BRIDGES BETWEEN MICROTUBULES

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ABSTRACT
Bridges between microtubules have been studied with the electron microscope in the axostyle of Saccinobaculus and in various tubule systems of chicken testis, including the helix of tubules surrounding the elongating spermatid nucleus and the flagellum of the sperm tail. In addition to the previously described periodic bridges, evidence is presented that nonperiodic bridges exist between certain tubules. An analysis of axial spacing between adjacent nonperiodic bridges suggests that these structures are attached to periodic binding sites on the microtubule wall, but that not all the binding sites are filled. The bridges appear nonperiodic as a result of random occupancy of some fraction of the periodic sites. The distribution of these binding sites is related to the substructure of the microtubule wall as seen with negative staining and optical diffraction.

INTRODUCTION
There are numerous examples in the literature of highly ordered arrays of microtubules. Reported studies include: the axostyle of certain flagellates (25, 26, 8, 29, 41, and 39); the axopods of heliozoa (34, 31, 33, 58, 57, 59, 56, 48, 49, 52, 55); the tentacles of suuctoria (50, 3); the cytoparyngial basket of some ciliates (60, 61, 62); the cortical fibers in other ciliates (2, 35 a, 32); transient structures in a variety of spermatids (10, 40, 46, 18); permanent structures in other sperm (5, 44, 45, 47); and the ubiquitous 9 + 2 structure of cilia and flagella (19, 23, 1, 51, 43, 30, 63, 64, 65). Studies on each of these systems have revealed thin bridges between the component microtubules. Several investigators have suggested that the bridges are important for maintaining the order of the microtubule arrays (34, 26, 40, 55, 48, 4, 56, 39). Evidence supporting this conjecture includes the observation that the interrelationship of the outer doublets in the 9 + 2 array can be maintained during solubilization of the other structural components of a flagellum until only the A sub-
tubules and periodic links between them remain (53). From this evidence and our general knowledge of how macromolecular aggregates are put together in biological systems (11, 12), it seems reasonable to conclude that bridges are important for the establishment of specific intertubule geometries in ordered microtubule arrays.

Intertubule bridges have attracted additional attention because of the possibility that some of them may be functionally analogous to myosin, and may play a direct role in certain examples of cell motility. A few arrays of microtubules are motile organelles (e.g., cilia and flagella, the axostyle, coccid sperm tubule bundles [44], and the mitotic spindle), and others have motion associated with them (e.g., nuclear migration in virus-induced syncytiu [29 a]; granule motion in chromatophores [6 a], in the arms of heliozoa [58], and of suuctoria [50]; and the elongation of certain spermatid nuclei [40]). It has been postulated that intertubule bridges and projections from tubule surfaces are transducers which hydrolyze...
high-energy phosphates and do mechanical work in a fashion analogous to muscle myosin (33). In the case of cilia and flagella, there is little question that the doublet tubules bind an ATPase called dynein which is essential for ciliary motility (24, 20, 22, 21). Dynein works to generate forces that slide microtubules parallel to their neighbors (54). The case for a similar model in the protozoan axostyle is weaker, but in this organelle, the tubules definitely slide over one another (36), and there is a dynein-like enzyme present as part of the axostyle (41). Sliding processes have been implicated in other motile systems built from microtubules (40, 2, 37, 38), but in these systems there is even less information available about the structure and function of the intertubule bridges.

Some interconnected tubules show regularity in the position of their bridges both around and along the microtubule axis. As a simple example, the tubules of the axostyle of the flagellate *Saccharomyces* are connected into rows by links attached at sites separated by about 180° of arc around the tubule axis. In longitudinal view, the links are periodically arranged in straight rows parallel to the tubule axis, and show an axial spacing of about 150 Å (26, 41, 38). The intertubule links in cilia and flagella are considerably more complicated. Chasey has shown that the inner pair of singlet tubules displays an axial period of about 160 Å (13). The outer doublet tubules have two dynein arms bound to the A subfibril at a separation of about 90° of arc. The axial period of the dynein is usually reported as about 150 Å, but values ranging from 120 Å to 240 Å are to be found in the literature (15). The A subfibril of the doublet tubules also binds a protein called nexin which is distinct from dynein and which binds each A subfibril to its two neighboring A subfibers. The axial period of the nexin binding site is 1,000 Å (53). The A subfibers of *Chlamydomonas* bind “spokes” which are separated by alternating intervals of about 300 Å and 700 Å (30). A similar arrangement has been described for the spokes of *Tetrahymena* cilia (15) and for various sperm flagella, although there is some variation in the placement of the long spacing (63, 64). Clearly, the microtubules of the 9 + 2 array possess a variety of binding sites for diverse bridges and projections, and there is a plethora of periodicities.

Other microtubule aggregates show evidence for regular bridges only when the tubules are seen in cross-section. The tubules of the cytobranchial basket of *Nassula* are interconnected by bridges which show sixfold rotational symmetry in their point of attachment to the microtubule surface. In longitudinal section, however, the bridges are not evident, and Tucker has suggested that the interconnections between tubules are sheetlike, with the plane of the sheet parallel to the tubule axis (60). In the axonemes of the heliozoa, the cross-section image is more elaborate, but still clear: there are two different lengths of intertubule bridges, one about 20 Å and one about 280 Å long (48). Each length of bridge seems to attach only to particular positions on the tubule circumference. The regularity of bridge placement in the cross-section image of the axoneme has prompted Roth and his coworkers as well as Tilney and Byers to suggest specific interactions between the bridges and the structural subunits of the tubule wall (48, 49, 55). When seen in longitudinal section, however, these intertubule bridges are not obviously periodic. Selected areas do indeed show regular bridging (49), but the case for an axial periodicity is not as strong as one might expect from the clarity of the cross-section images.

