10. S. E. Malawista and K. G. Bensch, *Science* **156**, 521 (1967).
11. G. G. Borisy and E. W. Taylor, *J. Cell. Biol.* **34**, 525, 535 (1967); M. L. Shelanski and E. W. Taylor, *ibid.*, p. 549; L. Wilson and M. Friedkin, *Biochem.* **6**, 3126 (1967).
12. S. E. Malawista, *Arthritis Rheumat.* **11**, 108 (1968); K. G. Bensch and S. E. Malawista, unpublished observations.
13. This work was done in part at the Marine Biological Laboratory, Woods Hole, Mass.; it was supported by grants from the John A. Hartford Foundation, PHS (AM-10493, AI-271, CA-10171, A-5514, GM-14834), and NSF (GB-5120). S.E.M. is a senior investigator of the Arthritis Foundation. We thank Miss S. Lesnieski and Mrs. S. Branson for technical assistance. We thank Ayerst Laboratories, New York, N.Y., for supplying the griseofulvin (microsize).

13 February 1968

Cupulae in Shark Neuromasts: Composition, Origin, Generation

Abstract. *Cupulae on the surface of the sensory epithelium of canal neuromasts of the shark lateralis system have been demonstrated. They are mounds of mucoid material secreted primarily by the supporting cells of the neuromast epithelium. Individual columns of mucus in fixed, stained sections produce striations perpendicular to the neuromast surface. The hair of the sensory cell is embedded in mucus at the bases of coalescing columns produced by a group of surrounding supporting cells. There is evidence that mucus production is continuous and is accompanied by regeneration of secretory cells and elimination of necrotic cells at the distal surface. It follows that cupular material is being discarded either intermittantly or continually, along with trapped cell debris and other detritus.*

We have found cupulae (Figs. 1–4) on the surface of neuromasts of the head and lateral canals of juvenile *Sphyrna lewini* (60 cm, total length), near-term pups of *Carcharhinus melanopterus* (39 cm), and adult *C. menisorrah* (133 to 152 cm). These delicate gelatinous structures have been demonstrated in several orders of teleost fish (*1*) but not with certainty in elasmobranch canal organs. In free neuromasts (pit organs) of sharks, we found only one of many preparations which showed a cupula (*2*), and this lacked the typical striations usually associated with cupulae and may have been an anomalous mucus secretion.

The cupular material of shark canal neuromasts, apparently of viscous rather than jelly-like consistency, is lost in freehand sections of fresh material but is occasionally retained in frozen preparations. It is often retained in permanent preparations of carefully handled, rapidly fixed (Bouin's, Susa's, 10 percent formalin) material, embedded and sectioned in paraffin (7 to 10 μm) and stained in various dyes (Mallory's, Giemsa, toluidine blue, periodic acid-Schiff, hematoxylintriosin, and so forth), but it is shrunken by dehydration and frequently is torn from the surface of the epithelium in sectioning.

The cupula is an elongated mound of mucoid material covering the surface of the neuromast epithelium and extending into the lumen of the canal. Its base is an elongate oval in shape and plane, convex or concave in contour, conforming to the shape and contour of the epithelial surface. In permanent sections its maximum height is about two to three times that of the sensory epithelium, and it occupies only about 20 percent of the area of the canal lumen. In fresh-frozen sections the cupula is much higher and of much greater cross-sectional area; it nearly fills the canal lumen. In permanent sections, the height decreases abruptly at the ends of the sensory epithelium, but not necessarily to zero. In some longitudinal sections, the cupulae of adjacent neuromasts are continuous across the peripheral layers of inwardly curved supporting cells (mantle cells) which separate the two neuromasts at their ends (Fig. 1).

In the permanent sections, the cupula has a free, irregular distal surface without any detectable limiting membrane (Figs. 1–3). The cupular material reacts positively with periodic acid-Schiff reagent, showing the presence of polysaccharides; it stains metachromatically in Giemsa and toluidine blue, showing the presence of mucopolysaccharides. It consists of numerous columns of dark-staining mucus separated by more lightly staining granular mucus, producing the striated appearance. When the cupula is cut at an angle, the cut ends of the columns present an areolar appearance. Trapped in and among the columns are nuclei and other cellular debris, which apparently are being discarded from the sensory epithelium, hairs torn from the sensory cells, occasionally blood corpuscles from hemorrhages, and sometimes sand grains which must have entered through the canal tubules.

