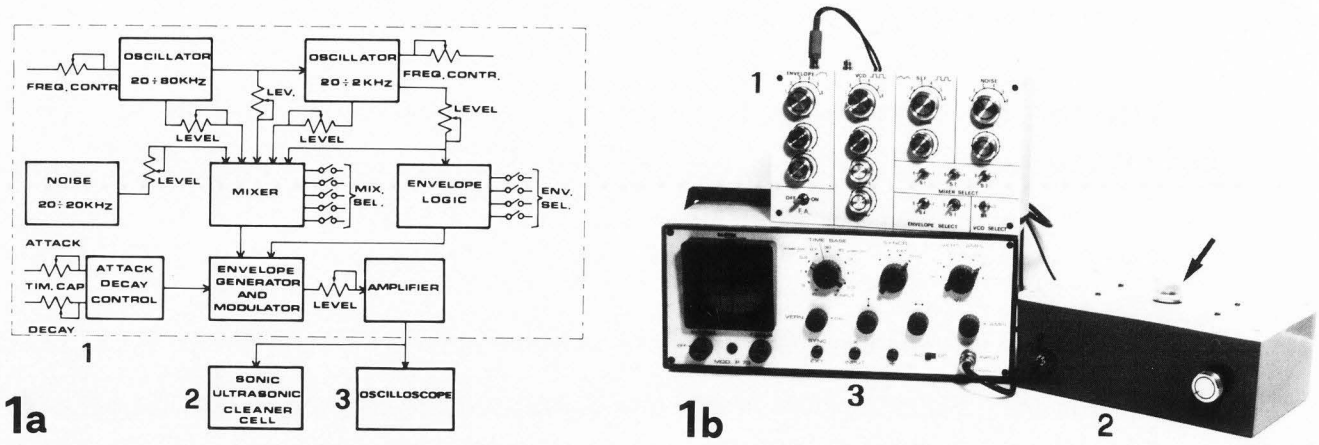


Figure 1. The instrumentation: **(a)** Block diagram of the sonicator/ultrasonicator; **(b)** photograph of the apparatus showing energy control system (1); sonic/ultrasonic cleaner cell (2), with specimen (arrow); and oscilloscope (3).

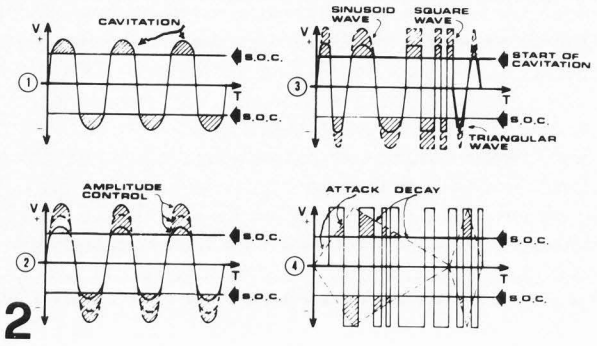


Fig. 2. Waveform diagram: **[1]** conventional ultrasonic waveform; **[2]** conventional ultrasonic waveform with power control; **[3]** waveform with amplitude, frequency and waveform control; and **[4]** envelope control system.

With the intent to obtain a fine cleaning of the mucosal surface without altering the ultrafine morphology of epithelial cells, a new model of sonicator/ultrasonicator is herein presented. The instrument was planned to allow a precise regulation of all physical parameters and of the energy applied to the sonic or ultrasonic wave.

In this study, with the application of variable energies, we used sonic frequencies and distilled water that yielded a fine exposure of the epithelial cell surface of trachea and small intestine in rats.

Materials and Methods

The instrument

The sonicator/ultrasonicator was provided with a control system for wave frequency, amplitude and form.

The device consists of two oscillators: the first (with a frequency ranging from 20 Hz to 80 kHz) producing two waves: a square wave applied to a mixing device and a triangular wave modulating the frequency of a second oscillator (with a frequency ranging from 20 Hz to 2 kHz).

A mixer system mixes the signal of the second oscillator with the square wave of the first one. The mixer system outlet is regulated by an envelope generator and modulator of the waveform by which it is possible to change the waveform symmetry and the "attack" and "decay" times of the envelope. The apparatus is also provided with an oscilloscope for monitoring the waveform (Figs. 1 and 2).

