

EVIDENCE FOR "TWISTED PLANE" UNDULATIONS IN GOLDEN HAMSTER SPERM TAILS

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ABSTRACT

Motile spermatozoa from the golden hamster have been arrested by rapid freezing and then fixed with glutaraldehyde at low temperature after substitution with ethylene glycol. As far as can be judged, the flagellar waveforms thus stabilized are similar to those seen in living sperm; in contrast, fixation in glutaraldehyde, without prior freezing, induces agonal changes in flagellar conformation. The characteristic waveform after freeze substitution contains three bends. Approx. half of these flagella are entirely planar. The rest are three-dimensional, with the third bend displaced in a regular way from the plane containing the proximal two bends. From the geometry of these flagella, it is concluded that the plane of action of a given bending cycle undergoes a clockwise twist (from a forward viewpoint) as the cycle is succeeded by new bending cycles. This "twisted plane" undulation is quite different from helical movement. The twisting seems to occur abruptly, between cycles, as if each bending cycle has a preferred plane of action. The mechanism underlying the twisting is uncertain. However, on the basis of the angular displacements between the preferred planes, and the findings from electron microscopy, the following idea is presented as a working hypothesis: that, if the most proximal plane of bending is topographically determined by peripheral doublet 1, then successive distal planes of action are influenced predominantly by doublets 2, 3, etc., in clockwise sequence. The merits and weaknesses of this hypothesis are discussed.

KEY WORDS spermatozoa · motility · flagella · axoneme · freeze-substitution

Although there have been several detailed studies of the movement patterns of mammalian spermatozoa, in which increasingly fast cine cameras have been used, no agreed description of the flagellar waveform has evolved. The bull sperm flagellum was reported to perform planar bending proximally and a more complex figure-of-eight movement at its tip (13). Alternatively, this fla-

gellum was said to execute waves of helical form but with an elliptical section (18), and planar beating was dismissed as pathological or artefactual (18, 21). Elliptically helical movement has also been suggested for rotating ram sperm, though, because of the flatness of the ellipse, it was conceded that the proximal bending might be planar (6). The idea that a planar bend might become helical as it is propagated was expressed in an earlier review (2). Thus, while there is agreement that rotating spermatozoa have a

waveform which is, over its entire length, three-dimensional, the detailed geometry has been in dispute.

Taking the view that a more exact account is unlikely to come from cinephotography (principally because three-dimensional waveforms must always be partially out-of-focus) the author has adopted the approach currently used in studying ciliary bending, namely to attempt the "instantaneous" fixation of the axonemes. If this can be achieved, the flagellar geometry can be studied at leisure, and it should be possible, by arranging the individual waveforms thus preserved, to put together an account of how the configuration of the typical flagellum is changing with time. However, rapid chemical fixation with osmium tetroxide, the method generally used for cilia, only sometimes arrests the metachronal wave satisfactorily (1) and so, for a suspension of spermatozoa, where there is no equivalent pattern of co-ordination which can be checked, a primary chemical fixation was excluded as being likely to produce a significant number of agonal flagellar configurations. Instead, "instantaneous" fixation has been attempted by rapid freezing. The configurations thus physically preserved have been chemically stabilized at low temperatures by substitution fixation of the cells in a glutaraldehyde containing ethylene glycol solvent. The development of this freeze-substitution procedure has already been reported in detail (22), and only minor technical changes have been made, except for the decision to omit a cryoprotectant from the sperm diluent. The first conclusion drawn from the work, for the rat spermatozoon, was that the most proximal flagellar bend is planar, occurring in a plane perpendicular to that containing the two central microtubules of the axoneme. In a second report (23) it was provisionally concluded that distal regions of the rat sperm flagellum also had a planar beat but that the plane of action had become angled, apparently without an accompanying twist in the flagellum. Since electron microscopy was used, these were high resolution studies but of very few spermatozoa.

In the present study mainly made by light microscopy, resolution has been sacrificed for the sake of studying a larger sample of cells, and golden hamster sperm have been preferred to those of the rat on account of their greater viability. Initially, a further check has been made on the validity of the method by comparing the proximal waveform of living hamster spermatozoa

with that obtained after freeze substitution or after glutaraldehyde fixation. Then, from a more detailed study of the frozen-substituted cells, a description has emerged of a twisted plane pattern of bend propagation and a hypothesis is proposed which relates the geometry of the twisting to the internal structure and chirality of the axoneme. The concept of a twisted plane waveform, though absent from reviews of flagellar function (19, 4, 20) has in fact appeared once previously, in a description of bull spermatozoa (3).

MATERIALS AND METHODS

Mature golden hamsters (*Mesocricetus auratus*) were killed by cervical dislocation and left at room temperature for 30 min. Then, from each animal, a single suspension of spermatozoa was prepared by adding some of the contents of a cauda epididymidis to 2 ml of Hanks' solution at room temperature. 5 min later, when the spermatozoa had become dispersed, successive aliquots were withdrawn and subjected either to (a) flash photography, (b) conventional glutaraldehyde fixation, or (c) rapid freezing (followed later by substitution fixation). These operations could be completed within about 10 min.

For flash photography, several drops of suspension were placed between a siliconized slide and cover slip, the latter supported by two hairs. Without delay, exposures were made of several fields using a 10 \times objective, dark ground condenser, Leitz microflash illumination (flash duration approx. 0.001 s) and Ilford FP4 35-mm film. The analysis of the data was done from large prints and consisted of making a decision, for every sperm where a decision was possible, whether the most proximal flagellar bend was in the principal or the reverse direction. (The terminology, taken from Gibbons and Gibbons (12), is discussed later.) The distinction between principal and reverse bends was easily made by relating the curvature of the bend to the disposition of the asymmetrically shaped sperm nucleus (Figs. 1-3).

To compare the normal flagellar geometry with that obtained after chemical fixation, samples of the sperm suspensions were fixed in relatively large volumes of 2.5% glutaraldehyde in 0.1 M *s*-collidine buffer (pH 7.4). Small volumes of the sperm suspension were taken as films on aluminum foil and quickly submerged in the fixative. After about 2 h the sedimented spermatozoa were carefully withdrawn and mounted on slides as deep, sealed, wet preparations. The same simple datum was recorded, for 300 spermatozoa, namely, the identification of the most proximal bend as being either in the principal or the reverse direction.