To extend our knowledge of intertubule bridges beyond the simple acknowledgment of their existence, we need structural and biochemical information as well as correlations between the two. This paper is a structural study of the distribution of intertubule bridges in several systems ranging in order from the paracrystalline (the protozoan axostyle) to the largely disordered (the mitotic spindle). In the axostyle there are nonperiodic bridges as well as the periodic ones previously described (26). Other systems, such as the pruninuclear helix of the chicken spermatid (40), also contain nonperiodic bridges. Accurate measure of the axial spacing between adjacent nonperiodic bridges reveals that they are separated by approximate integral multiples of the minimum interbridge spacing. Our findings suggest that all tubules possess periodic sites for binding bridges, but that in many systems, some of the binding sites are not filled when viewed in the electron microscope, so the bridges randomly occupy a fraction of the periodic sites and appear nonperiodic. We present evidence from negative staining studies of different microtubules to support the idea that the periodicity of the bridge binding sites is systematically related to the microtubule surface lattice.
|    | A           | B           | C           | D           |
|----|-------------|-------------|-------------|-------------|
| 1  | 1           | 6           | 4           | 1           |
| 2  | 2           | 1           | 1           | 1           |
| 3  | 3           | 7           | 3           | 3           |
| 4  | 4           | 4           | 4           | 4           |
| 5  | 5           | 7           | 7           | 7           |
| 6  | 6           | 6           | 6           | 6           |
| 7  | 7           | 7           | 7           | 7           |
| 8  | 8           | 8           | 8           | 8           |
MATERIALS AND METHODS

The polymastigote flagellate Saccinobaculus was obtained from the hind gut of the wood-feeding roach Cryptocercus punctulatus, generously supplied to us by J. J. Murray of the University of Virginia. Chicken testis was obtained from roosters killed by intravenous injection of Nembutol or by cervical dislocation. All material was prepared for electron microscopy by methods described earlier (for Saccinobaculus [39], for chicken testis [40]). Electron microscopy was performed on a Siemens 1A or a Philips 300 microscope. The microscopes were calibrated with a replica grating; experimental error is estimated at less than 8%. For some studies, very thin sections were necessary (about 200 Å). When contrast was inadequate, the negatives were contact-printed onto DuPont CRW film (E. I. DuPont de Nemours & Co., Wilmington, Del.), to make a medium-contrast reversal transparency.

The principal experimental problem in this investigation was establishing what was to be called an intertubule bridge, and what was to be disregarded as a precipitate of cytoplasm which lay by chance near a microtubule. This discrimination was made on the basis of three criteria: (a) size (35-70 Å in thickness and 100-250 Å in length); (b) staining intensity (pale in comparison with the cross-section image of a tubule wall and about the same as the longitudinal image of the wall); and (c) regularity of form (no sharp bends, no jagged edges). The table of bridge morphology (Table I), together with its legend, provides examples of the application of these criteria.

Most measurements of interbridge distance were made on the negatives using a Nikon microcomparator (Nikon, Inc., Div. of EPOI, Garden City, N. Y.). The design of this instrument permits measure of spacings with no feedback to the operator of the system (precision of the calipers, dδ0.1 mm = 5 Å, better than the resolution of the micrographs). Bridge periodicity in the chicken spermatid tubules was also studied by optical diffraction. (Optical diffraction studies of axostyle tubules are presented elsewhere [36]). To minimize noise, the negatives were printed and the microtubules were covered with thin white tape, leaving only the edges of the walls and the space between the tubules showing. The boundaries of the area to be transformed were then defined by a sawtooth mask, to throw the mask diffraction off into regions of no interest. The picture was then photographed on 35-mm Kodak Direct Positive film (Eastman Kodak Co., Rochester, N. Y.), and

All interbridge spacings in Saccinobaculus were measured by an assistant who was trained in the criteria for bridge recognition, but who was unfamiliar with the material and the results obtained from other systems. Measurements on microtubules from chicken testis were made with the comparator, but in this system several other methods were used as well, since the bridges are faint and sufficiently infrequent to make it difficult to find them on the screen of the comparator. Interbridge distance was measured with vernier calipers on high contrast prints at X 200,000 (precision of the calipers, ±0.1 mm = 5 Å, better than the resolution of the micrographs).

In an effort to minimize the subjectivity involved in deciding what constituted a bridge, tracings were made between adjacent tubules with a Joyce-Loebel recording microdensitometer (Joyce, Loebel & Co., Inc., Burlington, Mass.). The resulting record of optical density contained many peaks of diverse sizes (Fig. 5 c). An optical density peak was empirically defined as a bridge when it fulfilled two criteria: its half-width at half-height was between 35 and 70 Å, the width taken for bridges; and its height (the difference between the peak value and the average of the two adjacent relative minima) was equal to the average signal recorded when tracking across a microtubule in longitudinal orientation ± 25%.

Bridge periodicity in the chicken spermatid tubules was also studied by optical diffraction. (Optical diffraction studies of axostyle tubules are presented elsewhere [36]). To minimize noise, the negatives were printed and the microtubules were covered with thin white tape, leaving only the edges of the walls and the space between the tubules showing. The boundaries of the area to be transformed were then defined by a sawtooth mask, to throw the mask diffraction off into regions of no interest. The picture was then photographed on 35-mm Kodak Direct Positive film (Eastman Kodak Co., Rochester, N. Y.), and

Table I Table of bridge morphology. Examples of bridges between microtubules. The numbers on adjacent tubules indicate my characterization of the labeled structures according to the criteria described in the Materials and Methods section. The black bars obscure certain intertubule spaces to make the designation of the numbers unambiguous. 1, a bridge; 2, incomplete, not counted; 3, too faint, not counted; 4, too diffuse, not counted; 5, too dark, not counted; 6, too big, not counted; 7, nothing at all. (A) A longitudinal section from the axostyle of S. isai. The line of sight is down the tubule rows (see text). The section is about 200-Å thick, so that certain regions such as the tubule immediately below the first black bar show the intertubule links which stick up at the observer as sharp periodicities, but little microtubule material. The thin section allows high resolution, but reduces the number of clear intertubule bridges by slicing through some and excluding others entirely (X 325,000). (B and C) Cross-sections of the microtubules from the perinuclear helix of a chicken spermatid. All intertubule spaces are labeled following the conventions defined above (X 260,000). (D) A longitudinal section of spermatid helix tubules. Numbers as above (X 270,000).
used as a specimen for diffraction. The design of the diffractometer has been previously described (36).