Experimental protocol

Primary fixation. Samples of rat trachea and intestine were immersed in 2.5% glutaraldehyde in phosphate buffer (0.1 M at pH 7.4) for 1-3 days. Then, half of the samples was sonicated and the other half was used as control.

Sonicated samples. The following schedule was used:

[1]. Washing in distilled water with sonication at frequency of 5-15 kHz modulated by 200 Hz for 2 hours at room temperature (20-25°C).

[2]. Post-fixation in 1.0% osmium tetroxide in distilled water with sonication at frequency of 5-15 kHz modulated by 200 Hz for 2 hours at room temperature (20-25°C).

[3]. Washing in distilled water with sonication at frequency of 5-15 kHz modulated by 200 Hz for 20 minutes at room temperature (20-25°C).

[4]. Impregnation with 1.0% thiocarbohydrazide and 1.0% osmium tetroxide in distilled water according to Kelley *et al.* (1973).

[5]. Dehydration in a series of ascending concentrations of acetone.

[6]. Critical point drying in liquid carbon dioxide.

[7]. Observation in a field emission scanning electron microscope (SEM, Hitachi FE-S4000), operating at 5-10 kV using secondary electrons and with a working distance of 5-9 mm.

[8]. After SEM observation, selected samples of both trachea and small intestine, were cut in smaller size, immersed in pure acetone for 2 hours, embedded in