The cupular material originates in the supporting cells (including the mantle cells) of the neuromast epithelium. The supporting cells extend from basal membrane to distal surface and, within the peripheral layer of mantle cells, surround the shallower sensory cells. Metachromatic staining shows the presence of mucopolysaccharides within the supporting cells, extending from their distal ends to about the level of the nuclei of sensory cells where the supporting cells are greatly constricted (Fig. 6). In some sections, slender threads of mucus project from their necks and merge with the cupular material torn from the surface (Fig. 4). Some of the cells which are discharging mucus appear senescent, with partially vacuolated interiors and pycnotic nuclei lying close to the distal surface of the epithelium (Fig. 5). Necrotic cells are seen frequently among the sensory cells. Mitotic figures are seen occasionally. These observations give evidence that the secretory supporting cells are being continually formed by cell division, and are being discarded through the surface of the epithelium, contributing to the cell debris in the cupula. Whether a cell discharges mucus just once or several times before becoming necrotic is uncertain. Cross striations, occasionally seen in the mucus column, could represent periodic discharge of one cell or successive discharge of several.

Facial sections of both free and canal neuromasts show one sensory cell neck surrounded by four or five supporting cell necks, with the distal cell membranes joined by intercellular cement. The arrangement is such that each short sensory hair (about 6 μm long, Fig. 5) is completely surrounded by the bases of coalescing columns of mucus.

Continual production of cupular material proximally implies continual or intermittant loss distally. Erosion of the material, and, incidentally, removal of accumulated detritis, may be accomplished by a slow head-to-tail flow of water through the canals, which we have demonstrated in a juvenile *S. lewini* (*3*).

The cupula of the shark neuromast, described here as a mound of mucus, may differ from that of teleosts in viscoscity (degree of polymerization of the mucopolysaccharides) or in composition. In living *Lota vulgaris*, the cupula can be displaced as a unit for a short distance (10 μm) over the surface of the neuromast, and toward its tip it is plastic, bending when probed (*4*). In living *Rhyncocymba nystromi*, it

is a membranous elastic structure which, unlike the mucus of the canal wall and unlike the cupular material of the ear ampulla of sharks, resists digestion by hyaluronidase (*5*). We have not applied this digestion technique to shark material. However, metachromatic staining does not distinguish between the mucus of the cupula and that produced elsewhere in the canal epithelium, suggesting a similar composition.

Apart from possible differences in viscosity and composition, cupulae of the canal neuromasts in sharks and teleosts are remarkably similar. In teleosts, also, the "well-known parallel vertical striation of several cupulae has been ascribed to the pattern of secreting cells at the base; each cell will produce a slender pillar of the jelly-like substance" (*6*). In sections of *Fundulus heteroclitus,* the canal neuromast cupula is always jagged and irregular as if torn from an attachment, possibly the roof of the canal (*7*). In shark sections, also, the top surface is usually jagged and irregular, but there is no evidence of distal attachment. Our evidence for elimination of necrotic supporting cells at the distal surface of the neuromast is similar to that for the elimination of necrotic sensory cells of mormyromasts in *Gnathonemus,* described as a holocrine process (*8*). However, we have not found any references to the presence of either necrotic cells or mitotic figures in teleost neuromasts.