Bridge periodicity of the helix tubules was also investigated with the technique of translational superposition. The micrometer stage from a Nikon microcompator was used as an easel under the photographic enlarger. Each translational event could then be accomplished with high precision (±0.02 mm on the prints). In studies of the chicken meiotic spindle, only measure with the microcompator was used, because the disordered arrangement of the tubules eliminated all indirect methods.

To investigate the role of bridges in the stability of tubule aggregates, I have homogenized chicken testis in a variety of buffers to see whether the bridges can be broken without disrupting the microtubules. The tissue was homogenized with a rotating-pestle homogenizer in a given buffer and allowed to stand for varying periods of time at various temperatures. The homogenate was then spun at 30,000 g for 30 min, and the pellet was resuspended in 10 vol of 3\% glutaraldehyde buffered with 0.1 M sodium cacodylate to pH 7.2. After a 30-min fixation, the material was spun at 100,000 g for 30 min and the pellet was processed for electron microscopy as if it were tissue.

Isolated chicken testis microtubules were micrographed using uranyl acetate as a negative stain for electron microscopy. Testis was homogenized with a Potter-type homogenizer in buffers which stabilized the microtubules, and was then fractionated by differential centrifugation. Spermatid helix and manchette tubules were found in twice-washed nuclear pellets resuspended in buffered glutaraldehyde and put on carbon-coated Formvar films (polyvinyl formal, Ladd Research Industries, Inc., Burlington, Vt.) for negative staining. Sperm tail tubules were separated from the microsomes of a high-speed pellet by isopycnic banding on 1.2-1.4 g/ml linear density gradients of iothalamic acid (Mallincrodt Chemical Works, St. Louis, Mo.).

**Observations and Discussion**

**Axostyle**

Two large species of *Sacrinobaculus* were studied, *S. ambloaxostylus* and *S. lata* (16). Both organisms possess a motile, paracrystalline axostyle, but the distribution of the component microtubules and the pattern of bridging between the tubules are different (39). *S. ambloaxostylus* is the more ordered of the two. Fig. 1a shows a cross-section of this axostyle in which three kinds of attachments to the tubules can be recognized: arms (the 50 × 100 Å straight projections labeled A), links (the structures labeled L that connect one arm to the near side of the next tubule thereby arranging the tubules into rows), and bridges (the occasional connections labeled B which join adjacent rows, sometimes running from tubule to tubule, and sometimes from tubule to arm). We reserve the word "link" for the intertubule connections which bind the tubules into rows (intrarow links) and use the word "bridges" for connections between tubules in adjacent rows (interrow bridges).

Every tubule in the cross-section image is linked to its neighbors in the row, but only about one-half of the tubules are bridged to an adjacent row (Fig. 1a). The links are always in place, whereas the bridges only occasionally occupy their sites on the tubule wall. Therefore, there are at least two kinds of intertubule connections in the axostyle, based simply on the morphological criterion of frequency of observation. The positions of the link and bridge sites seem to be related by a symmetry operation. When bridges are present with the links and arms, they are bound to the tubule surface at points which show approximately sixfold rotational symmetry (Fig. 1a).

In longitudinal section, the links and arms are seen to be strictly periodic along the tubule axis at about 150 Å (26, 41, 39). Fig. 1b is a longitudinal view of the axostyle tubules of *S. ambloaxostylus* with the line of sight along the tubule rows. The intrarow links are projected onto the surface of the tubules and thus show as periodicities on the tubule surfaces. Fig. 1c is the same view for *S. lata* in which there are only links, no arms, so the periodicity is less clear. The interrow bridges appear in these pictures as connections between the tubules. The arrows in Figs. 1b and c point out examples of the bridges which we recognized. Others are visible upon close inspection.

At first sight the interrow bridges did not appear to be periodic, but given the regularity of the intrarow links, it seemed reasonable to look for order in the distribution of the bridges as well. Measurement on the microcompator of the axial distance between the points of attachment of adjacent bridges to the tubule's surface in *S. ambloaxostylus* gave a mean value of 370 ± 160 Å (N = 100), which is tantamount to no periodicity at all. When the data are presented in a histogram, however, plotting axial spacing between adjacent bridges against number of observations, several...
FIGURE 1  Fig. 1 a is a cross-section of the axostyle of *S. ambloaxostylus*. Arms (A), links (L), and occasional bridges (B) are seen affixed to the tubule surface with approximately sixfold symmetry. (× 310,000). Fig. 1 b is a longitudinal section from the same organism. The arrows mark some of the interrow bridges; others are evident upon close inspection. (× 280,000). Fig. 1 c is a similar picture from *S. lata*. (× 200,000).
modes can be seen. The data appear to fall into four groups (Fig. 2 a). If we define boundaries between groups as places where the population first drops to zero on either side of a mode, the mean and standard deviation for each group is $159 \pm 20 \text{Å}$, $294 \pm 30 \text{Å}$, $434 \pm 25 \text{Å}$, and $580 \pm 24 \text{Å}$.

The means of these groups approximate integral multiples of the minimum group mean. Since the minimum group mean for the interrow bridges is close to $150 \text{Å}$, the average value for the periodicity of the intrarow links as measured by optical diffraction (36), there appears to be a systematic relationship between the sites which bind the

links and those which bind the bridges. Scrutiny of Figs. 1 b and c will show that most, but not all, of the interrow bridges attach to the tubule surfaces at points marked by an intrarow link. About 90% of 150 interrow bridges examined in S. ambloaxostylus were so attached; the remainder lay between links. In S. lata, the equivalent figure is 80%.