The third technique used in the triple comparison was fixation by freeze substitution. The method employed differed in only a few details from that previously described (22). A rectangular piece of silver foil (6 \times

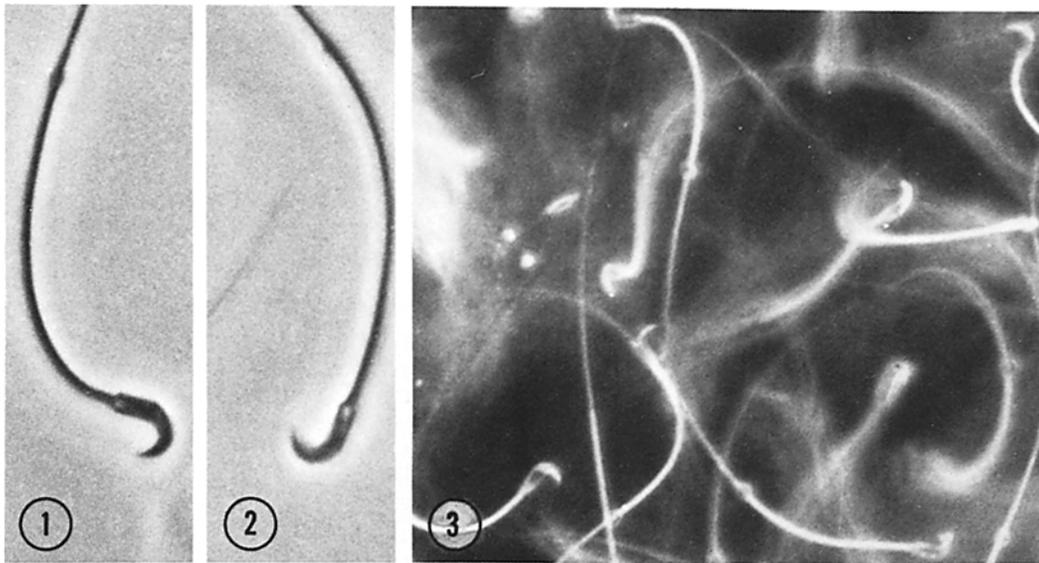


FIGURE 1 Phase-contrast micrograph of a golden hamster spermatozoon showing its asymmetrical head and the proximal part of its flagellum. This configuration of the flagellum is defined as a principal bend. $\times 1,000$.

FIGURE 2 Similar to Fig. 1 but showing the alternating flagellar configuration, which is defined as a reverse bend. $\times 1,000$.

FIGURE 3 Principal and reverse bends visible in motile spermatozoa. Flash illumination. Dark ground optics. $\times 500$.

20 mm; Goodfellow Metals Ltd., Esher, England) supported in a cleft applicator stick, was dipped into the sperm suspension and then quickly dropped into Freon-22 maintained at its freezing point (-146 to -147°C) by liquid N_2 . After remaining under the Freon for 5 s, the metal foil was quickly transferred to a liquid N_2 storage bath before being put into the substituting fluid. This fluid, based on a formula of Pease (14), was prepared in 100-ml lots in screw-capped Pyrex tubes. It is essentially a eutectic mixture of ethylene glycol containing a small amount of glutaraldehyde (ethylene glycol, 70 ml; 0.133 M phosphate buffer, pH = 7.3, 25 ml; 24.6% glutaraldehyde (TAAB Laboratories, Emmer Green, Reading, England), 5 ml). This solution is less acidic than that used earlier (22), owing to its lower glutaraldehyde concentration and greater buffering capacity. The tubes of substituting fluid were immersed in an acetone bath and cooled with dry ice to -50°C . For freeze substitution the sperm films were quickly transferred from liquid N_2 to the substituting fluid (2 films to each tube), and the tubes were immediately sealed and maintained at -50° ($\pm 2^{\circ}$) until the opacity of the frozen films had cleared (about 24 h). Thereafter, the preparations were left undisturbed until they had warmed to $+10^{\circ}\text{C}$ (about 24 h). From experiments with gelatin models (22), it was known that glutaraldehyde behaves

as a fixative in ethylene glycol and that it acts at temperatures at least as low as -20°C .

Further processing involved transferring the spermatozoa to a deep slide in which they could sediment freely before examination by phase-contrast microscopy. The supernate in the tubes above the level of the silver foils was removed by suction. Then the foils were gently agitated and removed. 3 days later, the sedimented spermatozoa were withdrawn by pipette and placed within a wax ring on a slide. A cover slip was sealed in place with wax (Vaseline, 2 vol; and beeswax, 1 vol). The chamber contained a depth of 2 mm of fluid. It became apparent that the geometry of a sperm flagellum influences its orientation in the fluid and ultimately the position in which the cell comes to rest on the slide. Therefore, to allow all the spermatozoa to reach an equilibrium orientation during sedimentation, the preparation was first inverted so that all the cells fell to the cover slip; then it was abruptly turned back over and placed on the microscope stage. 9 h were allowed for each sedimentation. Spermatozoa in these preparations were initially examined for the direction of curvature of the most proximal flagellar bend. Then a more complete study was undertaken in which each sperm's geometry was recorded using a notation which indicated (*a*) whether the sperm was lying with its left (*L*) or right

(R) side uppermost, (b) whether the most proximal bend was in the principal or reverse direction (p_1 or r_1), (c) whether further bends existed in the flagellum (p_2, r_2, p_3 , etc.), and (d) whether any of the bends were displaced upwards, out of the focal plane (denoted by p_2 , etc.). Photographs were taken using a $20\times$ objective and phase-contrast illumination. Many of the spermatozoa were photographed several times, focusing first on the plane of the first bending cycle (p_1r_1 and r_1p_1) and then focusing on the crests of bends displaced from this plane. The vertical displacements of the microscope stage between exposures were recorded from the $1\ \mu\text{m}$ calibrations of the fine focus knob; these values were adjusted by a small correction factor obtained by measuring the stage's movement (with a micrometer) for the entire travel of the fine focus control. Further measurements on the spermatozoa (as shown in Fig. 8) were made from photographic prints. The curvilinear length (l) was measured with a map measure read to 1/16 inches.

Spermatozoa from one of the hamsters were examined by electron microscopy. Pieces of cauda epididymidis were fixed in 2.5% glutaraldehyde in 0.1 M *s*-collidine buffer (pH 7.4) for 2 h, rinsed in buffer for 30 min, and postfixed in 1% OsO_4 in the same buffer for 1 h. After dehydration in a graded ethanol series, the tissue was embedded in Epon 812. Serial silver sections were mounted on slotted grids (supported by a Formvar-carbon film), stained successively in 3% aqueous uranyl acetate and 0.2% lead citrate, and examined in an AEI 6B electron microscope. To ensure a true reconstruction from serial sections, the order in which the sections left the block was always noted, and care was taken not to produce mirror-inversion during the microscopy and printing. For comparative and more extended observations, epididymal spermatozoa from a rat were processed in the same way and examined in serial sections.

RESULTS

Since the golden hamster spermatozoon has not previously been used in quantitative studies, it is worth reporting first of all the dimensions of its flagellum. From a series of photographs of 15 moribund sperm from a single male, the length of the middle piece is $50.9 \pm 0.3\ \mu\text{m}$ (mean \pm SE), and the total length of the flagellum is $179.9 \pm 0.8\ \mu\text{m}$. This last measurements includes the end-piece, approx. $3\ \mu\text{m}$ long.

As in the other mammalian species (15) the bending cycle of the proximal middle piece in spermatozoa from the cauda epididymidis is asymmetrical. And in the hamster, by reference to the nuclear asymmetry, the principal and reverse bends, as defined ostensively in Figs. 1 and 2, are readily distinguishable. The name "principal" has been given to the bend which has the longer

duration (12). Therefore at any moment more sperm are to be found executing principal first bends (p_1) than reverse ones (r_1), and this proportion, expressed as $p_1/(p_1 + r_1)$, is a parameter of the population which can be obtained by photography using flash illumination (Fig. 3). Any fixation method which fails to preserve this ratio cannot be affecting all the cells in the same way and must be of questionable value.