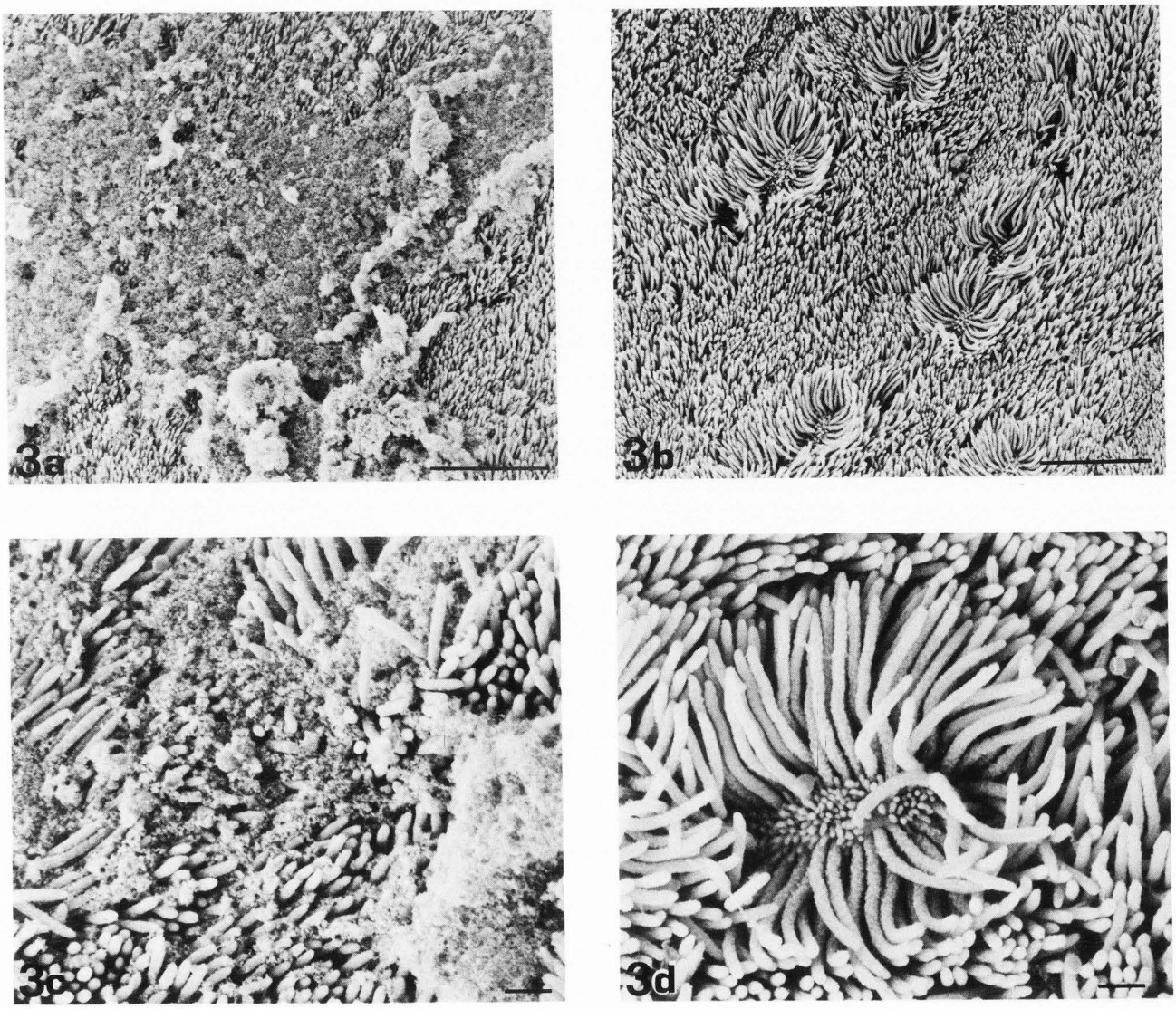


Figure 3. Rat trachea; scanning electron micrographs. (**a, c**). Control samples: a fine coating material partially covers the mucosal surface. (**b, d**) Sonicated samples: the mucosal surface is completely cleaned without superficial damage. Bars = 10 μm (in a, b) and = 1 μm (in c, d).

an epoxidic resin, and sectioned in a Reichert ultramicrotome. Unstained ultrathin sections were observed in a Zeiss EM-150 CR transmission electron microscope (TEM).

Control samples. The following steps were used.

[1]. Washing in running tap water for 2 hours at room temperature (20-25°C).

[2]. Post-fixation in 1.0% osmium tetroxide in distilled water for 2 hours at room temperature (20-25°C).

[3]. Washing in distilled water for 20 minutes at room temperature (20-25°C).

Steps **4-8** were the same as in the schedule for sonicated samples.

Results

Control samples

Usually, using conventional scanning electron microscopy techniques, only a few areas of the epithelial surface of both intestine and tracheal mucosae appeared clean (Figs. 3a and 4a). In fact, coating mucous material was present on the major extension of the epithelium surface, having the aspect of a fine superficial veil.

Such material, at high magnification, showed a fine meshwork covering the tips of the microvilli and the cilia, masking their fine morphology (Fig. 3c). Cell borders appeared as slight depressions and were detected with difficulty; goblet cells in the intestinal mucosa were often filled with mucous secretion (Fig. 4c).