Given the sharpness of the link-dependent layer lines in diffraction patterns taken from electron micrographs of axostyles (36), and given the apparent coupling of bridge and link binding sites, one might expect the distribution peaks in Fig. 2 a to be sharper than observed. To prepare comparable data for links, I measured directly on the microcomparator the axial distance between 100 intrarow links and prepared a histogram of the data. Fig. 2 b shows that the mean distance between links is $153 \text{Å} \pm 17 \text{Å}$. The standard deviation is similar to that of the first group in the data shown in Fig. 2 a. The greater spread in the groups for larger interbridge distances is not surprising, because distortions generally behave as a percent of total length.
The interval distribution of 500 interrow bridges from *S. lata*. Distance along the tubule is expressed in numbers of intrarow links counted in going from one interrow bridge to the next.

Since 10–20% of the interrow bridges were connected to the tubule surface at points between intrarow links, I looked to see whether these less frequently observed bridges might show a preference for a particular portion of the tubule surface between the intrarow links. Fig. 2c is the distribution of axial position of attachment for bridges lying between intrarow links. The data from both species are combined and axial position is expressed in percent of the distance from one intrarow link to the next. There is no evidence for preferential placement, considering that the judgment that a bridge is not opposite a link will be increasingly frequent when the bridge is in the middle of the space between the links. We interpret bridges lying between links as a result of nonspecific bridge-binding to the tubule surface, though there may be specific but weak binding sites between the ones which predominate.

From these data one can infer a pattern relating intertubule bridges to the microtubule wall. I suggest that the tubule wall contains periodic sites which can bind bridges. In transverse view of the axostyle tubules, the sites are arranged with approximately sixfold rotational symmetry; in longitudinal view, the sites show 150-Å translational symmetry parallel to the microtubule axis. There are at least two different kinds of sites: those which bind arms or links in a periodic fashion, and those which bind the interrow bridges in a nonperiodic manner. The difference between the two patterns cannot be due solely to the bridges and links themselves, because the links and arms of *S. ambulocystis* bind only to three of the six sites and never to the other three. Arms and links bind to their sites strongly enough to fill all available sites, so the links and arms appear periodic. The nonperiodic distribution of interrow bridges both around and along the tubule axis can be accounted for by the hypothesis that the binding of bridges to their sites (as opposed to the binding of links to their sites) is not strong enough to saturate the sites. A related possibility, of course, is that fixation is only partially effective in the case of the bridges.

The strength of binding may be less than that for the links, and/or the concentration of bridges may be a smaller fraction of the concentration of their binding sites. The apparent lack of periodicity is envisioned simply as a result of the random occupation of individual sites making up some fraction of the periodic sites available (Fig. 2d).

Studies of the axostyle, using the technique of rapid freezing and then freeze-cleaving as preparation for electron microscopy, show an image of the axostyle which is strikingly similar to that of the fixed, embedded material. The fraction of sites for nonperiodic bridges which are filled is higher, but the bridges are still not periodic, suggesting that fixation effects play a part in the relative frequency of the two kinds of microtubule connections, but that they do not account completely for the observed differences.

If this model is correct, then the frequency of finding an interrow bridge expressed as a function of spacing between adjacent interrow bridges should follow the Poisson interval distribution, \( F = \mu e^{-\mu X} \), where \( F \) is the expected frequency, \( \mu \) is the average number of bridges per unit distance, and \( X \) is distance along the tubule axis. Distribution data have been obtained by counting the number of intrarow links from one bridge to the next in *S. lata*. These data are presented in Fig. 2e. They conform well to the expected negative exponential (the \( \chi^2 \) test for goodness of fit shows

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1 Bloodgood, R. A., and K. Miller. Manuscript in preparation.
that the probability of this distribution's arising at random is far less than 0.005). The most noticeable departure from expectation is for bridges one intrarow link apart. This departure may reflect a capacity of one bridge to reduce the probability of another bridge's binding immediately adjacent, but the possibility of a systematic observation error cannot be excluded.

**Microtubule Helix**

Given the structural similarity of microtubules from diverse systems, one might expect to find similar patterns relating tubules and bridges in tubule arrays which do not possess a periodic set of intertubule links, for instance the helix of microtubules which surrounds the elongating nucleus of a chicken spermatid (40). When these tubules are seen in cross-section, the bridges appear as fine threads 30-50 Å in thickness and 100-200 Å in length (Fig. 3 a, arrows, and Fig. 3 c and d). Occasionally, small structures which look like bridges but which are bound to only one tubule are seen (Fig. 3 b, arrows); we call these structures arms.

When tubules are clustered together, one tubule is often bridged to several near neighbors. Although multiple interconnections of this kind are occasionally seen as a result of imperfections in a spermatid helix, they are more common in the spermatid manchette which surrounds the nucleus at a later stage of development (40). Fig. 3 e-g show portions of manchettes with several examples of multiple bridges connected to a single tubule (arrows). When a tubule has more than one bridge attached to its wall, the two bridges are usually separated by about 180° of arc; approximately 90° and 60° are also common, and we have occasionally seen bridges spaced by about 30° (Fig. 3 e, arrow). In chicken testis microtubules, the bridges are therefore spaced in multiples of about 30° of arc, but the uncertainty in these data is too large to permit a discrimination between 11-, 12-, or 13-fold rotational symmetry of bridge binding.

Information concerning the structural stability of intertubule bridges can be obtained from a study of the stability of tubule aggregates in vitro. Both the helix and the manchette can survive the shearing forces of vigorous homogenization when the physical-chemical conditions are chosen correctly (Fig. 3 a and i for the helix, and Fig. 3 j for the manchette). Table II presents a summary of the effects of the various homogenization media upon the helix microtubules and bridges. Tubules and bridges from an early manchette have stability properties similar to those of the helix, whereas the tubules from the manchette of an almost mature spermatid have thicker walls and considerably greater stability: they behave like the doublets in the sperm tail (20).