Data from the comparison of living, frozen-substituted, and glutaraldehyde-fixed spermatozoa are given in Table I. χ^2 analysis confirmed that in each experiment the ratio $p_1:r_1$ in the living sample had been significantly altered by glutaraldehyde fixation but not significantly changed by the freeze-substitution procedure.

One clear effect of the rapid freezing, however, is that some of the sperm tails are either partially or completely fractured. In the three experiments, 27%, 28%, and 23% of the sperm were damaged. These cells were not excluded from the data in Table I provided the shape of the proximal bend could be seen.

Undamaged sperm from the frozen substituted samples were studied further in a search for patterns in the distribution of flagellar configurations. It was noticed, firstly, that most of the flagella contained 1.5 cycles of bending; secondly, that some of these wave forms were three-dimensional and some planar; and thirdly, that the sperm did not sediment such that equal numbers came to lie right-side or left-side uppermost. This last irregularity was accentuated if the planar wave forms were not considered. These initial impressions were established quantitatively in a classification of spermatozoa from two of the experiments (Fig. 4). The classification used the descriptive notation explained in the preceding section. The side of the sperm head defined as the "right" side is that which is seen to be upper-

TABLE I

The Values Obtained for the Proportion of Principal First Bends ($p_1/(p_1 + r_1)$) in the Sperm Population after Different Fixation Procedures

Exp no.	Flash photography	Freeze substitution	Glutaraldehyde fixation
S32	0.75 (338)	0.78 (300)	0.53 (300)
S33	0.78 (277)	0.75 (300)	0.41 (300)
S40	0.66 (122)	0.62 (300)	0.40 (300)

The numbers of spermatozoa examined are given in parentheses.

most in Figs. 5-7. From Fig. 4 it will be seen that sperm with the common planar wave form $p_1r_1p_2$ sediment about equally as $R p_1r_1p_2$ and $L p_1r_1p_2$ whereas those having an elevated third bend ($p_1r_1p_2$) sediment such that $R p_1r_1p_2$ greatly outnumber $L p_1r_1p_2$. In the third experiment in which only these last two categories were analyzed, the same pattern was seen: 48 sperm of the type $R p_1r_1p_2$ were counted, and only two of the type $L p_1r_1p_2$. The likely explanation of the sedimentation behavior of the three-dimensional flagella is discussed later.

While recognizing that there is a diversity of wave forms in the frozen-substituted preparations, attention has been concentrated on the geometry of the largest class, $R p_1r_1p_2$. Two such sperm, having different bend amplitudes are shown in Figs. 5 and 6. In examining the vertically displaced bends, p_2 , it was found that their maximum vertical elevations coincided with their maximum projected amplitudes, suggesting that they were still planar bends but now had a plane of action twisted with respect to the plane of action of the

more proximal bends, p_1 and r_1 , i.e., it appeared that a principal bend, from beginning in a plane parallel to the microscope slide (as bend p_1) must then, as it propagates (and becomes p_2), move through a certain angle, θ_2 , away from this initial plane. Viewed from the sperm head, this angular movement is clockwise. The angle has been calculated by relating the projected amplitude a_p , of bend p_2 (defined in Fig. 8) to the upward displacement, d_2 , of the center of the bend (Fig. 9). These parameters are closely correlated ($r = 0.59$). The mean angle subtended by the centers of bends p_2 at the axis of progression was 46.6° with a 1% confidence interval of $\pm 6.0^\circ$. A more appropriate analysis, based on normalized data, gave the same value for the mean angle and will not therefore be presented in detail.

Figure 9 also includes a few data for the rarely seen waveform which contains three principal bends. One such sperm is shown in Fig. 7. The estimation of the angle through which the center of bend p_3 must have moved is complicated by the fact that bend r_2 , displaced in the opposite

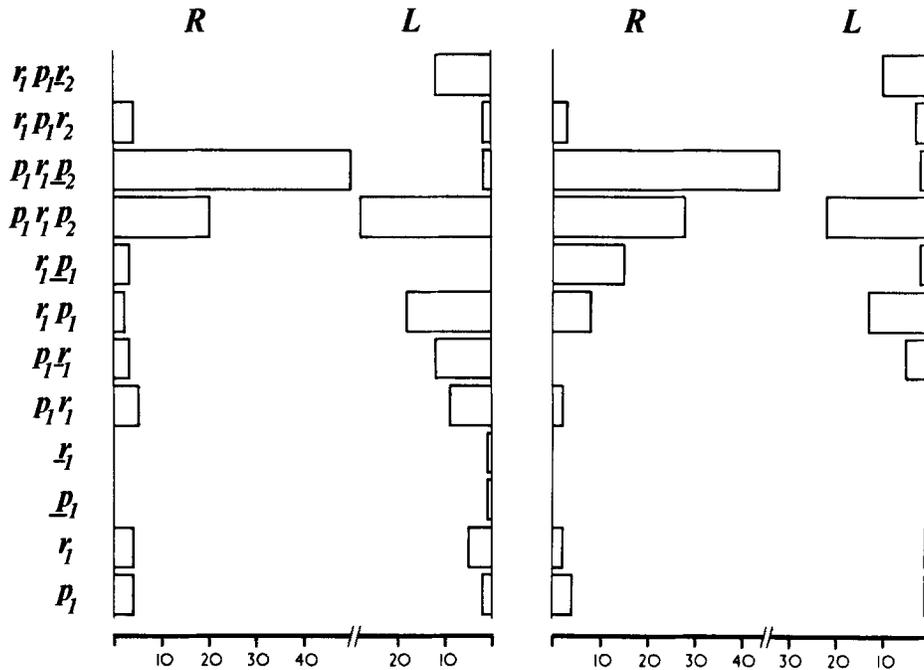


FIGURE 4 Waveform classifications from two experiments (S32 and S40). The histograms depicting the number of spermatozoa which landed right (R) and left (L) side uppermost are presented side by side for comparison. The descriptive notation, at the left of the figure, is explained in the text. Sperm numbers may be read from the horizontal scale. Detailed analysis of flagellar geometry has been confined to the largest single class ($R p_1r_1p_2$).