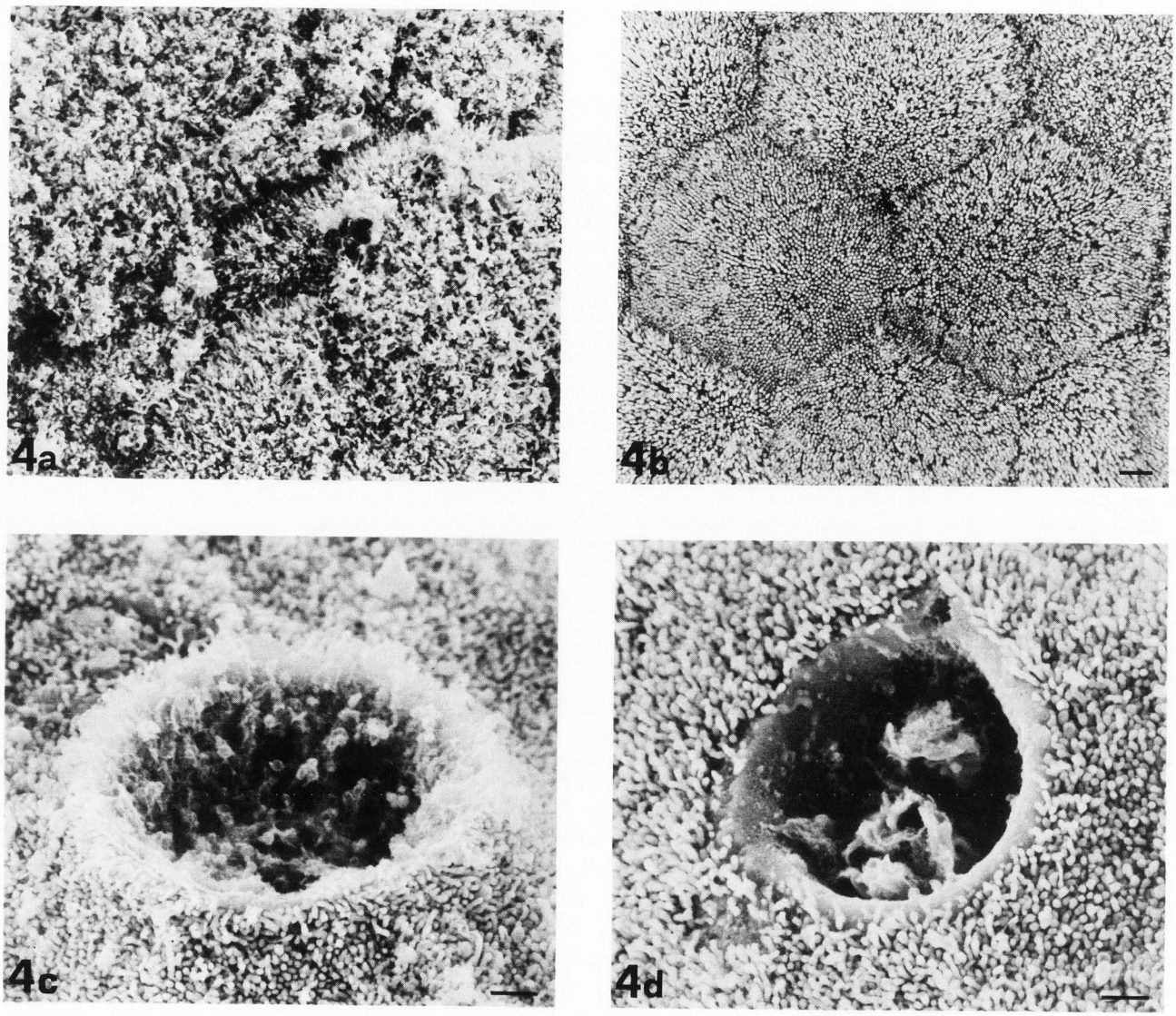
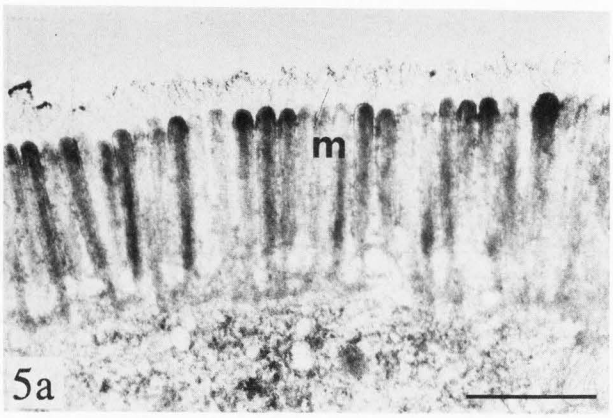


Figure 4 (above). Rat small intestine, scanning electron micrographs. **(a, c)** Control samples: a fine coating material masks the surface tips of microvilli. A goblet cell opening (in Fig. 4c) is filled with mucous secretion. **(b, d).** Sonicated samples: The mucosal surface is completely cleaned. Note in Fig. 4b the hexagonal shape of the enterocytes. Goblet cell opening (in Fig. 4d) is devoid of secretion allowing the exposure of the epithelial cell surface; within the opening some cellular residuals are evident. Bars = 10 μm (in a, b) and = 1 μm (in c, d).

Figure 5 (facing page, top). Rat small intestine. Transmission electron micrographs of samples processed after SEM observation. **(a, c)** Control samples; microvilli (m) with their filamentous extracellular coat. **(b, d)** Sonicated samples; microvilli devoid of the extracellular coat. At higher magnification, note the integrity of the double-layered plasma membrane. The electron-dense area at the top of microvilli is due to the thiocarbohydrazide-osmium impregnation. Bars = 1 μm (in a, b) and = 100 nm (in c, d).