Under favorable circumstances, the microtubule aggregates can be stripped away from the spermatid nucleus and all visible cytoplasm, showing that their mechanical strength is due to intrinsic bonds rather than to support from other structures (in Fig. 3 i there are bridges still interconnecting the helix tubules). The conditions which stabilize the tubule aggregates are also those conditions which preserve the tubules themselves. No conditions have been found which will separate all the tubules from one another but leave the tubules undamaged. Violent mechanical homogenization divides the aggregates into small bundles, but the tubules are likewise broken into short segments. I presume that the bridges are bound to the tubule's surface by noncovalent forces, but the nature of these forces remains undefined.

Intertubule bridges may also be seen in sections which graze the surface of the helix and show short segments of microtubule in longitudinal section (Fig. 4). Even thin sections will generally contain stain density from the nearby nuclear envelope, so it is not easy to see the bridges over large areas. Nonetheless, in each field of Fig. 4, several bridges are visible (arrows indicate examples of structures which I identify as bridges). We have used the microcomparator to measure the distance between adjacent points of attachment of these bridges to the surface of the tubules. Fig. 5 a is a histogram of the data analogous to Fig. 2 a for the axostyle. If groups are defined as in the graphs from Saccinobaculus, the means and standard deviations of the groups are 128 ± 16 Å and 214 ± 15 Å. At greater spacings, the peaks do not appear to be above noise level. I have also measured the axial spacing between bridges with calipers on high magnification prints where the recognition of the bridges is considerably easier and more data can be collected. Fig. 5 b shows a histogram of these data. Using the relative minima between neighboring modes to define group boundaries, the group means and standard deviations are 107 ± 16 Å, 209 ± 19 Å, 308 ± 34 Å, and 429 ± 26 Å.

While the pattern of axial spacing between
FIGURE 3  Figs. 3 a–d are cross-sections of tubules from the perinuclear helix of chicken spermatids. Representative bridges are marked by arrows in Fig. 3 a. Representative arms are so marked in Fig. 3 b. (× 110,000). Figs. 3 e–g are cross-sections of tubules from the spermatid manchette. A pair of bridges separated by only about 30° is marked with an arrow in Fig. 3 e; arrows in the other pictures indicate additional examples of multiple bridges connected to a single tubule. (× 120,000). Fig. 3 h and i are spermatid helix tubules in fixed, embedded homogenates where the stability of the helix is shown not to depend upon other cytoplasmic structures. (Fig. 3 h, × 100,000; Fig. 3 i, × 87,000). Fig. 3 j is a manchette from an homogenate. (× 130,000).
bridges observed in the axostyle is thus clearly present in the spermatid helix, these data are heavily dependent upon the reliability of bridge recognition. In the absence of periodic links which serve in the axostyle as an internal standard of interbridge spacing, more objective measurement techniques have been sought to obtain comparable data in different ways. A microdensitometer scan down the space between adjacent helix tubules as marked with stars on Fig. 4 yields the trace shown in Fig. 5 c. Using the criteria for discriminating signal from noise described in the Materials and Methods section of this paper, the peaks labeled with stars on Fig. 5 c are identified as bridges. Fig. 5 d is a histogram of all data thus obtained from the spermatid helix. The groups are much less well defined than in the histograms of data collected by eye, but group boundaries can still be identified. The group means are similar to those obtained with calipers. The increased standard deviations are attributed in part to tilted bridges and in part to the inclusion of more nonbridge material in these measurements.

If regions of sufficiently high bridge density could be found, the periodicity revealed by direct measure should be observable by optical diffraction. I have scanned many small areas, such as those of Fig. 4, with a diffractometer, but the resulting patterns contain much random scatter, and the evidence for periodic bridges is not compelling. When the region used to diffract is reduced to an area containing obvious bridges and little else, the expected spots are obtained, but a principal virtue of diffraction methods, their capacity to average, is subverted. I therefore undertook to reduce the random scattering by masking out appropriate areas. Using a 1,000-Å section which grazes the surface of the helix, 1/2-μm segments of tubule can be obtained (Fig. 6 a). Tubules were marked out as described in the Materials and Methods section to produce the specimen shown in Fig. 6 b. Fig. 6 c shows the diffraction from a mask like the one which defines the top and bottom of the field in Fig. 6 b (the horizontal spike results from knife edges used to define the sides of the specimen in this experiment). Fig. 6 d is the diffraction pattern obtained from Fig. 6 b, again with knife edges defining the vertical sides.
Figure 4 A group of fields showing grazing sections of the perinuclear helix. The arrows indicate examples of intertubule bridges. The stars define the intertubule space which was traced with the densitometer to produce Fig. 5 c. (× 95,000).
FIGURE 5 a. The distribution of axial distance between adjacent intertubule bridges in the spermatid helix as measured with the microcomparator.

FIGURE 5 b. The distribution of axial distance between adjacent intertubule bridges in the spermatid helix as measured with vernier calipers on high magnification prints.

FIGURE 5 c. A densitometer trace of the space between the tubules marked with stars on Fig. 4. The insert is a trace across two adjacent tubules in the same picture to define the range of peak heights to be considered as bridges (see Materials and Methods section). The peaks marked with stars were included as bridges in the distribution data shown in Fig. 5 d.

FIGURE 5 d. The distribution of axial distance between adjacent optical density peaks defined as bridges by the criteria described in the Materials and Methods section. Data from the perinuclear helix of the spermatid.

The peaks marked with stars were included as bridges in the distribution data shown in Fig. 5 d.

