STUDIES ON CILIA

The Fixation of the Metachronal Wave

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ABSTRACT

Upon excision into spring water, the lateral cilia of the gill of the freshwater mussel *Elliptio complanatus* (Solander) stop beating, but 0.04 M potassium ion can activate the gill so that these cilia again beat with metachronal rhythm. One per cent osmium tetroxide quickly pipetted onto a fully activated gill fixes the lateral cilia in a pattern that preserves the form and arrangement of the metachronal wave, and permits the cilia to be studied with the electron microscope in all stages of their beat cycle. Changes are seen in the fixed active preparation that are not present in the inactive control, *i.e.*, in the packing of the cilia, the position of the axis of the ciliary cross-section, and the diameter of the ring of peripheral filaments. Analysis of these parameters may lead to new correlations between ciliary fine structure and function.

INTRODUCTION

Metachronism represents the coordinated activity of multitudes of cilia. Each cilium beats slightly out of phase with its neighbor and the result is a series of waves of activity—metachronal waves—which move over the ciliated surface with the frequency of ciliary beat. The apparent motion of the metachronal waves may be stopped photographically or stroboscopically (*cf.* 10). Then, within one wavelength of the metachronal wave, the cilia are captured in a distinct pattern which, as expected, is the projection of the ciliary beat in the plane of the wave motion.

Studies of ciliary motion rely on an accurate description of the morphology of the motion, and one way to obtain such a description is to stop the movement of the metachronal wave and examine the resulting pattern.

Electron microscope studies have demonstrated a rather complex substructure within the cilium (2–4, 7). It would be extremely interesting to know what sorts of changes occur in the substructural units of the cilium during the ciliary beat, preliminary to a discussion of the molecular mechanism of ciliary motion. The beat of a single cilium cannot as yet be photographed in the electron microscope, and so another approach must be found if this problem is to be attacked.

There is another way to stop the metachronal wave motion, that is—fixation. Gelei (5) ably demonstrated this in 1926, and Parducz (12–14) has extended Gelei's original demonstration by analyzing the details of the form of ciliary beat from the fixed metachronal wave of *Paramecium*, but the studies stop with the light microscope. Nevertheless, the pathway is clear: fix the metachronal wave with a standard osmium tetroxide fixative for electron microscopy and it should be possible to examine all phases of ciliary motion with the electron microscope. This paper will attempt to demonstrate the feasibility of this approach. It will show that the metachronal wave can be fixed for electron microscopy in certain instances and that the ciliary beat form can be preserved for study. Since the ciliation of

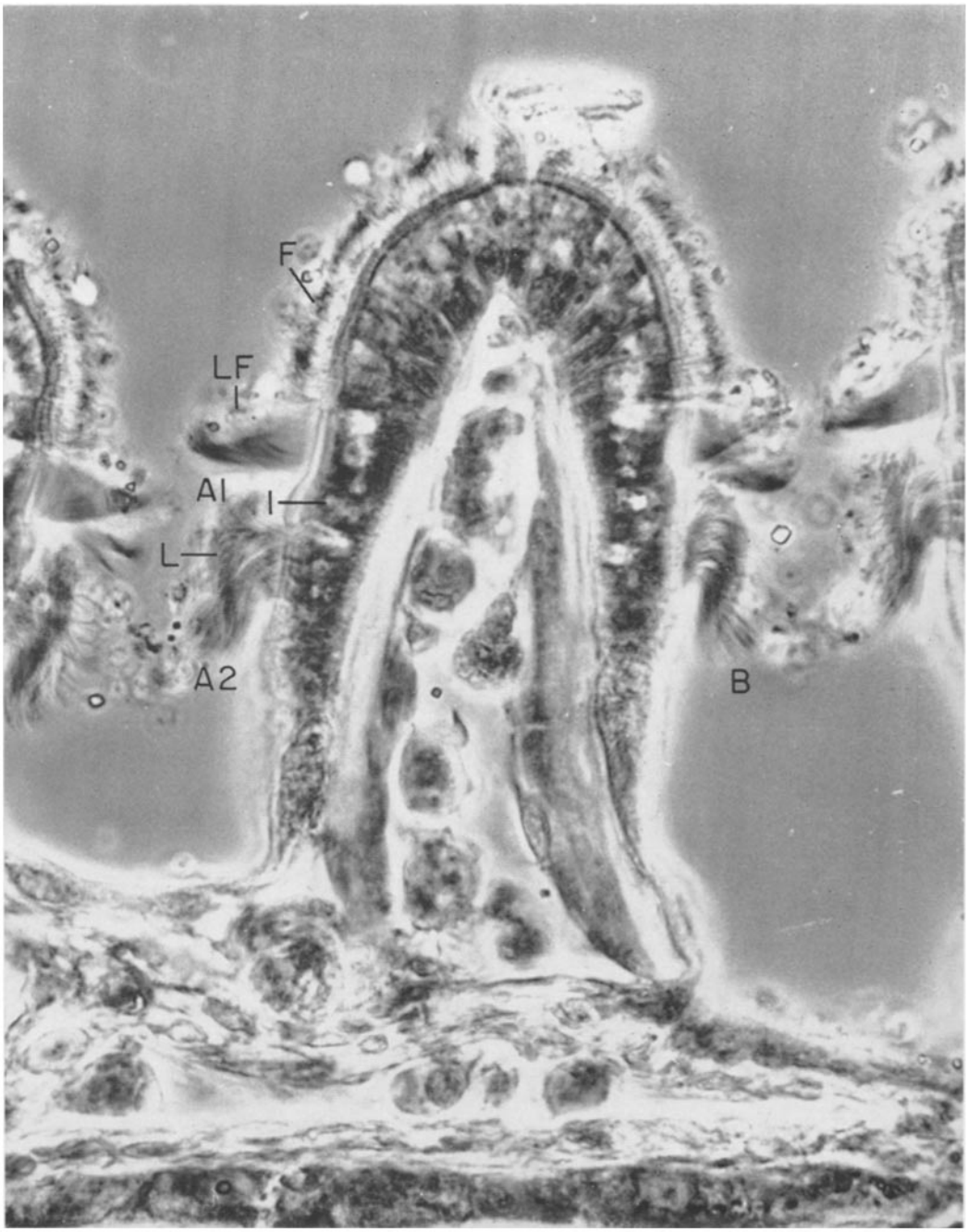


FIGURE 1 Transverse section of fixed activated excised *Elliptio* gill filament, cutting the lateral cilia (*L*) in plane of beat. During the effective stroke, these cilia move in an abfrontal direction from *A*1 to *A*2. The form of the curving recovery stroke is seen at *B*. The metachronal wave moves in a plane perpendicular to this section. Note the non-ciliated cell (*I*), intermediate between the lateral cell and the laterofrontal cell bearing the sail-shaped cirri (*LF*). Frontal cilia (*F*) can also be seen. 0.04 M K_2SO_4*. × 1300.

* In Figs. 1 and 8 to 15 the penultimate part of the figure legend indicates the solution to which the preparation was subjected prior to fixation.

Paramecium is sparse for electron microscopic study, the gill of the freshwater mussel, *Elliptio complanatus* (Solander), became the experimental object of choice for these studies. The numerous lateral cilia on the gill filaments beat with metachronal rhythm in the intact animal. The mussels are easy to obtain, keep, and handle. Gills of related genera (*e.g.*, *Anodonta*) have been studied extensively with the electron microscope (2, 4, 6). Lastly, the mussel gill has long been a favored object for physiological experiments on ciliary motion (1, 8–10), so that considerable information is already available linking ciliary structure and function in the gill. Because the observed morphology is complex and differs somewhat from that seen in other studies, emphasis is placed on following the tissue from living state to embedded and sectioned end-product.

METHODS

(a) PREPARATION OF THE GILL: *Elliptio complanatus* was obtained from the streams of Alamance County, North Carolina, from the Carolina Biological Supply Company, Elon College, North Carolina in periodic shipments. The mussels could be kept in an aquarium in spring or tap water, and they remained in good condition, as judged by their ability to close their valves tightly if disturbed, for many weeks.

When an experiment was to be run, an animal was opened and its gills excised by severing the connections with the body wall. The gills were then placed in a dish containing 1 to 3 ml of spring water (generally containing 0.2 ml of 0.05 per cent veratrine sulfate per 5 ml. H_2O) or tap water. The gills were spread out in the dish so that a maximum number of filaments had ready access to water and air. The two gills from one side of the animal remained attached *via* their dorsal margins, so that the tissue when spread out resembled an opened notebook whose unbound side was the ventral edge of the gill and the terminal edge of the gill filaments. One frontal face of each gill was exposed, the second rested on the top of the dish. In this state, the gill remained useable for many hours, and further dissection was easily accomplished.

Usually, the gill was stripped before it was used. This maneuver separated the two frontal faces of the gill (*i.e.*, the ascending from the descending lamella): a gill was held at its dorsal margin with a spatula and teased with a watchmaker's forceps until a few filaments of the exposed lamella could be torn away. This process was repeated until the greater part of the exposed lamella had been removed from the gill.

Since the exposed lamella was greatly disturbed during the stripping procedure, the pieces of tissue used in experiments were cut from the unexposed lamella with a small scissors. Many pieces of tissue, each approximately 0.5 cm square, could be obtained from one lamella. When a piece of tissue was to be used, it was inverted so that its frontal face was now exposed for easy observation. Stripping, then, produced a relatively undamaged piece of tissue containing a small number of gill filaments intact over a fairly long (0.5 cm) distance.