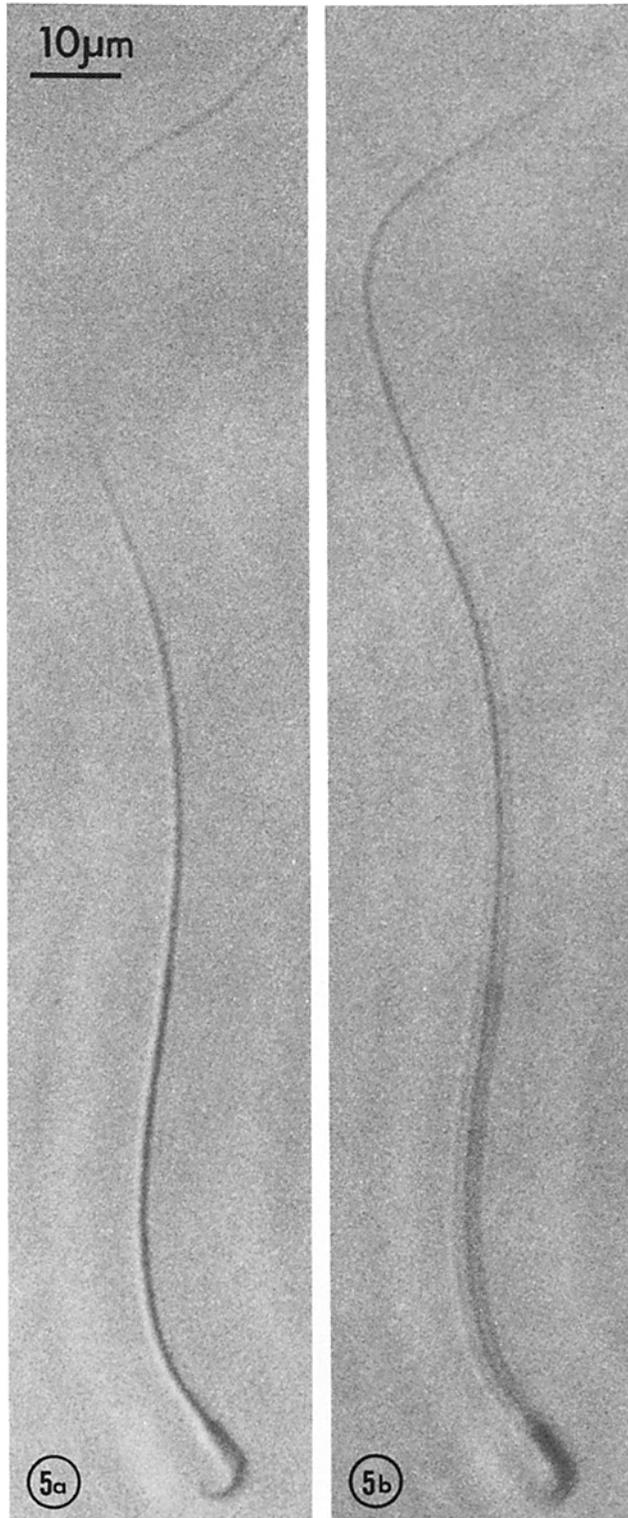


FIGURE 5 (a) Frozen-substituted spermatozoon having a planar first bending cycle and a vertically displaced second principal bend, lost from the plane of focus. (b) The same spermatozoon, with the second principal bend brought into focus. The plane of this micrograph is $6 \mu\text{m}$ above that of Fig. 5a. $\times 1,176$.

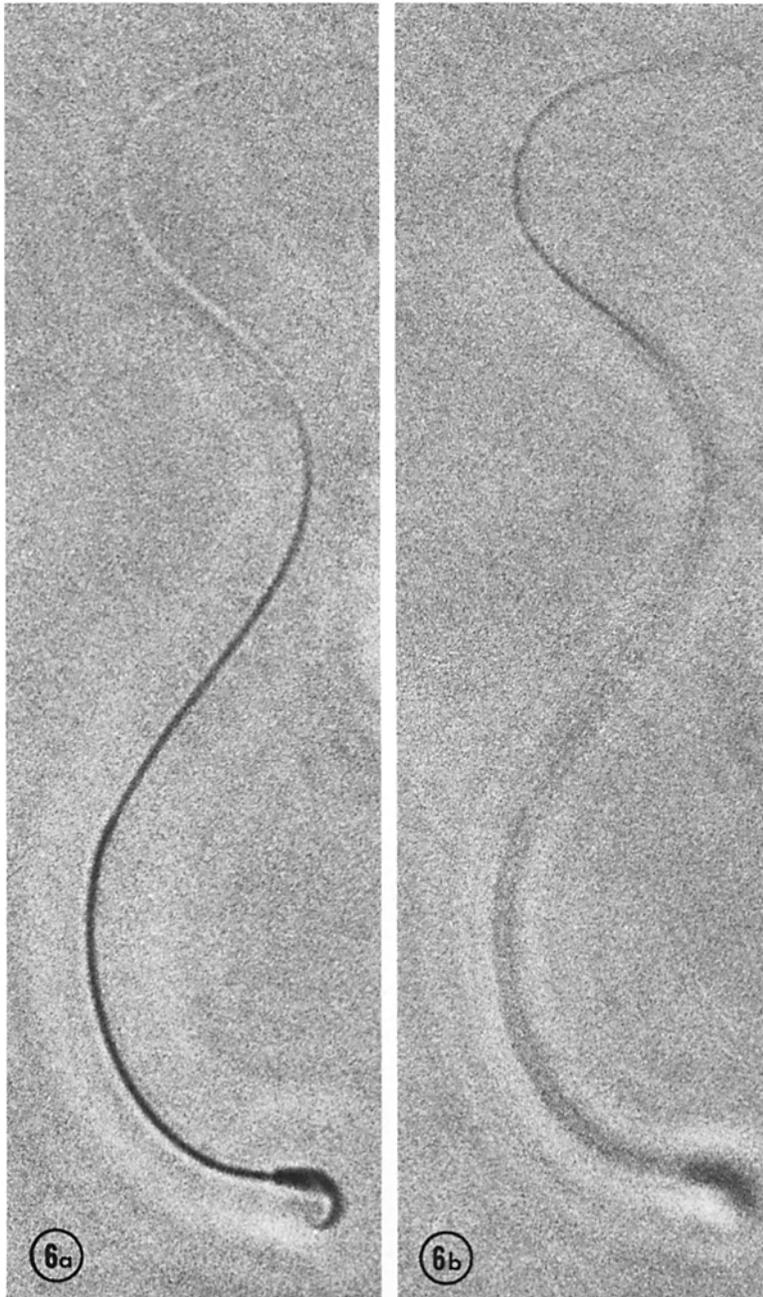


FIGURE 6 (*a* and *b*) Frozen-substituted spermatozoon of waveform $R p_1 r_1 p_2$ but having bends of greater amplitude than the flagellum in Fig. 5 *a* and *b*. The vertical displacement of the microscope stage between the two micrographs was $13 \mu\text{m} \times 1,176$.

direction from bends p_2 , p_3 , adds to the total upward displacement of these principal bends from the plane of the microscope slide. Assuming

that bends p_2 and r_2 have the same amplitude and supposing, as an approximation, that the displacements of p_2 and p_3 are equally augmented