We have evidence that the cupula in sharks is a dynamic structure with continual growth proximally and erosion distally. We gain the impression from the literature that the cupula of teleost canal neuromast is viewed generally as a static structure. However, Kuiper (*9*) has observed growth of cupulae in head canals of *Acerina cernus,* and Petraitis (*10*) suggests that the cupula of the canal organ of *Fundulus heteroclitus* may grow from the bottom as the top surface wears or degenerates. She also interprets the horizontal striations of the mucus columns as growth rings. Cell debris, which we interpret as the remains of necrotic supporting cells, has been observed frequently in teleosts, either in the cupula or on the surface of the neuromast when the cupula has disappeared (*5, 11*). Flock (*4*) states that the cupula substance can be partially removed by jets of water of traumatic velocity. We suggest that even a slow flow of water through the canal (*3*) would serve to remove discarded cupula material and expel it through the more posterior canal tubules. Smith (*12*) shows in some but not all teleosts a relatively rapid flow of water through the canals, accompanied by a discharge of mucus. It seems reasonable to assume that both sharks and teleosts have the ability to regenerate, or even to continually generate, this delicate structure which is so easily damaged and lost in living material and whose "gliding" movement, producing hair deformation and intracellular electrogenesis (*4, 9*), could be impaired by accumulation of detritus.

We have not yet found typical cupulae in free neuromasts of sharks. These occur in pits between the bases of modified scales and are well protected by the overhanging scale crowns (*2*). The supporting cells of the free neuromasts, like those of the canal neuromasts, are actively producing mucopolysaccharides. Frequently, unorganized mucus material is seen on the surface. In contrast to our lack of success with sharks, we have had no difficulty in demonstrating cupulae on