Pieces from the stripped gill were transferred to a perfusion chamber for the physiological and cinematographic control experiments described below, or they were transferred directly into various solutions without perfusion prior to fixation and embedding for electron microscopy. In the latter cases, the gill was placed under a microscope and observed until the lateral cilia had reached the desired physiological state. Then, 1 per cent osmium tetroxide in veronal acetate buffer was quickly pipetted onto the gill. The gill was again observed under the microscope immediately after fixation to see whether the cilia remained in their prefixation position, and, after this had been ascertained, the gill was washed with fresh fixative and placed in the refrigerator at $-1°C$ for 1 hour. The ordinary methods of dehydration and embedding were generally followed.

In the early experiments a visual control was exercised throughout. Sometimes the dehydration procedure was stopped at one point or another and whole mounts of the tissue were prepared (see Fig. 8). In any event, the gill remained undisturbed through the preliminary fixation and dehydration and was cut into smaller pieces for embedding only after it was placed in 95 per cent ethanol; by then, the tissue had hardened sufficiently so that cutting did not affect the position of the cilia. Each piece for embedding was followed further under the microscope until it was actually placed in its gelatin capsule; only those pieces that were intact were embedded. At first, material was embedded in methacrylate, and later, in Epon. In both cases, the desired ciliary pattern appears to be preserved.

Thick sections of the embedded material were cut on a Porter-Blum microtome for phase contrast micrography. Corresponding thin sections were also cut, for examination in the Philips EMU 100A or RCA EMU 3C electron microscope. Methacrylate sections were flattened with xylene, picked up on carbon-coated copper grids, stained with a saturated solution of uranyl acetate in 50 per cent ethanol (7), and sandwiched with carbon in the usual manner. Epon sections were stained as described above before examination in the electron microscope.

(b) PHYSIOLOGICAL AND CINEMATOGRAPHIC CONTROLS: The living excised gill and lateral cells which had come loose from gill preparations were

used to study the physiology of the lateral cilia for comparison and standardization of the fixed preparations. A series of perfusion chamber experiments served to quantitate the development of metachronism in the living gill under controlled chemical conditions. With a given perfusate, the per cent metachronism of the lateral cilia could be estimated by comparing the number of filaments whose lateral cilia beat with pronounced metachronal rhythm to the total number of filaments in a given field. The frequency of beat could be measured stroboscopically and motion pictures of the living lateral cilia could be obtained. Isolated lateral cells, identifiable with metachronal rhythm and in the isolated cell preparations. Observations with the stroboscope were used for routine confirmation of the motion picture results.

RESULTS

(1) COMMENTS ON GILL MORPHOLOGY: The morphology of the *Elliptio* gill is in most respects identical to that of *Anodonta*, summarized by Gibbons (6), but one important difference appears to be the absence of prolaterofrontal cilia in *Elliptio*. The general structure of a portion of

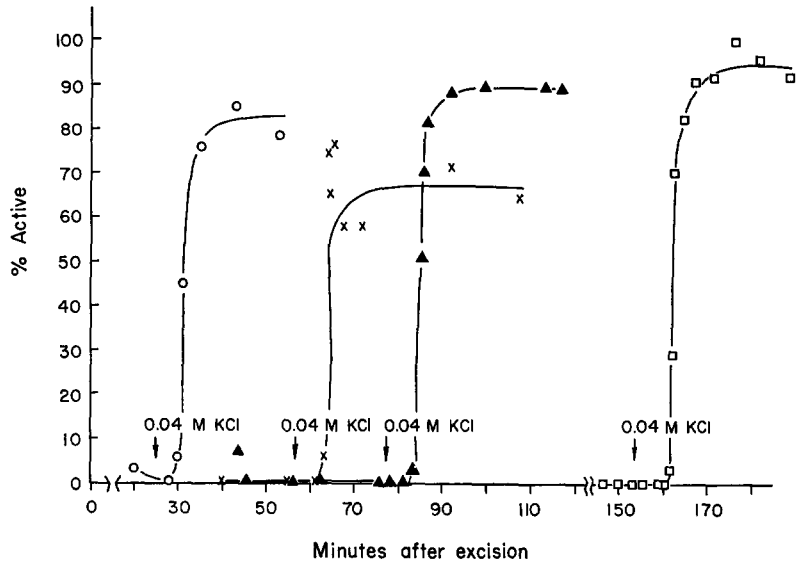


FIGURE 2 Activation of the excised gill by 0.04 M KCl. Four separate perfusion experiments are shown. In each case, the gill is first perfused with spring water and no activity is seen. When 0.04 M KCl is added (arrows), there is a short lag and then activity rises to nearly 100 per cent. The gill remains active if 0.04 M KCl perfusion is continued.

because of their numerous long cilia and large size, could also be photographed.

Motion pictures of the gill and of isolated cells were taken through a Zeiss standard phase contrast microscope equipped with an Arriflex 16 camera in a microcinematographic setup designed by H. Davies. The light source was a "zircon arc" lamp. The film speed was controlled by a tachometer and was 10 to 16 frames per second, depending on the experiment. All experiments were run at room temperature (generally 20–24°C).

Stroboscopic measurements were made using a Strobotac (General Radio Company, West Concord, Massachusetts). The illumination given by this instrument was sufficient so that the ciliary beat could be examined visually both in the gill beating

an excised gill is presented in Fig. 1. The conspicuous features commanding attention here are (1) the laterofrontal cells with their sail-shaped cirri (two/cell) composed of 30 adjacent cilia, (2) the four lateral cells with their long cilia—about 200/cell—which may beat metachronally, and (3) the non-ciliated intermediate cell separating them.

(2) ACTIVATION EXPERIMENTS: It has long been known that in the gills of marine mussels (*e.g.*, *Mytilus*) at least the lateral cilia come to rest within a few minutes of excision. Gray (9) found that exposure of these inactive gills to veratrine, to an excess of potassium ion, or to a reduced

concentration of magnesium ion, activated their lateral cilia so that metachronism was regained. Aiello (1) repeated Gray's work and found that 0.04 M KCl added to a quiescent gill in sea water activated almost all the lateral cilia. Since previous studies of mussel cilia (2, 4, 6) had failed to report fixation of the metachronal wave pattern of the lateral cilia, it was thought desirable that these experiments begin with an examination of the activation of the lateral cilia so that the presence of the metachronal wave throughout the gill could be assured before fixation was undertaken.

Metachronism is present in the *Elliptio* gill *in situ*, but, as in other mussel gills, excision results in an immediate cessation of metachronism on the majority of filaments. Generally almost all of the lateral cilia completely stop beating within 3 to 10 minutes after excision into spring water, although both laterofrontal and frontal cilia continue to move while the gill is perfused with water. Once metachronism has ceased, no spontaneous reversion to beat is observed, at least for several hours.

The lateral cilia may be reactivated by the addition of potassium ion to the perfusate. This is illustrated with several preparations in Fig. 2. At various times after metachronism has ceased, the perfusate is changed to 0.04 M KCl and, after a brief lag period, the lateral cilia begin beating again, the per cent activity rising rapidly to a maximum value that can be maintained for many minutes with continued perfusion. The cilia beat with an average frequency of 17 per second. The maximum per cent activity achieved appears to depend somewhat upon the individual experimental preparations, but not upon the time of addition of the KCl after excision, and not upon the length of time the gill has been perfused with water. In the best cases, close to 100 per cent activity is achieved. Full activation is also achieved with 0.02 M KCl, with 0.04 M K_2SO_4 or KI and with 0.04 M NaCl or NH_4Cl, but 0.04 M $MgCl_2$, $CaCl_2$ or sucrose fail to activate the gill.

The course of activation can be followed photographically in Figs. 3 and 4. Fig. 3 is a frame from a motion picture showing a field of filaments from an excised gill perfused with spring water. The gill is quiescent and even the laterofrontals lie still and clumped. The lateral cilia appear behind the laterofrontals as a faint line (arrow). No patterning of the lateral cilia is seen. No motion is visible when sequences of such frames are projected, indicating that the lateral cilia are stationary, and no metachronism is present in the unactivated preparation.

Fig. 4 shows another portion of the same gill after activation with 0.04 M KCl (a different preparation selected for superior viewing conditions). A sequence of frames is presented. The gill "opens up" as the interspace between the filaments widens. Then the patterning of the lateral cilia into a series of crests and troughs atop the filaments can be seen. This patterning

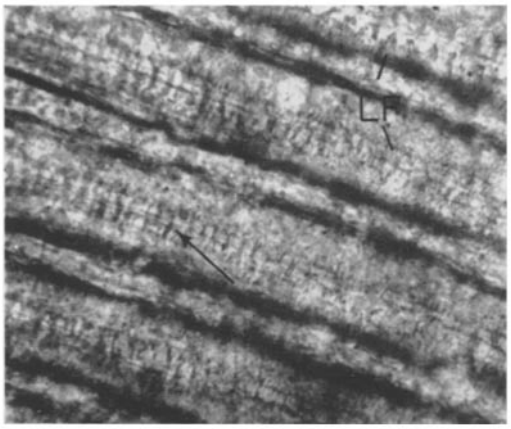


FIGURE 3 Light micrograph of an excised living gill before activation (from a motion picture sequence). Gill perfused with spring water. The gill filaments are close together and the interspace between them is nearly obliterated. Laterofrontal cirri (*LF*) appear as lines at both boundaries of each filament. Often several cirri may be clumped together. Behind them are the lateral cilia (long arrow), filling the interspace. The lateral cilia are not beating. × 300.

is a representation of the metachronal wave, so that from frame to frame in Fig. 4, the troughs are displaced (Table I); when these frames are projected, such displacement shows up as metachronal wave motion.