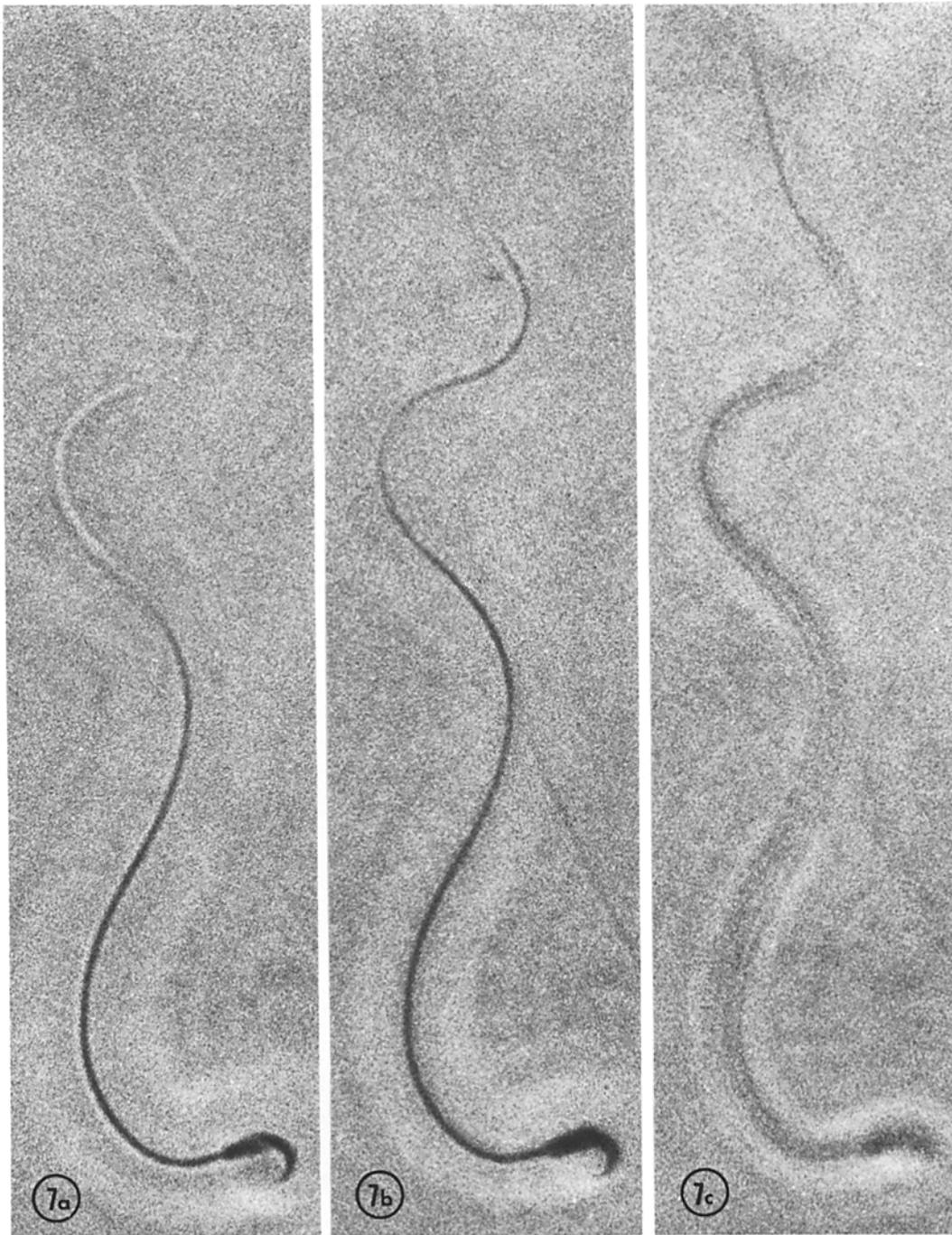


FIGURE 7 (a-c) A frozen-substituted spermatozoon having the rare waveform $R p_1 p_2 p_3$. Fig. 7a is focused in the plane of the slide; Fig. 7b is displaced upwards by $5 \mu\text{m}$, almost to the center of bend p_2 ; Fig. 7c is displaced a further $5 \mu\text{m}$ and brings bend p_3 into focus. $\times 1,176$.

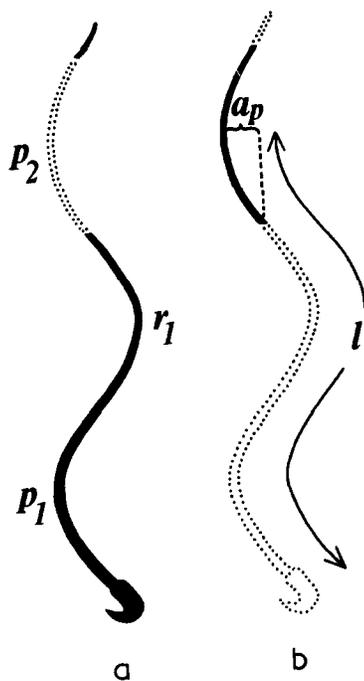


FIGURE 8 A diagram of the characteristic waveform ($R p_1 r_1 p_2$) seen after freeze substitution. The maximum upward displacement (d_2) of bend p_2 was recorded from the fine focus control. The projected amplitude a_p was obtained from a photograph after judging the center of the bend and locating the progression axis at the midpoint between bends r_1 and p_2 . An estimate of the distance (l) traveled by bend p_2 was obtained as shown in Fig. 8b, without making allowance for the upward displacement of bend p_2 .

by the height of r_2 , then a value for the displacement of p_3 from the progression axis can be obtained by subtracting from its total displacement one half of the total upward displacement of bend p_2 . The estimate of the mean angle subtended by the center of bend p_3 at the axis of progression, from five spermatozoa only, was $79.3 \pm 6.6^\circ$ (1% confidence interval).

Two attempts were made to account for the variation in θ_2 . Firstly, each value of θ_2 was plotted against the true amplitude of its bend, obtained from $\sqrt{d_2^2 + a_p^2}$. The relationship between these data can be appreciated indirectly from Fig. 9. The regression coefficient, $b = 0.015 \mu\text{m}$ of amplitude per degree, was not significantly different from zero ($P > 0.5$). The angle θ_2 was also plotted against the distance (l) travelled by the bend p_2 (see Figs. 8 and 10) but was found

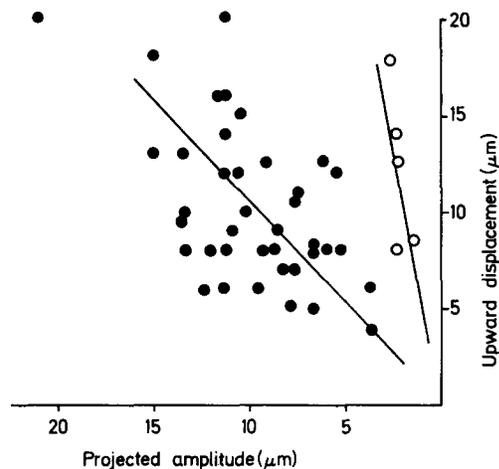


FIGURE 9 The relationship between the projected amplitude and the upward displacement of principal bends. The graph is drawn such that the origin represents the progression axis and the points represent the position in space of the centers of principal bends as if viewed from the sperm head. The mean angle subtended by each group of data at the origin is indicated by a line. The data have been pooled from three experiments after ruling out heterogeneity. (●) Second principal bends (p_2); and (○) third principal bends (p_3).

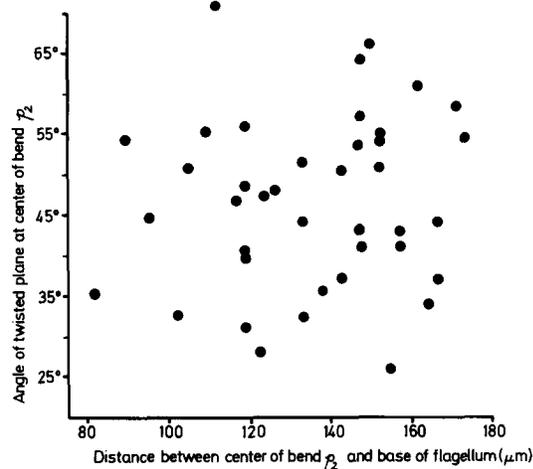


FIGURE 10 The relationship, within individual spermatozoa, between the angle (θ_2) of the twisted plane at the center of the second principal bend (p_2) and the distance travelled by this bend. The independence of the data indicate that the angle is not determined by whichever region of the flagellum happens to contain the bend.

not to be dependent on this distance ($b = 0.22$; $P > 0.5$).