Figure 6 (facing page, bottom). Rat trachea. Transmission electron micrographs of samples processed after SEM observation. **(a)** Control sample; cilia with their filamentous mucus covering. **(b, c)** Sonicated samples; cilia devoid of the mucous covering. At higher magnification the plasma membrane is preserved; a fine surface granularity is present (*arrows*). Bars = 1 μm (in a, b) and = 100 nm (in c).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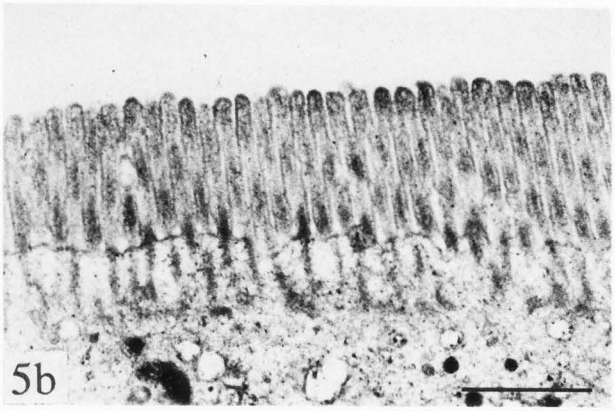
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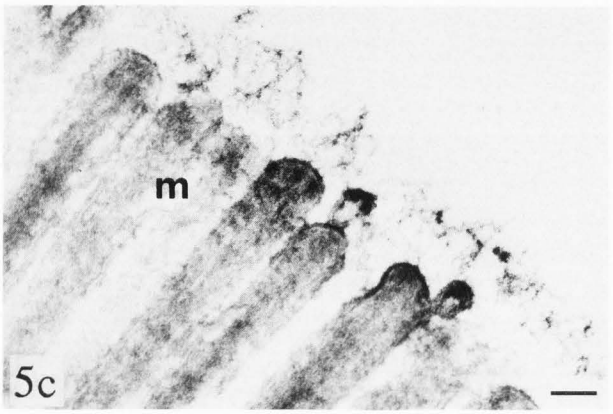
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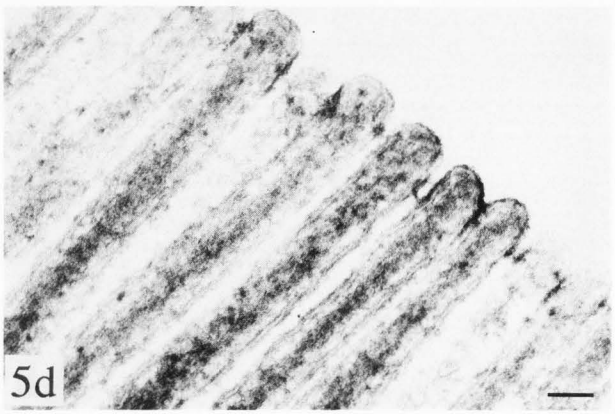
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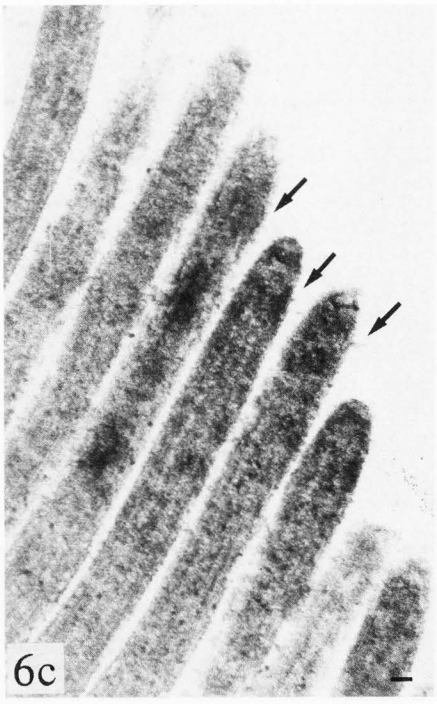
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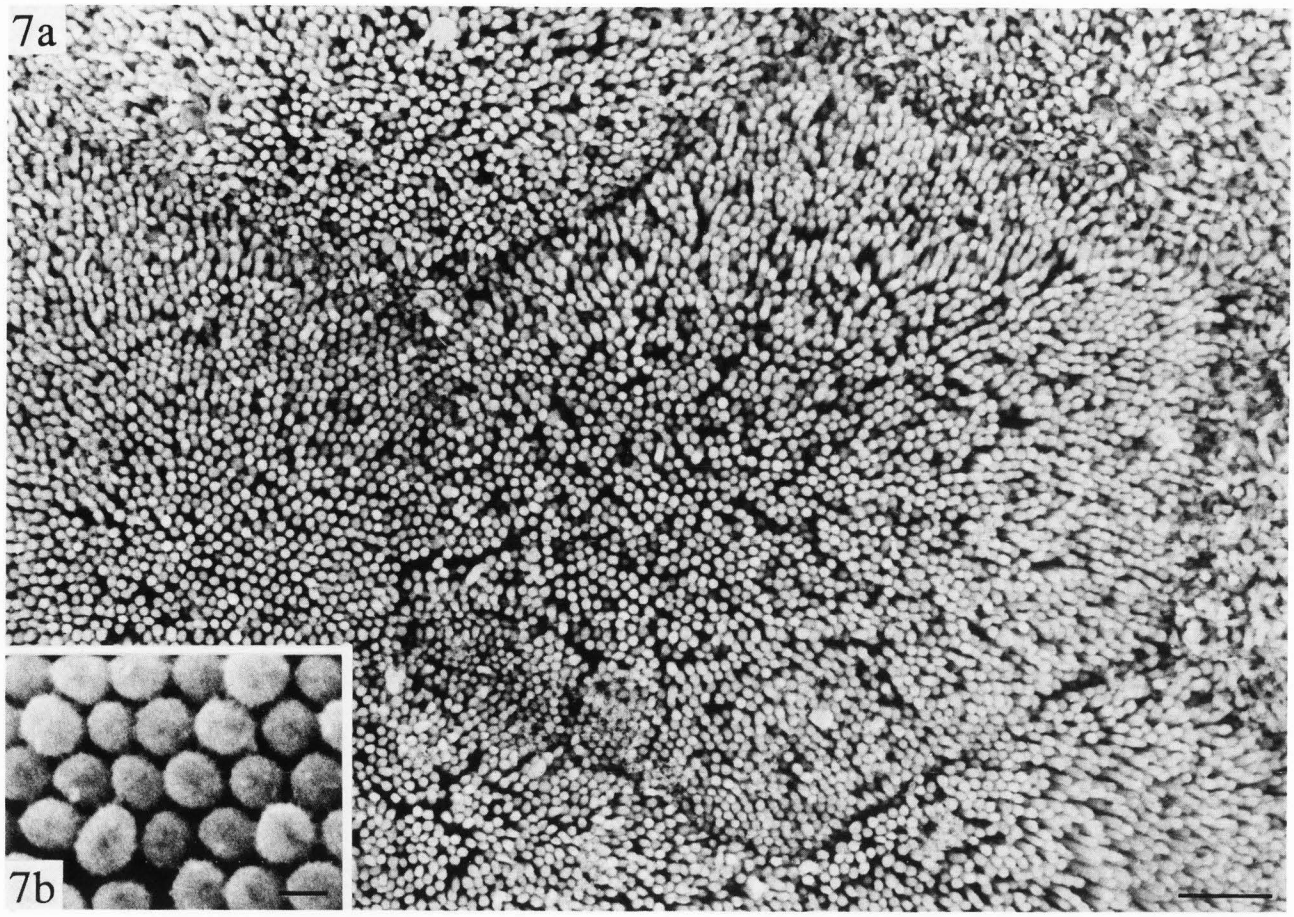