(3) THE CILIARY BEAT CYCLE: Correlation between metachronal wave pattern and ciliary beat in gill cilia rests on Gray's work (10). Gray compared the pattern of the lateral cilia of *Mytilus* to the beat of the long abfrontal cilium. In the present study, the correlation was reinvestigated. Lateral cells which had come free from the filaments were photographed in the hope that an

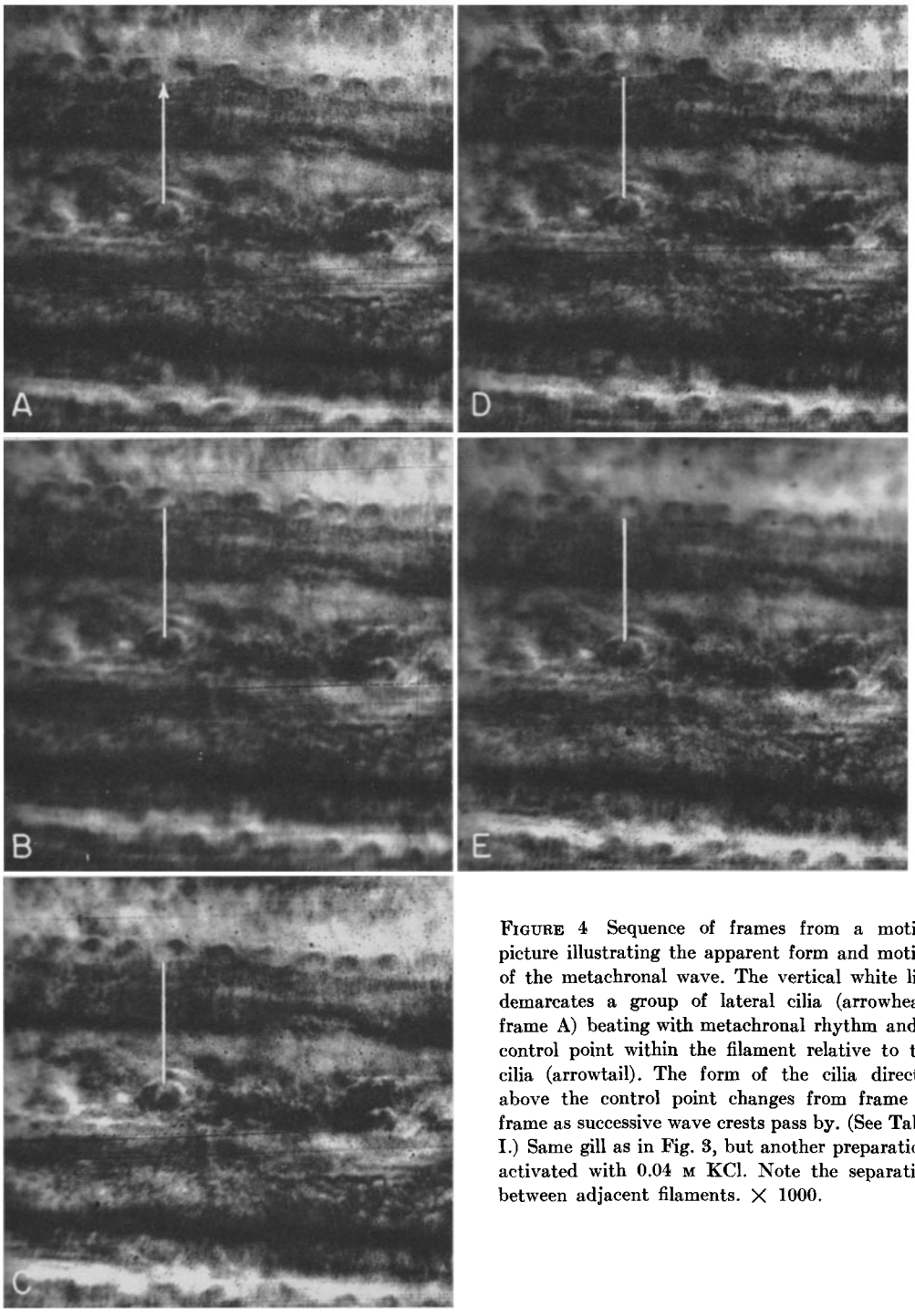


FIGURE 4 Sequence of frames from a motion picture illustrating the apparent form and motion of the metachronal wave. The vertical white line demarcates a group of lateral cilia (arrowhead, frame A) beating with metachronal rhythm and a control point within the filament relative to the cilia (arrowtail). The form of the cilia directly above the control point changes from frame to frame as successive wave crests pass by. (See Table I.) Same gill as in Fig. 3, but another preparation, activated with 0.04 M KCl. Note the separation between adjacent filaments. × 1000.

accurate comparison could be made with the lateral cells beating in the intact gill. A typical result is shown in Fig. 5.

Fig. 5 shows a sequence of motion picture frames illustrating the form of the ciliary beat on one such isolated lateral cell. The cilia appear in groups atop the cell. In frame A the group at the arrow is seen near the end of the recovery stroke. In frame B they have straightened further and completed the recovery. Frame C catches part of the effective stroke. Here the cilia appear to lash downward, moving their distal ends to a position parallel to the cell surface in frame D. In this position the effective stroke ends and the recovery stroke again commences. A flexible curve passes up the ciliary stalks in frames E to H, returning the cilia to their initial position (compare frames A and H).

The relationship of the beat cycle in Fig. 5 to the metachronal wave pattern is illustrated in Figs. 6 and 7. In Fig. 6 the maximum distance from the cell surface of the beating cilium of frames A to H of Fig. 5 is plotted against time (lower abscissa); the result is a segment of a sine curve that is repeated each time the cilium beats. The same curve is generated at any instant by the cilia along the intact gill filament (upper abscissa) beating out of phase with metachronal rhythm. A series of crests and troughs is seen which is comparable to that on the filaments in Fig. 4. The difference is that Fig. 6 presents only a two-dimensional projection of the wave, while in Fig. 4 the morphology is three-dimensional and, therefore, apparently more complex.

A model of the lateral cells and their cilia is shown in Fig. 7. Each of the ciliary forms from Fig. 5 is represented, spaced equidistantly and sequentially along the model cells. The result is again the metachronal wave form, which may also be seen in the projection (shadow) of the model. These projections (Figs. 6 and 7) have meaning in the interpretation of the essentially two-dimensional images in the micrographs shown below.

(4) FIXATION OF THE WAVE: In Fig. 8, a typical whole mount of an excised activated group of filaments is shown after the tissue had been fixed in 1 per cent OsO_4.[1] Focus is at the level of the lateral cilia and these appear in their typical metachronal positions: in several wavelengths, cilia may be seen standing upright while their neighbors are curled in other stages of their beat. The resemblance both to the model (Fig. 7) and to the living activated gill (Fig. 4) is striking, but it should be noted that in Fig. 4 the gill is stopped photographically, while in Fig. 8 the gill is permanently fixed. This is, after all, the result expected from the work of Gelei and of Parducz cited in the Introduction, but now, after dehydration and embedding, material similar to that of Fig. 8 is suitable for further light and electron micrograph study not possible with *Paramecium*.

The pattern seen in Fig. 8 is never seen in

TABLE I

Apparent Motion of the Metachronal Wave

Fig. 4 frame No.	Measurement cm: Experimental (edge to wave trough 4)	Measurement cm: Control (edge to stationary object)	Corrected experimental values	Δ‡	Apparent progress of wave in μ
A	2.45 (arrowhead)	2.43 (arrowtail)	2.45*		0*
B	2.75	2.38	2.70	+0.25	2.6
C	2.40	2.40	2.43	−0.27	5.4
D	2.70	2.45	2.68	+0.25	7.0
E	2.35	2.45	2.33	−0.35	10.6

* Corrected values (cm) and apparent progress of wave in μ relative to frame A (standard).
‡ Δ (cm) gives displacement of trough from frame to frame. Average Δ, 0.28 cm = 2.9 μ.

[1] In this one case, the tissue was further hardened with Bouin's fluid, stained with eosin, and mounted in glycerin. This further treatment was unusual, but did not appear to cause substantive changes in gill morphology.