In order to relate the apparent clockwise twist of the plane of bending to the structure of the axoneme, it was necessary to confirm that the hamster sperm flagellum conforms with others studied previously in having a "clockwise" axoneme. (This means that, when viewed from the base, the axonemal doublets numbered 1-9 appear in clockwise array.) The morphological transition at the distal end of the middle piece, where the axoneme complex is enclosed successively in mitochondria, the annulus and then the fibrous sheath (see Fig. 11), was identified in serial transverse sections and used as the means of knowing the true orientation of the cell. Thus, in the serially sectioned sperm in Figs. 12-15, the axoneme appears anti-clockwise but the series progresses proximally; therefore, the flagellum, by convention, is clockwise. This result was obtained in eight spermatozoa from one hamster.

Although the flagellar configuration $R p_1 r_1 p_2$ was the dominant three-dimensional waveform, there were a few flagella ($L p_1 r_1 p_2$) which were twisted anti-clockwise (Fig. 4). Did these few have anti-clockwise axonemes? The question could not be answered for hamster spermatozoa on account of the scarcity of suitable sections. However, in the rat a larger number of spermatozoa could be examined by taking advantage of a more easily found morphological marker, the posterior lobe of the spermatozoan nucleus (Fig. 16). 50 sperm from one epididymis were studied in serial sections (Figs. 17-18), and all had clockwise axonemes.

DISCUSSION

The significance of the flagellar waveforms observed after freeze substitution depends critically on the assumption that in freezing the cells, the time taken to arrest the flagellum is short in relation to the time scale of bend generation and propagation. There is at present considerable, though indirect support for this assumption. Rapatz et al. (16) have reported the cooling rates of blood samples rapidly frozen in capillary tubes. From their Fig. 3 the fastest rate of cooling, estimated over the freezing range (say to -10°C) was about 220°C/s . If such a figure applied, the sperm sample would be cooled from $+21^\circ\text{C}$ to -10°C in 0.14 s. Now, under the conditions specified, at room temperature, a few hamster spermatozoa swim extremely rapidly, but nearly

all of them have beat frequencies within the range 1.1-1.7 cycles/s (personal observation). The fastest of this group, therefore, would be arrested within 0.24 cycle. However, where Rapatz et al. used isopentane as their coolant, in these experiments Freon-22 has been used; published estimates of cooling rates indicate that Freon-22 is $1.6 \times$ more efficient than isopentane in cooling a naked thermocouple (17). Also, greater efficiency must have been gained from the higher surface-to-volume ratio of the sperm specimens compared with the blood samples and from the direct contact between the sperm suspension and the coolant. These considerations indicate that the actual time taken to stop the flagella was considerably shorter than the above estimate.

The comparison which has been made between frozen-substituted and living spermatozoa lends some support perhaps to the view that the sperm are stabilized nearly instantaneously, but the work is interpreted with caution, since, strictly, it reveals nothing about the speed of the quenching procedure; and, furthermore, the flash micrographs can reveal nothing of the important distal part of the flagellum. The results do indicate, however, that there is no time available for at least the proximal part of the flagellum to undergo gross agonal responses during stabilization. Furthermore, it has also been possible to show that a gross agonal response, in this case to immersion in glutaraldehyde, can be detected by this simple technique. Before discussing the waveforms in detail, some explanation is required of the way in which the frozen-substituted sperm sedimented: the relevant observation was that, in a flagellum in which a distal segment deviated from the plane of the first bending cycle (plane P_1), the sperm nearly always came to lie with this part of the flagellum projecting upwards from the microscope slide. If we consider a spermatozoon in a fluid, its equilibrium orientation is given by a line connecting the centers of gravity and buoyancy; and, if we assume the sperm head to have a higher specific gravity than the tail, the orientation of a planar tail would be vertical, as shown in Fig. 19a. Any deviation of the tail from plane P_1 would cause a greater lateral displacement of the center of buoyancy than of the center of gravity. Such a sperm would therefore adopt a different orientation (Fig. 19b and c), and this orientation would determine the most likely direction of fall after the sperm first touches the slide (Fig. 19d). In fact, gliding might well have occurred also, but

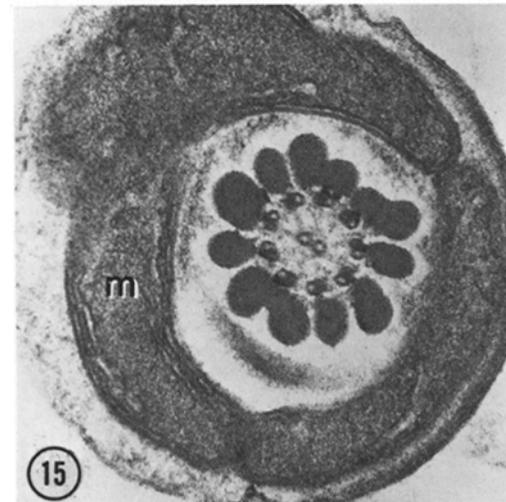
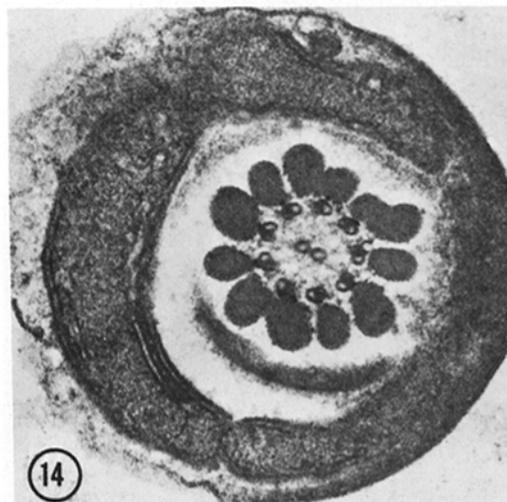
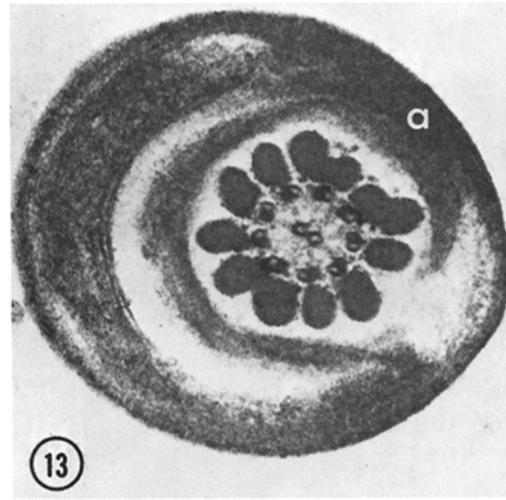
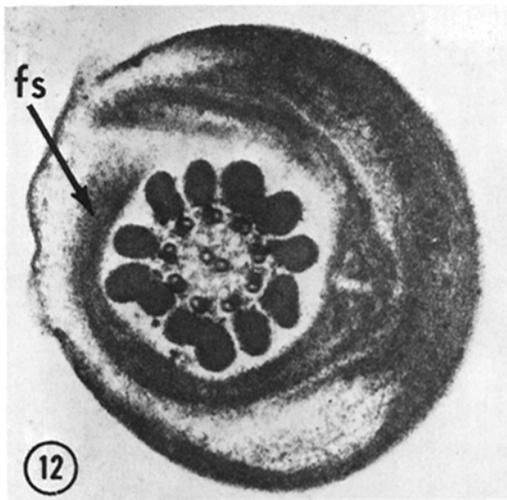
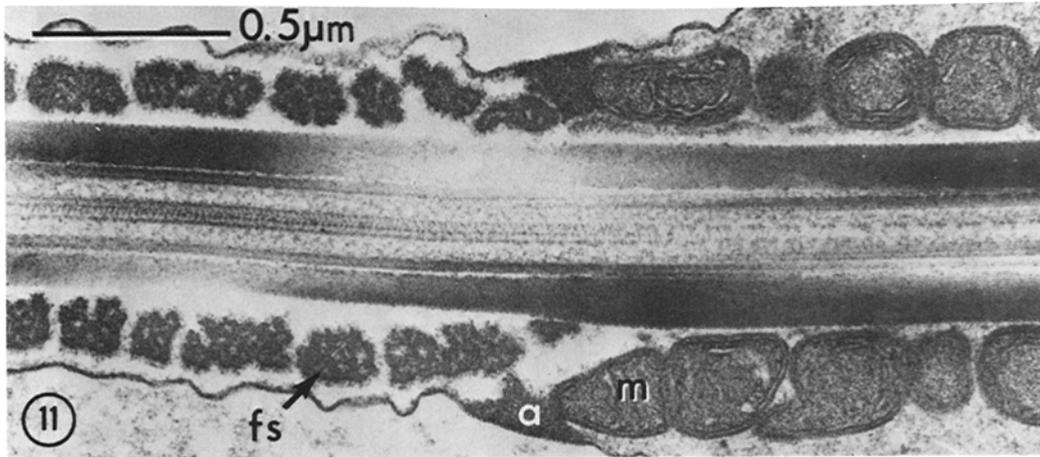