Figure 7. Rat intestine, scanning electron micrographs of sonicated samples. **(a, b)** At high magnification the ultrafine features of microvilli are easily identified. Bars = 1 μm (in a) and = 100 nm (in b).

By transmission electron microscopy, intestinal microvilli appeared evenly covered by a dense extracellular coat (Fig. 5a) that at higher magnification was filamentous in nature (Fig. 5c). The trachea samples showed a loose network of filaments covering the cilia (Fig. 6a).

Sonicated samples

Sonicated samples of intestine and trachea revealed large clean areas of the mucosal surface, in which no significant damage to the surface epithelial cells was observed (Figs. 3b and 4b). At high magnification, the effect of removal of the mucous coat covering the cellular surface was more evident. The ultrafine features of apical cilia and microvilli were easily identified. At higher magnification the cilia were preserved. They showed a fine surface microgranularity, that indeed, did not mask the three-dimensional structure and arrangement of cilia (Fig. 3d). The microvilli, even when seen at higher magnification, were not damaged and uniformly distributed on the surface of the epithelial cells (Figs. 7a and 7b). The intercellular boundaries were well exposed and the enterocytes appeared as regular hexagonal shaped elements (Figs. 4b and 7a). Goblet cells, in the intestinal mucosa, were devoid of secretion, and the goblet cell opening may be easily evidenced (Fig. 4d).