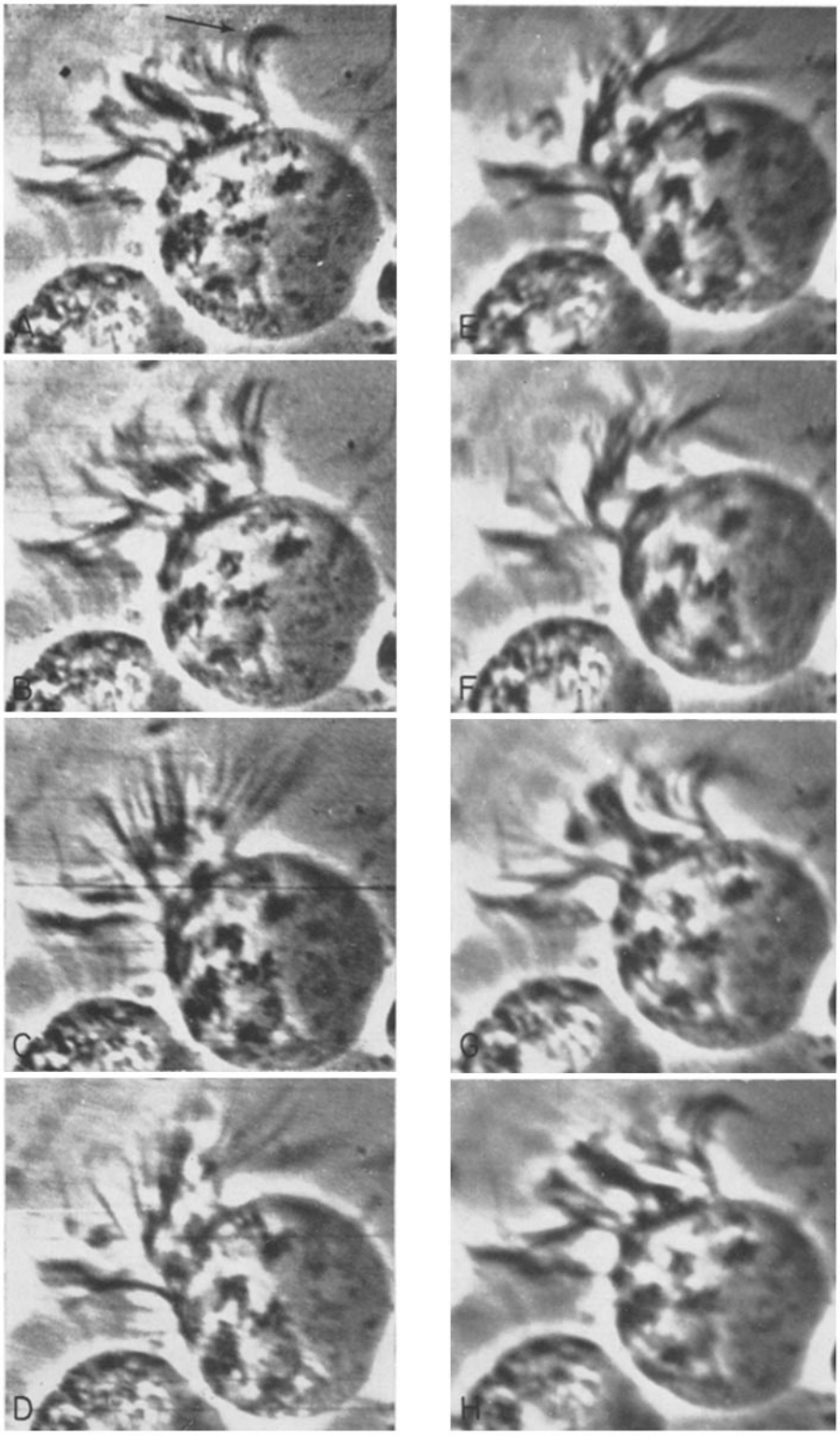


FIGURE 5 Sequence of frames showing beat form of lateral cilia in life. Note how the form of the group of cilia at the arrow (frame A) changes in each consecutive frame. See text for further explanation. × 1300.

control preparations, fixed without activation; in fact, it is rather difficult to preserve even after activation. Not all fixatives preserve the metachronal wave; osmium tetroxide itself gives good preservation only when applied rapidly. Rapid application probably prevents unfixed cilia from moving and destroying the regularity. These observations argue against the pattern's being an artifact unrelated to the physiological state of the

Close to 100 per cent metachronism was observed on the filaments of Fig. 9 before fixation, and now all the filaments show a repeating pattern after sectioning. Each unit of the pattern represents one wavelength of the fixed, sectioned metachronal wave. Because of the sectioning, the whole length of each cilium comprising the pattern is not seen. Note the wide interspace between adjacent filaments: this has been found to be

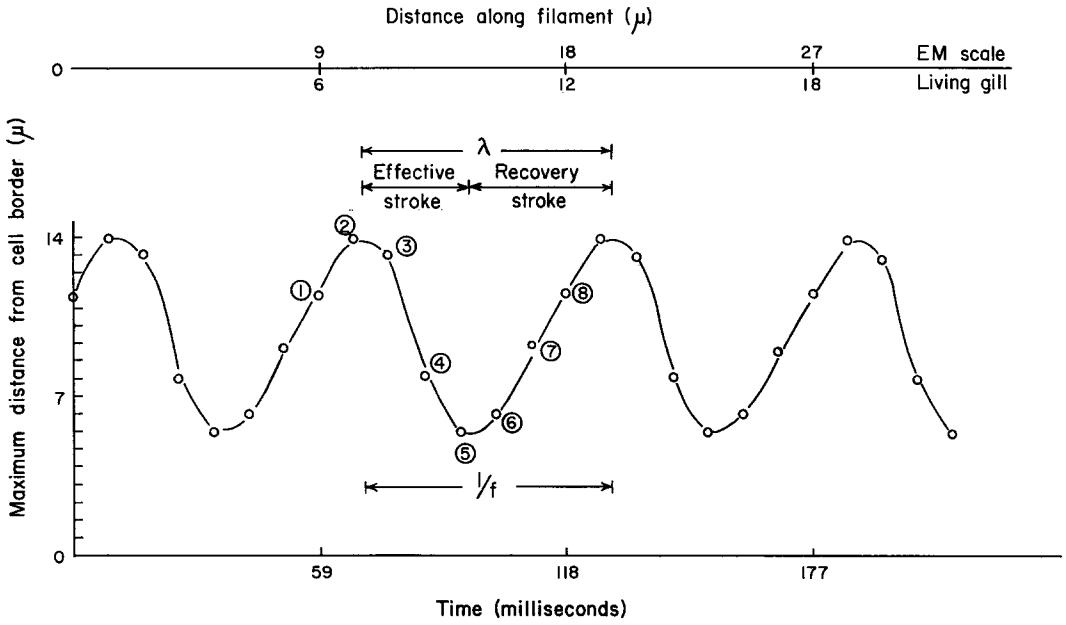


FIGURE 6 Construct of the envelope of the metachronal wave from the stages of ciliary beat. Lower abscissa plots time in milliseconds for stages of Fig. 5 (frames A to H). Ordinate plots maximum distance from the cell border (μ) of the cilia from each stage. The result is a segment (circle *1* to circle *8*) of the curve shown that is repeated each time the cilium beats. The same curve is generated at any instant by the cilia along the gill filament (upper abscissa) beating with metachronal rhythm. The scales for the abscissae are derived from the average beat frequency (17 BPS) and from measurements of Figs. 4 and 12. Wavelength (λ) and period (1/*f*) of the metachronal wave are indicated and apportioned between effective and recovery strokes.

gill or to the presence of the metachronal wave. The precise relationship of the fixed pattern to the form of ciliary beat will become clearer shortly.

Figs. 9 and 10 compare a fixed sectioned K^+-activated gill (Fig. 9) and its control (Fig. 10). Both figures represent filaments of the same gill sectioned at the level of the lateral cilia, but the filaments shown in Fig. 9 were activated by treatment with 0.04 M KCl before fixation, while the portion of the gill of Fig. 10 remained in spring water.

characteristic of well-activated gills (compare Fig. 4).

On the other hand, the picture seen in Fig. 10 is very different. The filaments are close together (compare Fig. 3) and the lateral cilia are packed so tightly that they appear as a continual grey mass in the interspace. This is consistent with the absence of the metachronal wave in the preparation before fixation. The important point is that the physiological differences between the living control and the activated gill are preserved in the

sectioned material so that cilia that were beating in life can be distinguished from those that were not.

The activated gill does apparently show all the stages of the ciliary beat. This may be seen on if the pattern preserves the ciliary beat, the entire end-on view of the beat should also be seen. This is seen from *A* 1 to *A* 2 in Fig. 1; the beat traverses an angle at 135° (compared to 113° in Fig. 5, a difference that is not considered significant).

FIGURE 7 Model of lateral cells of activated gill viewed along gill filament. The metachronal wave form is constructed by bending pipe cleaners into the forms of frames A to G of Fig. 5 and spacing these sequentially along the "cells." Shadow shows projection of the wave; a wavelength (λ) is indicated.

closer examination of Fig. 1. In Fig. 1, the filaments are far apart and the lateral cilia vary in position from one filament to the next. Where the section is relatively thick, a profile of the metachronal wave pattern should be visible, and The effective stroke is in the abfrontal direction, beginning at position *A* 1 and ending at *A* 2. During the recovery stroke as noted above, a flexible curve passes up the stalk; this is seen at *B*.