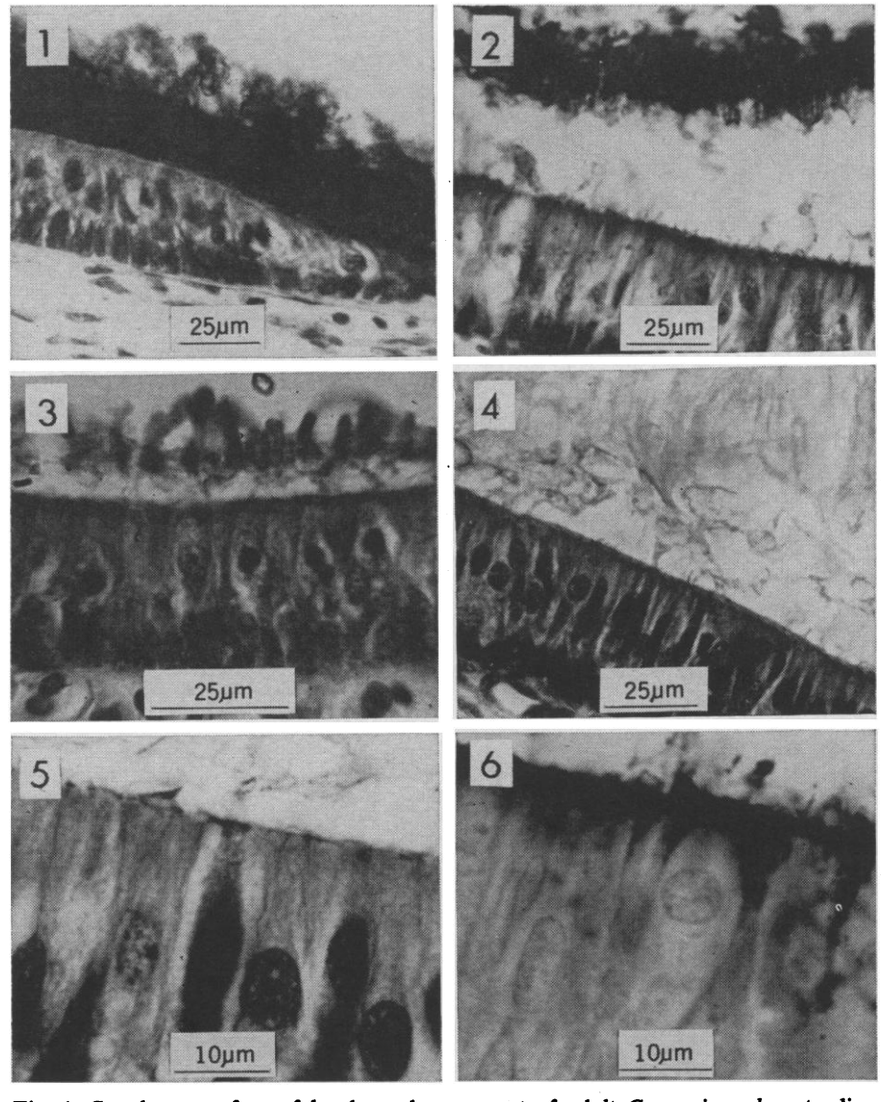

Fig. 1. Cupula on surface of head canal neuromast of adult *C. menisorrah,* extending over inwardly curved mantle cells to the right (formalin, toluidine blue). Fig. 2. Cupula torn from head canal neuromast of juvenile *S. lewini* (Bouin's, toluidine blue). Fig. 3. Cupula on lateral canal neuromast of near-term pup of *C. melanopterus,* showing vertical columns of mucus (Bouin's, toluidine blue). Fig. 4. Head canal neuromast of *S. lewini,* showing cupula, mucus emerging from supporting cell near center, and cell debris above neuromast at left (Susa, hematoxylintriosin). Fig. 5. Same preparation as in Fig. 4, showing discharging supporting cell with pycnotic nucleus (center) between two sensory cells, and inclined sensory hair (left of center). Fig. 6. Head canal neuromast of *S. lewini* showing mucopolysaccharides (black in photograph, lavender in preparation) in the compressed supporting cells surrounding a sensory cell (Bouin's, Giemsa).

free neuromasts of teleosts. These are elongated flexible structures with definite form, similar to those described by others (*1*, *13*). It is likely that the cupula of the free neuromast in sharks, like that of the canal neuromast, is a mound of mucus which is continually being replenished. Probably it differs in texture and is more readily lost during handling or preservation (or both) than the typical tongue-like cupula of free neuromasts in teleosts.

ALBERT L. TESTER
JAMES I. KENDALL
Department of Zoology,
University of Hawaii, Honolulu

References and Notes

1. P. H. Cahn and E. Shaw, *Copeia* **1962**, 109 (1962).
2. A. L. Tester and G. J. Nelson, in *Sharks, Skates and Rays*, P. W. Gilbert, R. F. Mathewson, D. P. Rall, Eds. (Johns Hopkins Press, Baltimore, 1967), p. 34.
3. In a juvenile *S. lewini*, forced to swim in a dilute solution of trypan blue in seawater, the dye penetrated the canal system from head to second dorsal fin in 50 minutes. It entered only from the anteriorly directed tubules of the anterior head canals.
4. Å. Flock, in *Lateral Line Detectors*, P. H. Cahn, Ed. (Indiana Univ. Press, Bloomington, 1967), p. 163.
5. Y. Katsuki, V. Mizuhara, S. Yoshino, *Jap. J. Physiol.* **2**, 93 (1951).
6. S. Dijkgraaf, *Biol. Rev.* **38**, 51 (1963).
7. W. A. van Bergeijk and S. Alexander, *J. Morphol.* **110**, 333 (1962).
8. R. Cordier, *Mem. Soc. Zool. Belg.* **68**, 72 (1938).
9. J. W. Kuiper, thesis, Groningen, Netherlands (1956).
10. R. Petraitis, *J. Morphol.* **118**, 367 (1966).
11. T. J. Walker, in *Lateral Line Detectors*, P. H. Cahn, Ed. (Indiana Univ. Press, Bloomington, 1967), p. 13; J. A. Suckling, *ibid.*, p. 45.
12. G. M. Smith, *Biol. Bull.* **59**, 313 (1930); *Anat. Rec.* **56**, 365 (1933).
13. T. Iwai, in *Lateral Line Detectors*, P. H. Cahn, Ed. (Indiana Univ. Press, Bloomington, 1967), p. 27; M. Denny, *J. Comp. Neurol.* **68**, 49 (1937).
14. Contribution No. 298, Hawaii Marine Laboratory. Project supported by ONR contract Nonr 2756(00).