FIGURE 5 e. A cluster of evenly spaced, ink-drawn tubules. I placed tiny periodic marks along either side of each tubule and then connected them at random. The tilt of the bridges results from random registration of these sites on adjacent tubules. Fig. 6 f is the diffraction pattern obtained from Fig. 6 e. Lines parallel to the equator are present, but even in this drawn structure there is sufficient disorder to make the "signal" very weak in the diffraction pattern. The optical "noise" in the diffraction pattern can be suppressed photographically with a simple trick. Diffraction from the noise is, by definition, random. The expected signal lies in lines parallel to the equator. I therefore printed the diffraction pattern while slipping the photographic paper parallel to the equator. The signal is not weakened by this technique, but the noise is spread out to an even gray. Signal-to-noise ratio is thereby improved, as shown in Fig. 6 g. Fig. 6 h is to Fig. 6 d as Fig. 6 g is to Fig. 6 f. A layer line at 120 Å and one at 240 Å can be seen rather clearly. Other lines corresponding to greater interbridge distances can just be detected amid the central noise. Using the horizontally translated plate as a guide, one can find the same lines in Fig. 6 d, but note that one is a mirror image and a photographic negative of the other.

Fig. 6 i and j are included to aid in the interpretation of these patterns. Fig. 6 i is a diagram of tubules connected by strictly periodic bridges. The registration of bridges in each row is at random with its neighbors to simulate adjacent helix turns which are believed to slide relative to one another (40). Fig. 6 j is the diffraction pattern obtained from Fig. 6 i. In this case the layer lines are very
FIGURE 6  Fig. 6 a is a section about 1,000 Å thick which grazes the spermatid helix. (X 36,000). Fig. 6 b–j are an analysis by optical diffraction of the intertubule bridges. For description, see text.

FIGURE 7  Fig. 7 a is a portion of a grazing section of the spermatid helix. Fig. 7 b is the translational superposition of a portion of Fig. 7 a made by 10 translational events corresponding to 330 Å each. Fig. 7 d is to Fig. 7 c as Fig. 7 b is to Fig. 7 a.
clear and more than one order is shown (eight orders were present on the original picture). No layer lines are seen nearer to the equator than the first strong reflection from the bridges drawn with periodicity simulating 240 Å. In the diffraction from the helix (Fig. 6 h), however, there are many lines close to the equator, corresponding to longer periodicities along the tubules. One gets a reasonable accounting for the placement of layer lines from the helix by assuming that they derive from bridges spaced at integral multiples of 120 Å, the value corresponding to the outermost layer line. (Predicted: 120, 240, 360, and 480 Å; observed: 120, 236, 340, and 486 Å.) Additional lines can be accounted for as the first, second, and fourth orders of a 1,080 Å period. (The 360- and 120-Å lines would be the third and ninth orders. The 480-Å and 240-Å lines are not accounted for by higher orders of 1,080 Å.)

Fig. 7 shows an application of the technique of translational superposition to the helix tubules. Fig. 7 b is the result of multiple printing of a portion of Fig. 7 a, Fig. 7 d of 7 c. The best reinforcement was obtained with a translation of about 230 Å.

Spindle Tubules

There are several reports of bridges between the microtubules of the mitotic spindle (66, 28, 7). The spindle tubule bridges are not periodic, but given the characteristics of intertubule bridges described above, I investigated the microtubules of the chicken meiotic spindle for evidence of systematically positioned intertubule bridges. The spindle from meiosis II is particularly favorable because of the tight clustering of the tubules near the metaphase spindle poles (Fig. 8 a). Figs. 8 b and c are transverse and longitudinal sections showing intertubule bridges.

Using the isolation techniques described above, one can prepare meiotic spindles, though the rough treatment of the tissue generally fragments the structure. Fig. 8 d is a cluster of chromosomes and microtubules from testis homogenized in 10% sucrose (wt/vol), buffered with 50 mM imidazole-HCl to pH 6.3. The tubules have clearly stuck together and a few bridges are visible.

The data for axial spacing of bridges between spindle tubules are presented in Fig. 8 e. In this distribution, one clear mode is seen at 210 Å. There is a suggestion of a shoulder on the left of this mode and if the relative minimum between the

Sperm Tail Tubules

Chicken sperm tails are similar to other flagella which have been described (see reference 65 for a review). It is of interest to compare the periodicities seen in this well-ordered tubule array with the binding-site periodicities found in other microtubules from chicken testis. Fig. 9 a shows the cross-section image of a sperm tail. It is much the same as many other sperm tails, except that the inner dynein arm is not evident, and the A subfiber of the doublets is electron dense. Fig. 9 b is a median longitudinal section containing the central pair and showing periodic links spaced at 150 ± 18 Å (70 arms from two sperm tails). Fig. 9 c grazes the 9 + 2 array and reveals the periodicity of the dynein arms. In chicken sperm, the fixed, embedded, and sectioned material yields a value of 230 ± 21 Å (180 arms from 10 sperm tails). The dynein periodicity value is close to the mode value of interbridge distance for the spindle, and to the second group mean for the helix bridges. The links between the inner pair of sperm tail tubules apparently follow a different logic, resembling more closely the periodicity of the links in the axostyle.
**FIGURE 8** Fig. 8 a is a spindle from Meiosis II in chicken testis showing the polar bunching of tubules which is favorable to the study of intertubule bridges. (X 18,000). Fig. 8 b and c are cross and longitudinal sections near the spindle poles. Arrows indicate bridges. (X 80,000). Fig. 8 d is an isolated meiotic spindle showing occasional intertubule bridges (arrows) (X 80,000).