(5) ELECTRON MICROGRAPHY: Fig. 11

shows an electron micrograph of a control preparation, similar to that of Fig. 10. Again, no metachronism was present in this preparation before fixation and no metachronal wave pattern can be seen in the section. A portion of a lateral cell and the interspace above it are visible; the grey

bridge and between the central pair, all lie in one direction in such a picture. This result is confirmed in Fig. 11; each axis of each cross-section makes an angle of about 70° with the surface of the cell, almost without exception (±6° average error). However, here the interpretation of this align-

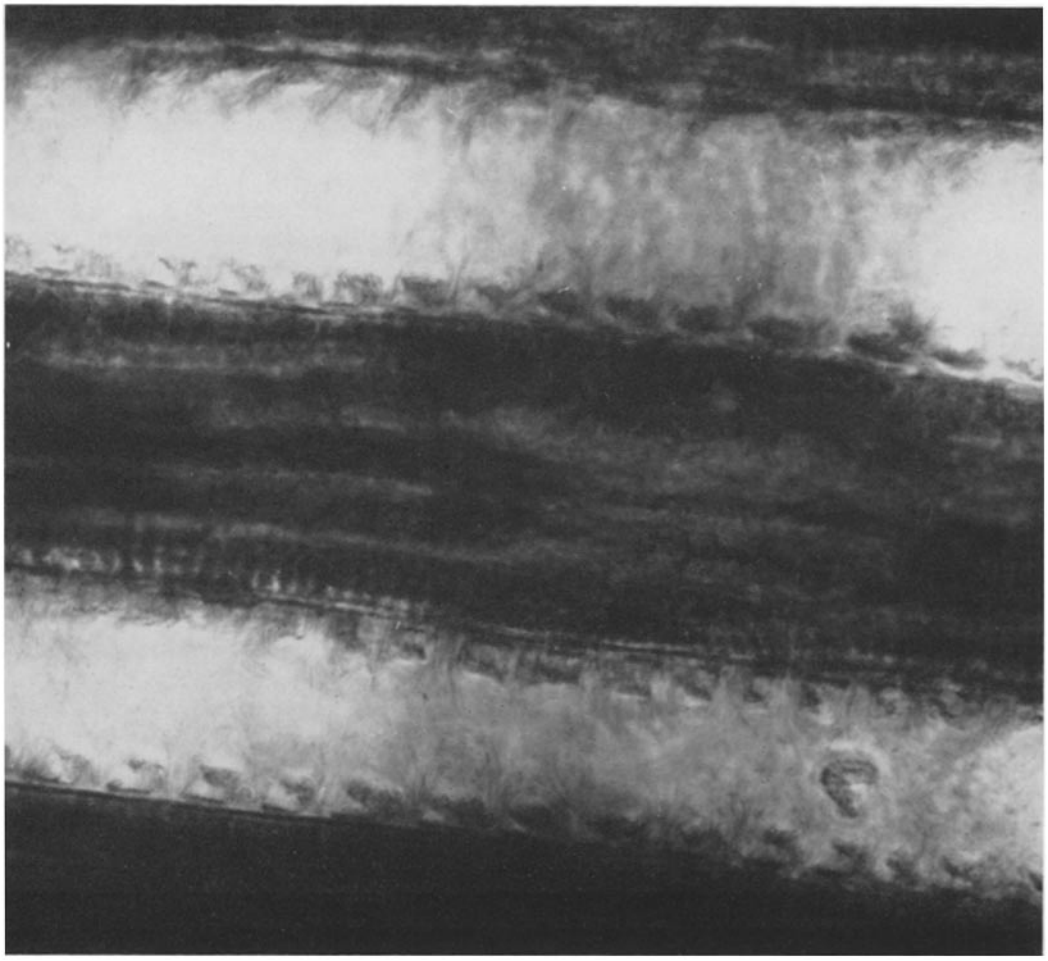


FIGURE 8 Optical section of a whole mount of a fixed activated gill. Focus at the level of the lateral cells showing repeating pattern of ciliary form. Glycerin-mounted preparation (see text footnote). 0.04 M K_2SO_4* × 1000.

mass, visible in the light micrograph, can be resolved into a multitude of regularly packed ciliary cross-sections filling the interspace. This resembles the picture of the lateral cilia presented by previous workers. Fawcett and Porter (4) and Gibbons (6) have ably demonstrated that the axes of the cross-sections, passing through the

ment of axes is somewhat at odds with previous interpretations. It has been determined that the cilia of the controls lie over the cells with their tips pointing frontally, that is, as if the cilia were about to beat effectively. As noted above, the gill filaments of the control are close together and this apparently causes a further bending of the ciliary

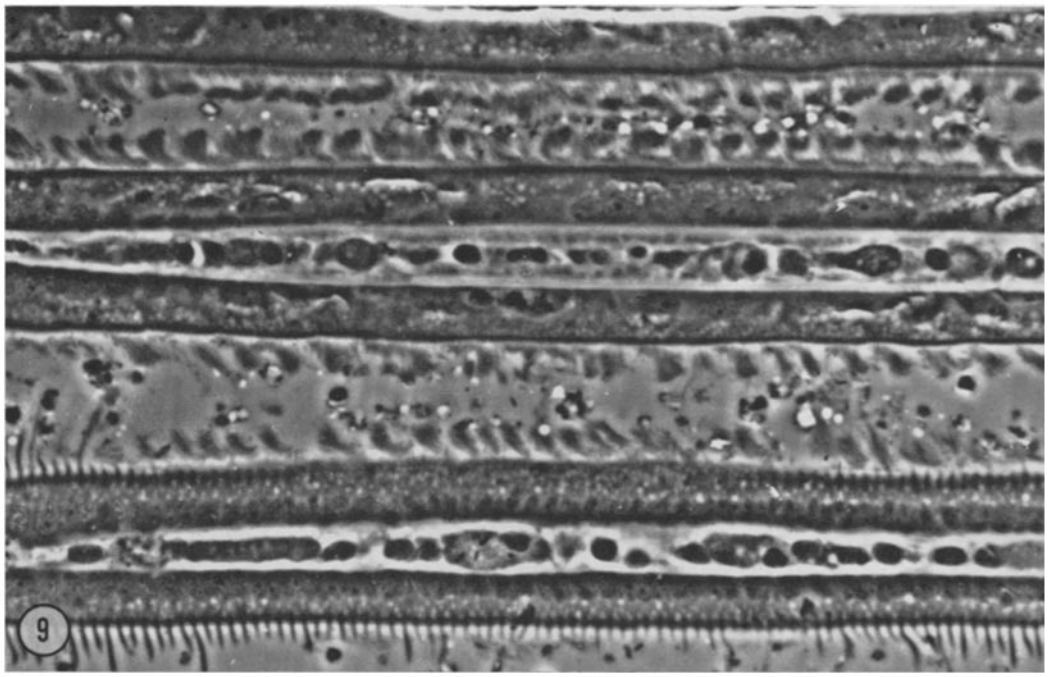
stalks so that they run almost parallel to the cell surface. The regular packing of the cross-sections results then from cuts through different levels of a package of parallel rods, the lateral cilia from adjacent gill filaments. The ciliary fibers of the 9 + 2 pattern run straight from base to tip, within these cross-sections, and so the axes fall identically. It must be emphasized again that this description is not to be correlated with the ciliary beat (see below), and need not apply in the least particular to active cilia: this is the control; the cilia are still and never move without activation.

The activated gill presents a more complex and entirely different picture. This is seen in Fig. 12. This figure shows an electron micrograph of a 0.04 M K_2SO_4-activated preparation. Several lateral cells are cut in longitudinal sections and their cilia are patterned into crests and troughs completely comparable to Figs. 6 and 7. The fit between the two-dimensional micrograph and the model speaks for itself, so that the time sequence of Fig. 5 is seen to be preserved in the distance sequence, the metachronal pattern here. Five wavelengths can be seen wholly or in part in Fig. 12. The crests represent cilia late in their recovery stroke, that is, cilia which stand upright from the cell surface (see Fig. 5, frame B). Oblique sections (arrow *A*) mark the effective stroke. These are seen first at some distance from the cell surface, then only very close to the surface (arrow *B*) if at all. The troughs correspond to cilia which have the same form as those of Fig. 5, frames D and E. The recovery stroke is outlined by the sweep of longitudinal sections from the troughs to the crests (rectangle) that represent the stalk pulled at more and more distal portions through the plane of the section as the recovery wave traverses it.

The section in Fig. 12 is a fortunate one because it passes directly through the lateral cell layer and cuts the bundle of cilia longitudinally. More often, if the section is more oblique or frontal, only part of the fixed pattern is seen, and then many of the cilia may be cut transversely. This is seen at intermediate magnification in Fig. 13. The fixed wave can be identified by the sloping front of ciliary sections that progresses away from the surface of the lateral cell. Most of one wavelength is visible, but the majority of transverse sections are apparently of cilia in their recovery stroke. This is a picture that points up the difficulties of correlating its inactive counterpart (Fig. 11) to the ciliary beat. First, the irregularity of the packing of the cross-sections should be contrasted to the regularity of packing in Fig. 11. In Fig. 13, the gill has "opened up"; the gill filaments have separated and the cilia are, therefore, seen further apart. As in Fig. 12, the adjacent filament is out of sight. Second, note the many ciliary axis angles (lines) as compared to the specific single angle which defines the control. The axes of the cross-sections of the activated gill vary from horizontal (upper left) to nearly vertical (lower left, upper right) and the shift appears to be somewhat systematic. The angle made with the surface of the lateral cell sometimes varies from one cilium axis to another by over 90°. This is completely outside the range of variation found for the control. Finally, the circle of filaments appears to be much closer to the cell membrane in Fig. 13 than in Fig. 11. This has been confirmed by planimeter measurements. In the control, the circle of filaments occupies about 54 per cent of the cross-

FIGURE 9 Light micrograph of fixed activated gill after embedding and sectioning, showing the metachronal wave pattern stopped and preserved in the lateral cilia of each filament. The lowest filament passes out of the plane of the lateral cells and shows laterofrontal cirri also. Note the relatively wide interspace between the filaments. 0.04 M KCl. × 600.