FIGURE 11 A longitudinal section through the end of the middle piece of a golden hamster spermatozoon. The mitochondria (*m*) are succeeded distally by the annulus (*a*) and then the fibrous sheath (*fs*). $\times 52,500$.

FIGURE 12-15 Serial transverse sections through the region shown in Fig. 11. The sections were cut from the block in the order in which they are presented. The progression of the series, therefore, is towards the sperm head. The peripheral doublet microtubules then, if viewed from the sperm head, would lie in clockwise array. $\times 52,500$.

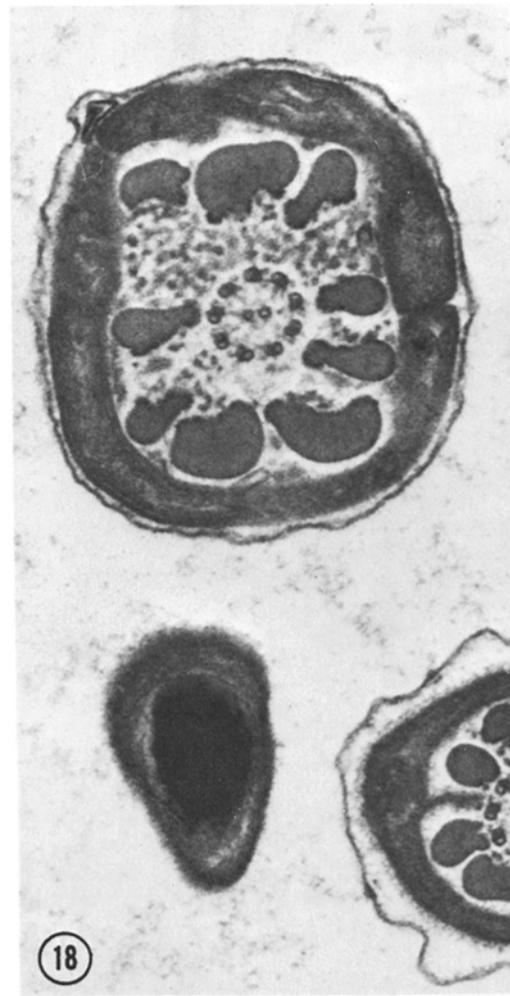
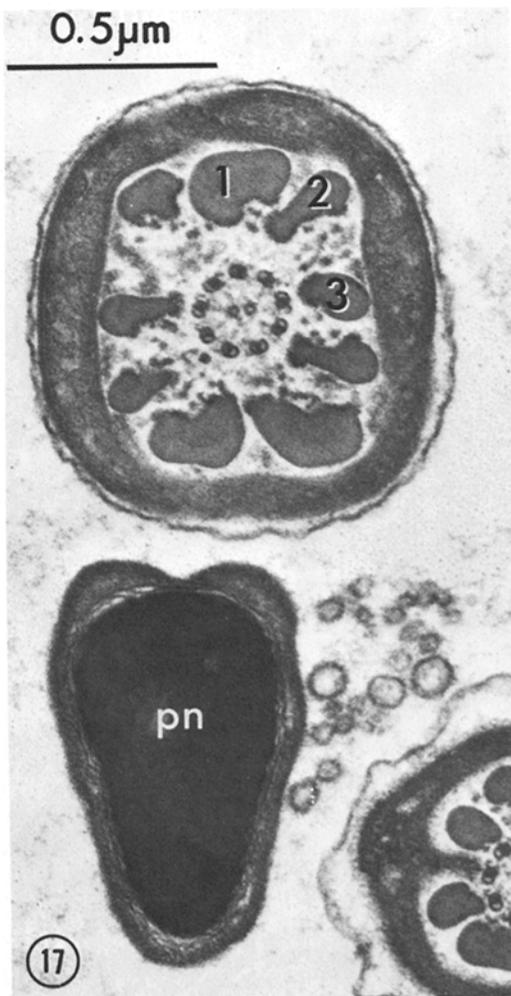
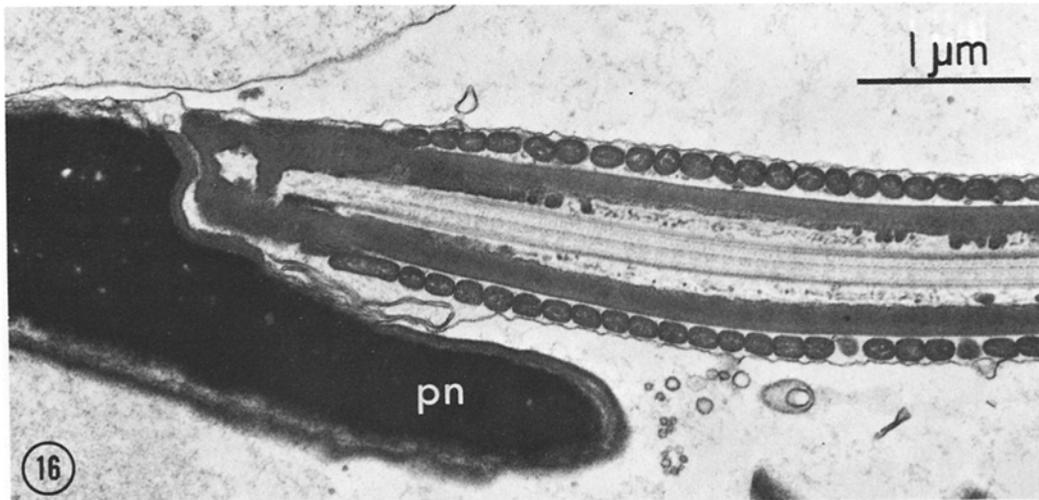


FIGURE 16 A longitudinal section through the neck region of a *rat* spermatozoon showing how the posterior nuclear lobe (*pn*) of this species lies alongside the proximal part of the flagellum. $\times 23,000$.