Transmission electron microscopy showed the intestinal microvilli devoid of their extracellular coat (Fig. 5b). At higher magnification, the plasmalemma appeared clean and well preserved (Fig. 5d). At low magnification, transmission electron micrographs of trachea showed regularly arranged cilia devoid of the superficial mucus (Fig. 6b). At higher magnification the cilia surface was preserved and well exposed, only a fine surface granular material could be seen (Fig. 6c).

Discussion

Variable sonic frequencies (from 5 to 15 kHz) modulated by 200 Hz generated by our instrument permit an effective cleaning of the mucosal surfaces without damaging their fine structures. The morphological aspects that we obtained by using these sonic frequencies resulted in better preparations than those provided by

other cleaning procedures (Takagi *et al.*, 1974; Zalewski and Moody, 1979; Wood and Dubois, 1981; Al-Tikriti *et al.*, 1986).

Several studies have used ultrasonic frequencies in order to microdissect biological tissues (Highison and Low, 1982; Low and McClugage, 1984; Low, 1989). The dissecting action depends on the alternation of positive and negative pressures owing to the passage of ultrasound through a fluid. However, both sonic and ultrasonic frequencies, when passing through a liquid, create within this liquid an alternating condition of pressure/depressure, graphically corresponding to the positive/negative tracts of the waves shown in Fig. 2. This action is more evident at the fluid-solid interface and varies with the frequency, amplitude, waveform, and the vapor pressure of the liquid in which the specimens are immersed (Apfel, 1981). When the pressure is negative (depressure phase) and the applied energy coincides with the cavitation threshold (the latter also related to the vapor pressure of the liquid phase), i.e, when the negative pressure is reduced beyond the vapor pressure of the liquid, microbubbles containing vapor develop and vibrate with the same frequency as the wave. When the pressure is positive (compression phase) and the applied energy exceeds the cavitation threshold, microbubbles implode and release a great deal of energy (under the form of thermic and pressure energy) in a very short period of time. In this way, the detachment of small particles from the samples surface and the cleaning or dissecting of the tissue is obtained (Coakley and Nyborg, 1978; Apfel, 1981).

The time of cavitation period and of the subsequent fluid resting time influences the microdissection of the samples. This time, i.e., the time of formation of the microbubbles (earning energy) and the time of their implosion (releasing energy) may be modulated by:

[1] Changing the waveform and symmetry, in a way that, at the same frequency, a square wave increases the time in respect to a sinusoidal or a triangular wave.

[2] Changing the amplitude, in a way that increasing the amplitude the level of pressure/depressure increases and then the time increases.

[3] Changing the frequency, in a way that increasing the frequency the time decreases.

[4] Changing the shape of the envelope of the waves, in a way that envelopes with a steeper attack and decay decrease the time. In fact, the envelope is the graphic expression of the modulation of the wave amplitude. It has a diamond shape that may be varied. The period of the amplitude increase is the ascending tract, called **attack**, the period of the amplitude decrease is the descending tract, called **decay**. Changing the steepness of the decay or attack results in a variation of the time of microbubbles formation and implosion.

[5] Changing fluids, in a way that different vapor pressures determine different cavitation thresholds.

[6] Changing the temperature of the liquid, in a way that its variations cause changing of the vapor pressure of a given fluid.

Usually ultrasonicators, commonly known as *tank cleaners*, have fixed frequency (from 20 to 80 kHz) and power, so that, at a given temperature, only the time of exposure may be varied. Other models are provided with a regulatory system for power, but this is often not sufficient to obtain a fine regulation of the wave energy.