FIGURE 10 Light micrograph of control preparation. Another portion of the gill of Fig. 9, but one that has not been activated, is shown. Sectioned at the level of the lateral cilia. The interspaces between filaments are much narrower than those shown above (Fig. 9) and they are packed with grey masses. No pattern can be seen. H_2O. × 600.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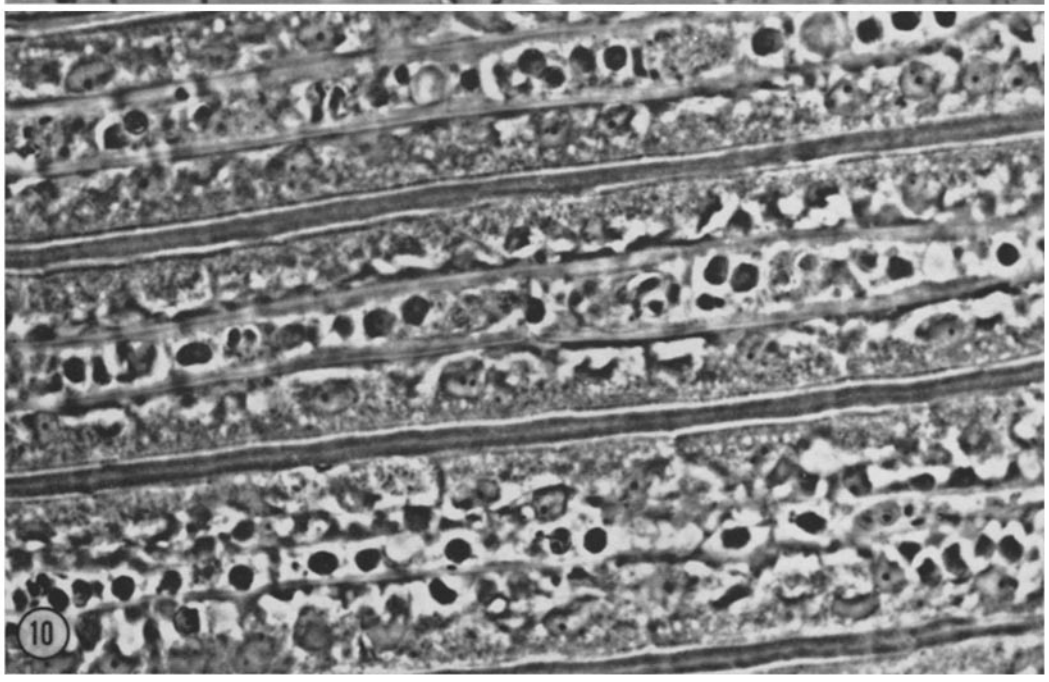
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section, according to preliminary measurements. The activated gill shows a slightly greater variability than the control, and the average diameter of the inner ring appears to be somewhat (10 per cent) larger than in the control.

Figs. 14 and 15 present the same findings in other preparations, at higher magnification. In the active preparation (Fig. 14), only a small portion of one metachronal wavelength is visible, but the variability of axes, as contrasted with the control (Fig. 15), is still seen. The contrast in packing arrangement of the cross-sections and in the relationship of the peripheral filaments and the membrane is also apparent.

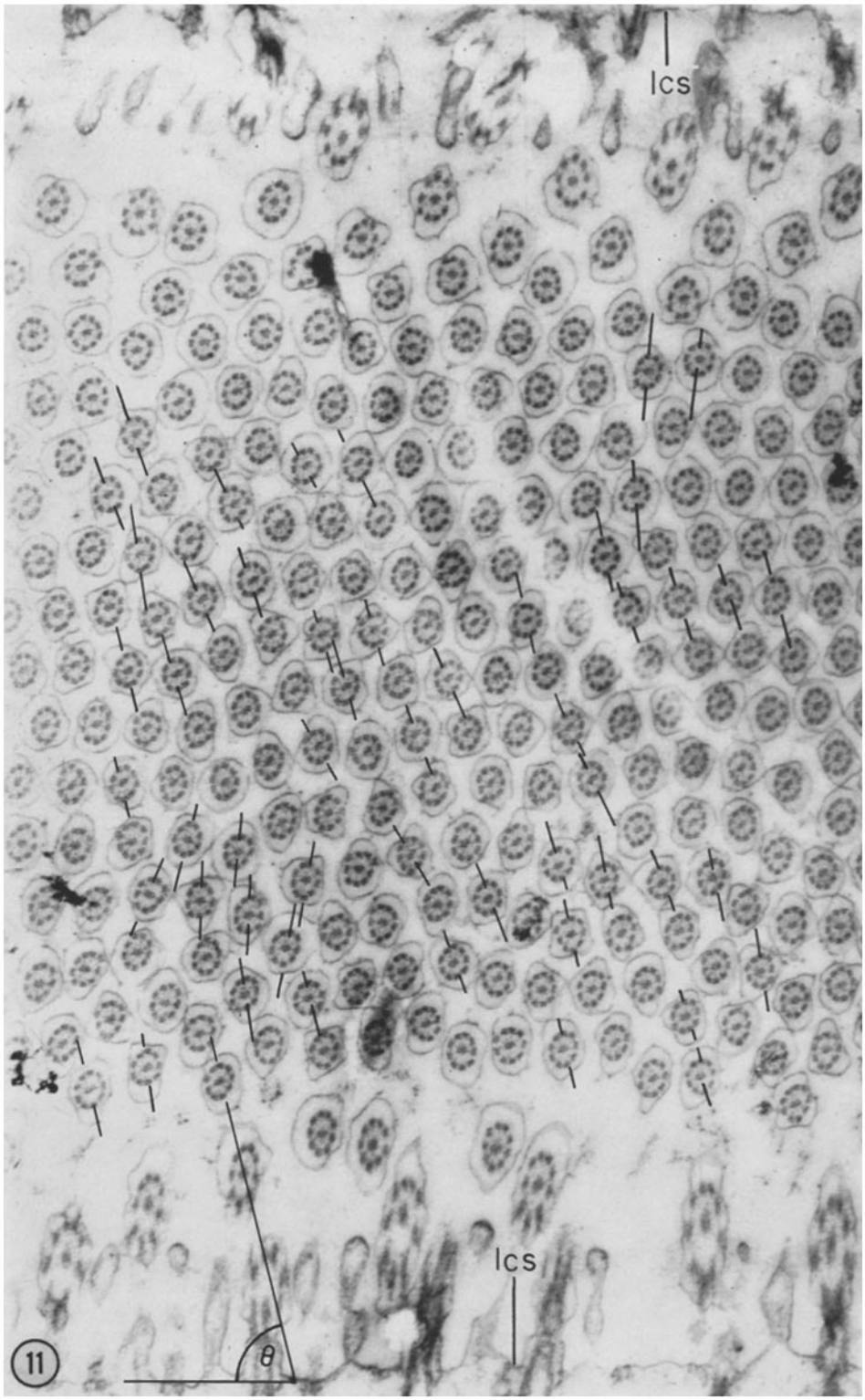
DISCUSSION

This study has demonstrated that when osmium tetroxide is quickly pipetted onto an activated *Elliptio* gill, the lateral cilia can be fixed in a pattern. This pattern can be shown to preserve many features of the ciliary beat in living lateral cells both in form (Fig. 1) and in arrangement (Fig. 12). The pattern has never been seen on unactivated excised gills where the lateral cilia do not beat, and it can be correlated with the presence of the metachronal wave in these cilia after treatment with potassium ion. Gray (10) has shown a correlation between the metachronal wave and the form of the ciliary beat in lateral cilia of *Mytilus* where the amplitude of the beat is about 180°. The amplitude of the beat here is somewhat smaller (135°), perhaps due to the artificially high potassium ion concentration (9), but, by following Gray's analysis, the same sort of correlation between wave and beat can be derived, although the curve (Fig. 6) is somewhat simplified. Therefore, it seems clear that the gross pattern seen here represents the fixed metachronal wave, and that the gross morphology is meaningful in terms of the ciliary beat.

The observation that the form of the metachronal wave can be preserved by osmium fixation has been generally neglected as a means of studying the ciliary beat. Parducz's work on *Paramecium* is the obvious exception to this neglect, but Parducz has stayed with the light microscopy of whole mounts, which are difficult to analyze. The more radical forms of ciliary beat that Parducz first claimed to have seen (12) he has later (13, 14) shown to be, at least, uncommon and possibly artifactual. Parducz now thinks (14) that the beat may have a three-dimensional component in *Paramecium*. However, his interpretation of the stroke form is more in accord with that seen by other workers (10, 15) in other instances and with that found in this study for *Elliptio* lateral cilia (Fig. 5).

There are relatively large mechanical forces present in a living, moving cilium that could cause appreciable distortion of the internal arrangement of filaments at the critical moment when the fixative hits the cell. On the basis of the present data, distortions at finer levels of organization cannot be excluded. Similarly, since potassium ion is present in high concentration in the medium surrounding all active preparations so far examined, changes in fine structure in the active gill may be related to the presence of this ion, and not to ciliary beat. Nevertheless, since the findings are consistent from preparation to

FIGURE 11 Survey electron micrograph from a control preparation. Note the regularly aligned ciliary cross-sections filling the interspace between adjacent filaments. The cilia come from lateral cells from both sides of the interspace; the surfaces of two such cells are indicated. The lines through the cross-sections pass through the central pair of ciliary filaments, through the bridge through filaments 5 and 6, and through filament 1. This defines the axis of the cross-section (2, 4, 6). The bridge is always above the central pair when the cilia are viewed from above the lateral cell of origin. An angle between the lateral cell surface (*lcs*) and the cross-section axis may be measured. One such measurement (θ) is shown. The near-parallel alignment of the axes as shown is reflected in a near-uniformity of axis angle measurements (see text). Also note the wide separation of the ring of ciliary filaments from the ciliary membrane. H_2O. × 25,000.


lcs
lcs
θ
11

preparation, any distortions that occur probably represent consistent interactions between the moving cilium, the ion, and the fixative, and, therefore, should prove of some later value in interpreting ciliary motion.