13 February 1968

Mitotic Synchrony in Mammalian Cells Treated with Nitrous Oxide at High Pressure

Abstract. *Mammalian cells grown in suspension or monolayer cultures were synchronized for cell division by the application of nitrous oxide under pressure. The metaphase block induced by nitrous oxide was dependent on pressure and was reversible. Exposure of HeLa cells to nitrous oxide had no significant effect on the synthesis of DNA, RNA, or protein. The progress of cells through the mitotic cycle was also unaffected. A high degree of mitotic synchrony was obtained in suspension cultures of HeLa cells treated with thymidine during exponential growth, resuspended in fresh medium, and then exposed to nitrous oxide.*

There are a number of procedures for obtaining synchronous (*1*) or synchronized (*2*, *3*) populations of mammalian cells in culture. I now describe a new method in which nitrous oxide (N_2O) applied at high pressures synchronizes cell division in mammalian cells grown as suspension or monolayer cultures.

Nitrous oxide at ordinary atmospheric pressures induced mitosis similar to that seen after treatment with colcemid (c-mitosis) in *Pisum sativum* but not in *Allium cepa* (*4*). The suggestion that similar effects might be produced in *Allium cepa* at higher pressures was proved to be correct by Ferguson *et al.* (*5*), who found that the threshold pressure for nitrous oxide to induce polyploidy was about 6 atm. I therefore tested N_2O for its usefulness in obtaining synchronous division in HeLa cells grown as suspension or monolayer cultures.

HeLa cells were maintained in exponential growth at 37°C in Eagle's minimal essential medium supplemented with nonessential amino acids, sodium pyruvate, and calf serum (5 percent). The cultures were gassed with a mixture of carbon dioxide (2.7 percent) and air. Monolayer cultures of HeLa (wild type) and a Fernandes line of human amnion cells were also maintained under similar conditions. The serum content of medium for human amnion cells was 10 percent, and 5 percent for others.

HeLa cells growing in spinner bottles or plastic tissue-culture dishes were exposed to N_2O in a stainless-steel pressure chamber (*6*). In experiments with former cultures, the cells were kept in suspension by a magnetic stirrer placed under the water bath directly beneath the pressure chamber. Optimum temperature was maintained by immersing the pressure chamber in a water bath kept at 38° ± 0.1°C for 1 day before and during the course of the experiment. One atmosphere of air, into which 20 ml (or 3 percent of the total volume) of carbon dioxide was injected, was allowed to remain in the pressure chamber before N_2O under pressure was introduced.

Cells fixed and stained as previously described (*3*) were examined microscopically. Nitrous oxide at high pressures blocked HeLa cells in exponential growth in metaphase. On microscopic examination these blocked cells presented typical c-mitotic configurations. The effectiveness of the N_2O block was dependent on the pressure. The metaphase block was incomplete between 2.72 and 4.42 atm and was ineffective at lower pressures. In the range of 2.72 to 4.42 atm, most of the cells could complete mitosis, which was anomalous in some respects and appeared to be similar to the phenomenon of chromatid nondisjunction (*7*). The optimum range of pressure for a complete metaphase block was 5.1 to 5.4 atm of N_2O. Increasing the pressure above 6.8 atm or the duration of treatment beyond 16 hours, even in the range of 5.1 to 5.4 atm, caused disintegration of some cells. Nitrogen applied at similar pressures caused no metaphase block.

The metaphase block was reversible. The reversibility was greatly influenced by the duration of exposure and pressure. A partially synchronized population of HeLa cells was obtained by treating an exponential culture with thymidine (final concentration, 2.5 mmole/liter) for 16 hours, centrifuging

Table 1. Effect of N_2O on the progression of cells through mitotic cycle; M.I., mitotic index; labeled, percentage of cells labeled with tritiated thymidine.

Duration of treatment (hours)	Nitrous oxide at 5.1 atm		Control with colcemid		Control without colcemid	
	M.I.	Labeled (%)	M.I.	Labeled (%)	M.I.	Labeled (%)
1.0	0.03	35.0	0.03	39.0	0.02	36.0
2.0	.08	51.0	.06	47.5	.04	49.5
4.0	.11	55.0	.12	55.0	.03	58.0
8.0	.27	78.0	.26	78.0	.03	79.5
16.0	.62	80.0	.65	81.5	.03	83.0