It has been shown (Low, 1989) that the lower ultrasonic frequencies (20 kHz) break-up the tissue samples without selectivity. On the other hand, higher frequencies (80 kHz) were gentler and more selective. Therefore, it was suggested that an "ideal" microdissection could be obtained with tissue samples immersed in pure acetone (225 mm Hg vapor pressure at 25°C) and exposed for 3-5 minutes at 80 kHz of ultrasound (Low, 1989). The tissue damage seen at 20 kHz (at a fixed amplitude) could be related to the fact that the frequency used had higher peaks of acoustic pressure than at 80 kHz. In fact, the higher the level of the peaks of the acoustic pressure, the earlier the cavitation threshold onsets (Apfel, 1981). However, by reducing the amplitude, it is possible to reduce the peaks of acoustic pressure; and by modulating the frequency, it is possible to choose the level of the peak of acoustic pressure. Our model of sonicator/ultrasonicator is supplied with a control system for the wave frequency, amplitude and form. This yields an ultrafine regulation of the wave energy, and in particular, also permits to control the levels of the peaks of acoustic pressure at any frequency.

In our study, we used sonic frequencies from 5 to 15 kHz modulated by 200 Hz, and water as the immersion fluid (water vapor pressure is 23.7 mm Hg at 25°C and consequently the threshold of cavitation is higher compared to acetone) for four hours. This procedure generated a gentle wave energy that was effective in obtaining a fine cleaning of the tissue treated. In fact, the cells we studied showed well preserved microvilli and cilia, not artificially flattened, with a regular microtopographic arrangement, and without significant surface changes also when they were seen at high resolution SEM. The preservation of the cells, as well as the cleaning effectiveness of this procedure, was confirmed by TEM observation.

Finally, we would emphasize the chances that a fine and slow cleaning effect of **hydrated** samples may imply, e.g., to associate this procedure to other technique of tissue exposure such as the maceration methods by means of NaOH, osmium, or others, and to histochemical/immunological staining.

Acknowledgments

This research project was partially supported by the *Consiglio Nazionale delle Ricerche* (Italy). A fellowship was awarded to Prof. L.J.A. DiDio from the *Conselho Nacional de Pesquisas e Tecnologia* (Brazil).

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Discussion with Reviewers

F.N. Low: If you are not already preparing a paper describing the sonic frequency apparatus used in preparing your tissue samples, please indicate were this apparatus is described in detail so that other investigators may obtain and use it.

E. Reale: An exact indication of the models types of oscillators, envelope generator and modulator, etc. would allow construction of the device in each interested laboratory.

Authors: This paper mainly intended to describe the applications of the technique. Therefore, an overly technical description of the instrumentation was avoided. The block diagram of Fig. 1 gives the technical information necessary to build the instrument. All the components described in the blocks are not specific and are easily commercially available. However, according to your suggestion, we are now preparing a manuscript in which a more detailed description of the instrumentation will be reported.

L.G. Friberg: As you mention, other techniques have been used to avoid the mucus layer and surface debris. Using a more biologic method, cleaning by 1% HCl was reported to give the same result as yours by Doran *et al.* (J. Anat. **110**: 507, 1971), i.e., your more physical method by ultrasonicator could, maybe after all, give some kind of damage at high magnification. Have you compared other techniques with your technique?

Authors: The paper you mentioned deals with the villous surface as studied by means of a thermoionic scanning electron microscope with a resolution of about 70 nm, as it was possible in the early seventies, of air dried and gold-palladium coated samples. Our study was intended for a field emission SEM with a resolution of about 5 nm and for critical point dried, uncoated, thiocarbohydrazide-osmium impregnated samples. The thickness of the mucus filaments ranges from 20-50 nm (Familiari *et al*,. Micr Res Techn **23**: 225-229, 1992) and the filaments could be resolved only with the current models of SEMs. The observation we made at high resolution did not show a significant epithelial surface damage. We did not compare our technique with others, but, as you suggested, it would be very interesting to combine our technique with those of others (for example that of Doran *et al.*, 1971) and to evaluate the results by means of the current standard SEM.

L.G. Friberg: Figures 3b, 4b and 7a illustrate the excellent results with your method at medium magnification. High magnification showing cilia in trachea and microvilli in the intestine seems to present rigid cilia with a granular surface in both locations. Because you get smoother microvilli and a clean, non-granular surface by a biological method, the question is whether this cannot be an effect of the used technique itself?

Authors: The fine microgranularity seen on the tracheal cilia both by high resolution SEM and TEM was not related to any known damage of the plasmalemma. Its nature could be related to some residue of the extracellular material, but it should be further investigated in a dedicated study in which all the treatments performed, including staining, could be individually evaluated.