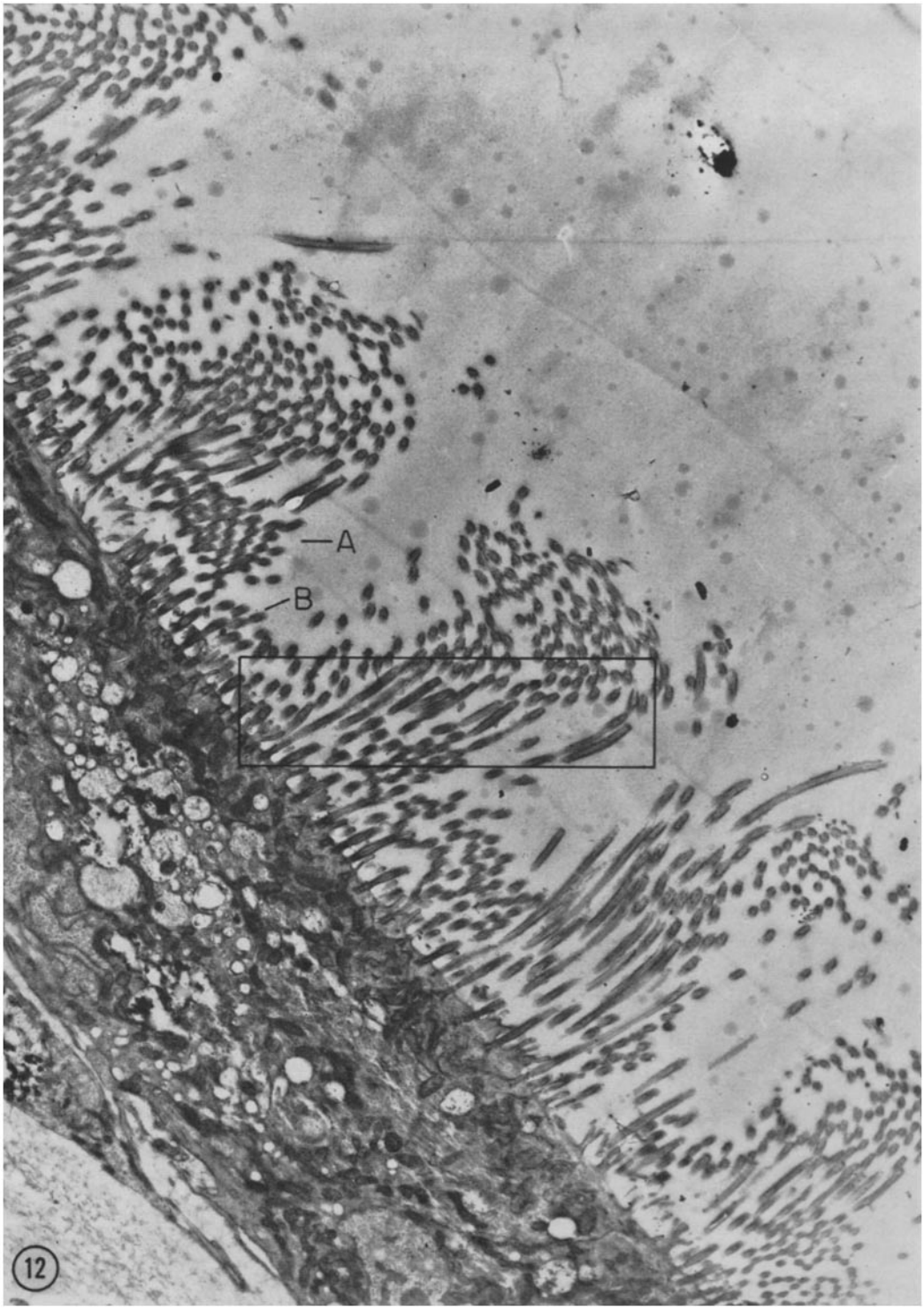
Attention has been drawn to the differing nature of this study and previous studies of ciliary morphology of lamellibranch gills with the electron microscope. The published pictures of Fawcett and Porter on *Mya* (4) or Gibbons on *Anodonta* (6) show no evidence of a fixed metachronal wave in the lateral cilia. As mentioned above, these pictures resemble the control micrographs presented here. These authors do not provide any information as to whether the metachronal wave was present in the gills before fixation, but, if the gills were excised from the animals, only a short time would have to elapse before metachronism was completely abolished if the situation is similar to that in *Elliptio*. Whatever the reason, on the basis of the published micrographs it seems reasonable to assume that in this earlier work the ciliary beat was not preserved; therefore, comments by these workers on correlations between ciliary fine structure and beat must be taken somewhat cautiously.

This study has defined three new parameters that distinguish the active gill from the control and that may prove useful in correlating fine structure and beat. Specifically, this study has shown that Fawcett and Porter's interpretation of the axis of the cilium with respect to the stroke is oversimplified. Fawcett and Porter have claimed that the ciliary filaments run straight, and that the ciliary beat in *Mya* is planar on the basis of finding that all cross-sections through *Mya* lateral cilia show parallel axes. Gibbons has shown an identical case for *Anodonta*, and the present study has produced evidence of the same phenomenon in *Elliptio* (Figs. 11 and 15). But this is the control situation in *Elliptio* at least: all the cilia lie pressed against the surface of the gill, straight and in one position. The activated gill shows a more complicated picture (Figs. 13 and 14); the ciliary cross-section axes are not aligned and are quite variable with respect to the angle they make with the surface of the lateral cell. This, in turn, may mean that the fibers do not run straight in beating cilia, or that the beat is not strictly two-dimensional.

Another difference seen here in the activated preparation *vs.* the control is in the per cent of the ciliary cross-section occupied by the circle of peripheral filaments. The filaments are close to the membrane in the active preparation (Fig. 14) while there are large gaps between filaments and membrane in the control (Fig. 15). As an initial hypothesis, changes in the inner diameter are taken to be an index of stiffness of the cilium, and changes in the axis angle, discussed above, are taken to be an index of progression of the recovery wave up the ciliary stalk, large angles occurring before recovery. The third difference in spatial arrangement of the ciliary cross-sections would seem to follow as a consequence of the "opening up" of the gill on activation. This may be of little significance in terms of mechanism of beat. However, the constancy of all these parameters in the control is taken to mean that this preparation has come completely to rest at an equilibrium position not in itself descriptive of any beat position.

Finally, this study has some presented evidence against Harris's hypothesis that the 9 + 2 pattern shifts to a hexagonal packing arrangement during activity (11). No such deformation has been observed (Figs. 13 and 14). On the contrary, all the evidence here favors the view that changes during the ciliary beat are rather more subtle shifts in position of the ciliary filaments. The interpretation remains somewhat difficult and incomplete at the moment; no doubt other useful parameters of

FIGURE 12 Low-power electron micrograph showing fixed metachronal wave. The interspace is wide and the filament adjacent to the one shown cannot be seen in this picture. Compare form of the pattern to Figs. 6 and 7. At *A* and *B*, cilia are sectioned in their effective stroke; in the rectangle, successive portions of the ciliary shaft are pulled through the plane of the section in the recovery stroke. With minor variation, this sort of stroke form is seen for 4 to 5 wavelengths. 0.04 M K_2SO_4. × 6000.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B
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change are yet to be discovered. However, preparations like the one presented in this study, where the metachronal wave has been preserved, and hence cilia fixed in all stages of their beat, should provide an excellent tool for testing speculations about the mechanism of ciliary movement in terms of ciliary fine structure.

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FIGURE 13 Survey electron micrograph from an activated preparation for comparison with Fig. 11. Part of one wavelength visible; cross-sections show cilia in recovery stroke. Note the wider spacing between cross-sections. The adjacent filament is not visible. The lateral cell surface (*lcs*) is indicated and axis lines and angle measurements are taken as in Fig. 11. Note variations in lines (and, therefore, in angle measurements) from left to right of figure so that at a level where large oblique angles are measured, middle left, smaller angles can be measured, middle right. Note, too, that the ring of ciliary filaments seems closer to the ciliary membrane than in the control. 0.04 M KCl. × 25,000.