**FIGURE 9** Fig. 9 a is a cross-section of a chicken sperm tail showing the 9 + 2 pattern in which the lumen of the A subfiber is electron-dense. A portion of a sperm head shows at the left. (X 73,000). Fig. 9 b is an axial longitudinal section showing the inner pair of tubules and the bridges between them. (X 93,000). Fig. 9 c is a longitudinal section containing a pair of outer doublets and showing the periodicity of the dynein. (X 100,000).
Negative Staining of Spermatid Microtubules

The similarity of the bridge periodicities in three different kinds of microtubules from chicken testis suggests some common underlying factor which establishes the distribution of the bridge binding sites. Most microtubules thus far studied are constructed as a regular array of globular proteins with a unit cell of about \(40 \times 50 \text{Å}\), short dimension approximately parallel to the tubule axis (27, 14, 15, 17). It is plausible that the periodicity of the bridge binding sites is directly related to the surface lattice of the microtubules. Unfortunately, the information currently available about the tubule surface lattice is not straightforward. X-ray diffraction reveals a different lattice from the one seen after negative staining for electron microscopy, but the X-ray patterns are not yet well enough developed to reveal the bridges or other longer range periodicities (80 and 160 Å), which show up with electron microscopy and optical diffraction (27, and even larger values in reference 15). Further, there is considerable discrepancy between the values reported for the periodicity of dynein binding sites in different systems (15, 23, 27). As a final complexity, recent results show that microtubules can contract (36).

It is not the purpose of the present study to pursue the question of the true tubule surface lattice in any detail, since X-ray and electron diffraction of wet specimens are more likely to be the methods of choice for that study. I have, however, looked for systematic relationships between the tubule wall periodicities and bridge binding periodicities for those few cases in which both wall subunits and bridges or arms can be observed in a single micrograph.

Figure 10 a is a chicken sperm tail negatively stained with uranyl acetate. Both singlets (labeled 1) and doublets (labeled 2) are present. This assignment is easily made in the case of all the doublets except the one marked with a star, because they show both subfibers. The starred tubule is identified as a doublet because it shows some projections from its surface which look like the “spokes” that stick to the doublets in all the negative stain studies of flagella in which they are described (30, 64, 15). The walls of both doublet and singlet tubules are made from paraxial strands of beads. The surface lattice in these tubules is based on a \(40 \times 50\text{Å}\) cell, short dimension approximately parallel to the tubule axis. Thus, the arrangement of the structure units in chicken sperm flagella is similar to that described in other electron microscope studies of flagellar tubules (27, 14, 15).

In considering the various possible relations between microtubule subunits and bridge binding sites, we are chiefly concerned with the long-range periodicities which show up in the optical diffraction patterns obtained from negatively stained tubule walls. Chasey has shown that the outer doublets of *Tetrahymena* flagella have a fundamental periodicity at 480 Å, with higher orders showing at 240, 160, 80, and 40 Å (15). Fig. 10 b is the optical transform of a portion of the tubule marked with a star on Fig. 10 a. Layer lines corresponding to axial spacings, of 40, 80, and 160 Å, are evident, but longer range periods are buried in the central spot. Direct measurement on the doublet tubules between the two white P’s on Fig. 10 a shows that there is an obvious periodicity at 240 Å. Given the location, the size, and the spacing of the structures that define this periodicity, I interpret them as dynein. The starred tubule also allows an estimate of the periodicity...
| Microtubule system          | Method of observation                 | Observed periodicity | Probable true value | Probable origin of periodicity                                                                 |
|----------------------------|--------------------------------------|----------------------|---------------------|------------------------------------------------------------------------------------------------|
| Sperm tail inner singlet tubules | Negative stain and optical diffraction | 40 Å                 | 40 Å                | A globular protein, single peptide                                                               |
|                            | Same                                 | 80 Å                 | 80 Å                | Pairing of dissimilar peptides to form the 6S dimer                                               |
|                            | Same and direct measure on negatives  | 160 Å                | 160 Å               | Binding sites for links between inner singlets                                                   |
|                            | Fix, embed, and thin section         | 150 Å                | 160 Å               | Same                                                                                             |
| Outer doublet tubules      | Negative stain and optical diffraction | 40 Å and 80 Å as above | 40 Å and 80 Å as above | As above                                                                                         |
|                            | Same                                 | 160 Å                | 160 Å               | Perhaps second order of 320 Å from spokes; perhaps result of systematic perturbation of 80-Å biochemical structure unit |
| Manchette singlet tubules  | Negative stain and optical diffraction | 240 Å                | 240 Å               | Dynecin                                                                                          |
|                            | Fix, embed, and thin section         | 230 Å                | 240 Å               | Same                                                                                             |
|                            | Negative stain and direct measure    | 320 Å                | 320 Å               | Spokes                                                                                            |
| Perinuclear helix singlet tubules | Negative stain and optical diffraction | 40 Å and 80 Å as above | 40 Å and 80 Å as above | As above                                                                                         |
|                            | Same                                 | 160 Å                | 160 Å               | Perhaps result of a systematic perturbation of the 80-Å unit.                                     |
|                            | Fix, embed, and thin section; direct measure with microcomparator | 128 Å and 214 Å      | 120 Å and 240 Å     | Integral multiples of the minimum interbridge distance of 120 Å; part of the cause of these values being lower than one would expect from the negative stain results is probably a shrinkage during dehydration |
|                            | Same, but direct measure with calipers | 107 Å and 209 Å      | 120 Å and 240 Å     | Same                                                                                             |
|                            | Same, but with indirect measure using the microdensitometer | 106 Å and 210 Å      | 120 Å and 240 Å     | Same                                                                                             |
TABLE III—Continued

| Microtubule System       | Method of Observation            | Observed periodicity | Probable true value | Probable origin in periodicity |
|--------------------------|----------------------------------|----------------------|---------------------|-------------------------------|
| Same, but with           | optical diffraction              | 120                  | 120                 | Same                          |
|                          |                                   | 236                  | 240                 |                               |
|                          |                                   | 340                  | 360                 |                               |
|                          |                                   | 406                  | 400                 |                               |
| Same, but with           | translational superposition      | 230                  | 240                 | Same                          |
| Meiotic spindle          | Fix, embed, and                   | 129                  | 120                 | Same                          |
| singlet tubules          | thin section; direct measure      | 228                  | 240                 |                               |
|                          | with microcomparator              |                      |                     |                               |

shown by the spokes. In this picture and four others, near-neighbor spokes show an axial spacing of 320 ± 20 Å, but they are too ill-defined to permit comment on the systematic absences described in other systems (63, 30, 15). While these measurements of spoke spacing may be imprecise for want of more examples, the 320 ± 20 Å is neither the same as nor a multiple of the dynein period as described by Hopkins for Chlamydomonas flagella where dynein is periodic at about 150 Å (30). (The two spacings may be compared directly on Fig. 10 a.)