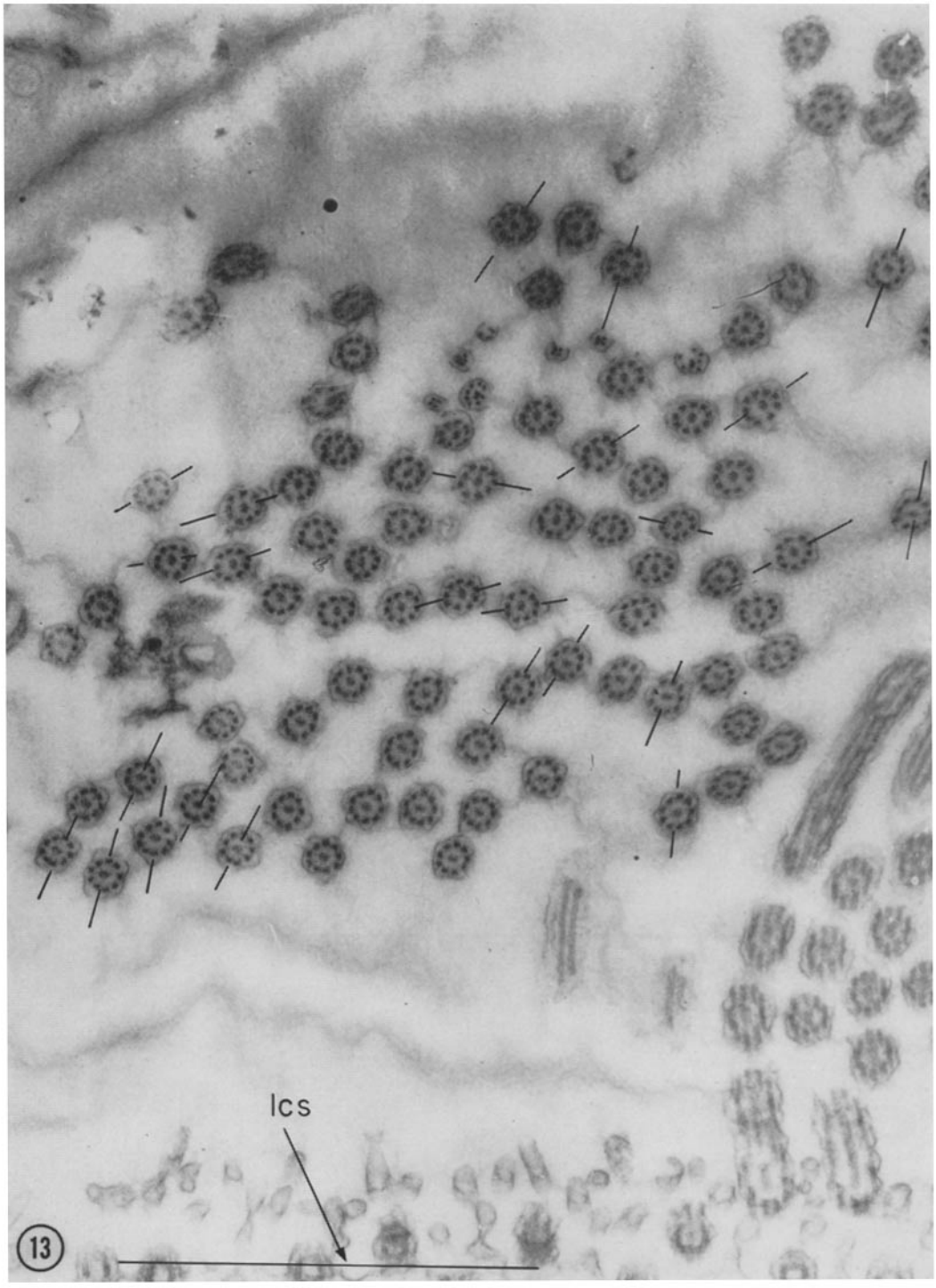
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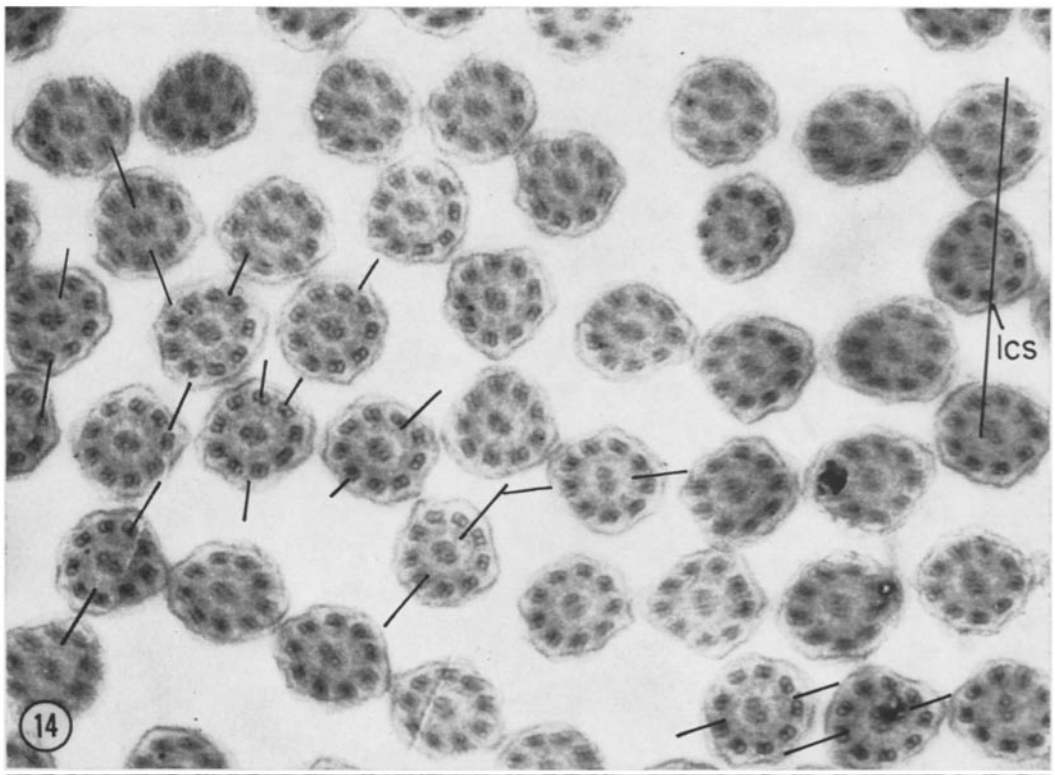
FIGURE 14 Ciliary cross-sections from an activated preparation. The irregular packing of cross-sections contrasts with the regularity of arrangement in the control. From cross-section to cross-section, differences of more than 90° in orientation of the central pair of filaments may be seen. The axes of the cross-sections (lines) are determined as above (Fig. 11), except that the numbering of the peripheral filaments is more difficult because of the variability in direction of the central pair. A line (*lcs*) parallel to the surface of the lateral cell is indicated. The ring of filaments is in close proximity to the ciliary membrane. 0.04 M K_2SO_4. × 58,000.

FIGURE 15 Ciliary cross-sections from control for comparison with Fig. 14. Although a greater number of cilia are shown, the axis alignment is less variable from cross-section to cross-section. Differences in orientation of the central pair do not exceed 20°. There are often wide gaps between the peripheral filaments and the ciliary membrane. H_2O. × 50,000.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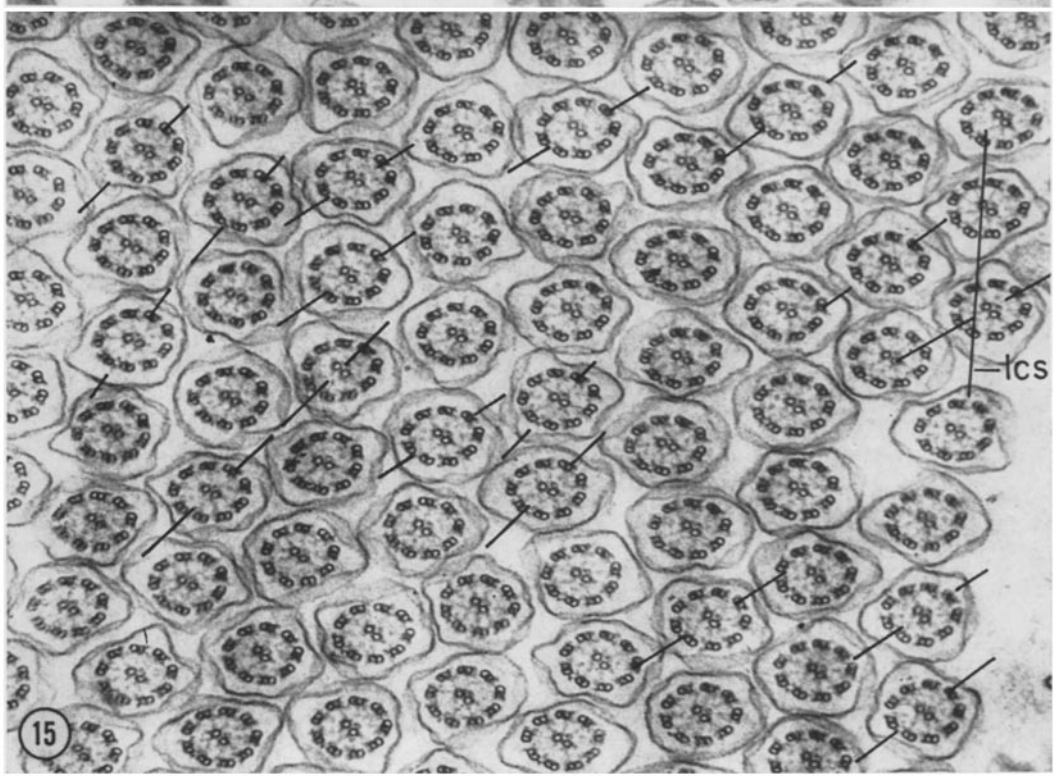
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