Fig. 10 e is the diffraction pattern obtained from the right end of the central tubule marked with the upper number 1 on Fig. 10 a. Layer lines at 40 and 80 Å are barely visible in this print, but the 160-Å line is reasonably clear. Indeed, the 160-Å periodicity can be measured directly on Fig. 10 a. I interpret it as the links that interconnect the central pair of singlet tubules (cf. 13).

To simplify a discussion of these diverse periodicities, a table is included (Table III). The shortest axial periodicity of chicken sperm tail tubules is 40 Å. As others have observed, the 40-Å structure unit probably corresponds to a globular protein made from a single polypeptide chain of about 55,000 Daltons (for review, see 42). The periodicity at 80 Å may depend upon the dissimilarity of the two peptides which comprise tubulin (9, 6). The biochemical difference would then account for the slight tilt in adjacent pairs of structure units which is probably the origin of the 80-Å periodicity (27). In chicken sperm tail tubules, we can identify the 160-Å and 240-Å layer lines with sites for additional material which affixes to the tubule wall. The links between the central pair of tubules show a 160-Å repeat on the surface lattice of 40 × 50-Å structure units. The periodicity which we interpret as dynein arms shows a 240-Å repeat on the doublet tubules which have approximately the same surface lattice as the singlets. The spokes show that the doublets also possess an additional binding site which is periodic at about 320 Å. All these longer range periods are integral multiples of the presumed structure unit of the tubule, the 80-Å dimer. It is thus clear that the bridge binding sites on chicken sperm tail tubules are systematically related to the surface lattice of the microtubule.

We can now ask whether a similar pattern can be seen in the less-ordered microtubule systems. Cytoplasmic microtubules will occasionally stay in bunches throughout the preparation for negative staining, and can then be distinguished unambiguously from sperm tail tubules. Fig. 11 a shows a bundle which is almost certainly a portion of a spermatid manchette. Fig. 11 b is the optical diffraction pattern of Fig. 11 a. The typical surface lattice of about 40 × 50 Å can be seen, and an 80-Å layer line is visible. Longer-range periods do not show up in this diffraction pattern, but by viewing Fig. 11 a from the side, one can get a hint of longer periods.

The negative stain preparations also include clusters of short segments of as many as eight tubules lying parallel at regular spacings. I interpret these as helix tubules, although they might derive from the spindle. Fig. 12 a is a group of three such tubules. There is some indication of bridges interconnecting the adjacent tubules, but
the images are too vague to permit direct measurement. Optical diffraction of these aggregates shows a hint of a line at 40 and 80 Å, and reasonably strong spots at 160 Å. Fig. 12 b is the transform of a single tubule from Fig. 12 a, and Fig. 12 c is the transform of the group of three.

SUMMARY AND CONCLUSIONS

Several kinds of arms and bridges between microtubules have been described. There are periodic bridges: the intrarow links of the axostyle, and the bridges between the central singlet tubules of the 9 + 2, both at 150-160 Å. There are periodic arms which may on occasion serve as a part of a bridge: dynein on the sperm tail doublets at 240 Å, the arms on the tubules from the axostyle of S. ambloaxostylus at about 150 Å; and the spokes on the sperm tail doublets at 320 Å. I suggest that all these periodicities are multiples of the 80-Å axial periodicity defined by the microtubule structure unit, and that departures from integral multiplicity of 40 Å, e.g., 150 Å for the links between the central pair as seen in thin sections, are due to a combination of preparative artifacts and experimental error (Table III).

The nonperiodic bridges show a range of spacings, but there is a strong tendency for all the measurements to cluster around multiples of the minimum interbridge distance. Bridges between axostyle tubules are grouped around multiples of 150 Å. Nonperiodic bridges from chicken testis tubules cluster around multiples of about 110 Å. In the translational superposition and the diffraction studies of testis tubule periodicities, the average value of the basic period is slightly larger. In the data from both the helix and the spindle, about 220 Å is the most common interbridge spacing. This periodicity is close to the 240-Å axial period of dynein. I suggest that 240 Å is also the correct value for the helix tubule bridges. Since the doublet tubules, which bind dynein at 240 Å, show a layer line at 160 Å, it is not surprising that the cytoplasmic tubules, which also show a 160-Å layer line, bind most bridges at a spacing near 240 Å.

The occurrence of nonperiodic bridges at a spacing near 120 Å is intriguing, because this is equivalent to only 1.5 × 80-Å subunits. Many workers currently believe that the microtubule is made from a heterodimer arranged in paraxial protofilaments (reference 9, and for review, see reference 42). With this model there should not be a point of equivalence along any given protofilament after only three subunits. There are many possibilities to account for our observation, e.g., neighboring bridges are on adjacent, half-staggered protofilaments, or interbridge distances are affected by surface lattice contraction, as observed in axostyle (36). There is not sufficient information available to identify the correct interpretation.

The negative-staining studies allow clarification of the hypothesis for the distribution of the nonperiodic bridges. I suggest that every tubulin dimer possesses a binding site for a bridge, but that steric hindrance or some more subtle factor, such as the "gradation" hypothesized by Roth and his collaborators (48), prevents bridge attachments at every 80 Å of axial length. Further evidence concerning this suggestion must await isolation of the bridge molecules and experimental manipulation of their concentration relative to a defined length of microtubule in order to see whether excess bridge material will occupy enough of the sites to render their periodic distribution obvious.

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