FIGURES 17-18 Transverse sections through the posterior nuclear lobe (*pn*) and the adjacent flagellum of the same spermatozoon. The imminent disappearance of the nuclear lobe from serial sections establishes the absolute orientation of the cell. In this case the series of sections progresses distally, and the flagellum is clockwise. This was the finding in all the 50 *rat* sperm examined. The pattern of the axonemal doublets, in less clear sections, could often be judged from the characteristic profiles of the nine dense fibers (numbered 1, 2, 3, etc. in Fig. 17). $\times 56,400$.

spinning cannot have occurred since it would have confused the outcome.

Although this study has emphasized the characteristic three-dimensional wave form $R p_1 r_1 p_2$, there was about an equal number of the two-dimensional wave forms $R p_1 r_1 p_2$ and $L p_1 r_1 p_2$ (considered together). This uniplanar type is illustrated in a perspective drawing (Fig. 20a). It is probable that such sperm are responding to confinement within a film of fluid (5). In interpreting the three-dimensional forms ($R p_1 r_1 p_2$) the problem was to decide between the "planar-becoming-helical" and the "twisted plane" models. The alternatives are shown in Fig. 20b and c. The decision was based on the fact that the maximum upward displacement of bend p_2 consistently coincided with the point of greatest lateral displacement of the flagellum from the projection of the axis of progression (Fig. 20c). For a helical wave form, this point of maximal displacement would lie directly above the axis of progression (Fig. 20b). The difficulty in drawing Fig. 20b also suggests the unlikelihood of the model, since it involves a transition between two sections of flagellum which would be executing quite different movements. The twisted plane model, in contrast, requires fundamentally similar movements to be made by different segments of the flagellum.

In Fig. 20c the plane of bending is shown twisting clockwise in a gradual fashion. A gradual twisting, however, is thought to be unlikely. It has been noticed that the proximal bending cycle $p_1 r_1$ appears to be exactly uniplanar (in plane P_1) and that twisting of the plane of action is most pronounced in the region between bends r_1 and p_1 . After this pronounced twisting, the p_2 bends clearly subtend a preferred angle at the progression axis (see Fig. 9), as if the cycle $p_2 r_2$ is also essentially uniplanar (plane P_2). Therefore a twisted plane model is suggested in which each cycle of bending has a preferred plane of action (Fig. 20d). There was no indication that the plane of action depends on the distance travelled along the flagellum nor was it related to the amplitude of the bend. Generally, the angulation of the plane of bending seems to depend on the number of following bending-cycles existent in the flagellum; some of the less common types of wave form, however, are exceptions to this generalization (e.g., $p_1 r_1$, $r_1 p_1$; see Fig. 4).

From the electron microscope study, it seems likely that all the spermatozoa conform to the rule (8, 10) that axonemal doublets lie in a

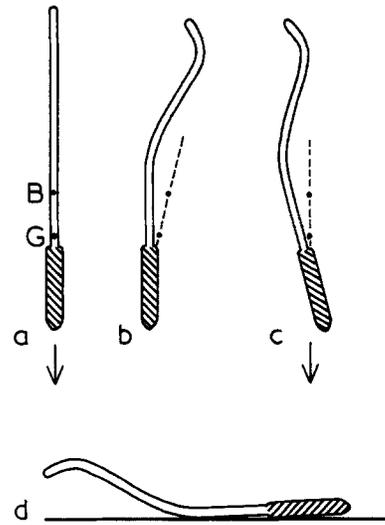


FIGURE 19 An explanation of the sedimentation behavior of the waveform $R p_1 r_1 p_2$. A displacement of the distal flagellum from the plane of flattening of the sperm head (Fig. 19b) causes a change in its equilibrium orientation (Fig. 19c). This predisposes the sperm to fall such that the displaced region of flagellum lies above the level of the microscope slide (Fig. 19d). G = center of gravity; and B = center of buoyancy.

clockwise pattern when viewed from base to tip. Therefore, in the proposed model, the clockwise twist in the plane of action follows the direction in which the doublets are numbered. The plane (P_1) in which the proximal flagellum beats has not been related to the axes of symmetry within the flagellum for the golden hamster. In rat and mouse sperm, however, plane P_1 is defined by a radial line passing through doublet 1; and the sperm head attaches to the flagellum such that its hook-shaped projection and axonemal doublet 1 are on the same side of the cell (23). Assuming this to be true also for the golden hamster, it would follow that the convex edge of bend p_1 would contain doublet 1, and the convex edge of bend r_1 , doublets 5 and 6. This would make the principal bend analogous to the recovery stroke of a cilium (9).

In considering subsequent planes of bending (P_2, P_3), there are two main possibilities, neither of which can yet be eliminated: (a) the entire flagellum might be twisted in these regions such that the successive planes of action might all still contain doublet 1; or (b) the flagellum itself might not be twisted, but its planes of action might have twisted with respect to doublet 1.

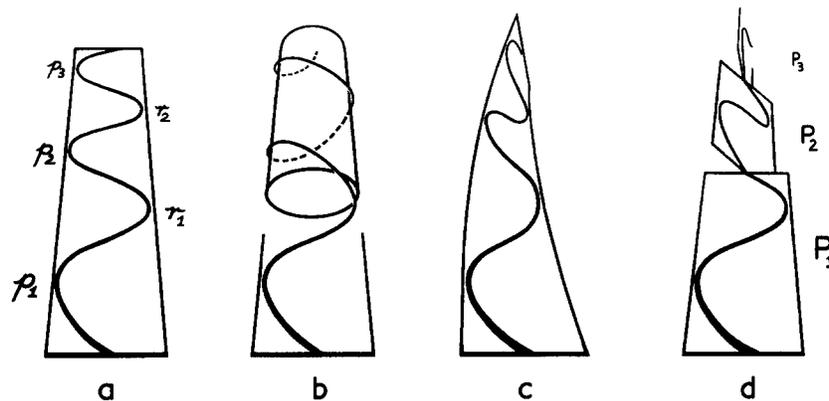


FIGURE 20 Perspective drawings of the possible waveforms discussed in the text. In each drawing the flagellum is seen from a position in front of and above the sperm head (not drawn). Very few hamster sperm actually contain a third principal bend. (a) The frequently observed planar waveform, with the bends individually labeled. (b) An attempt to envisage a transition from planar to helical bending; arguments against this waveform are presented. (c) A waveform which follows a gradually twisting plane. Each small segment of the flagellum bends in only one plane yet the undulation, overall, is three-dimensional. (d) A twisted plane waveform in which most of the twisting occurs between bending cycles and each cycle tends to lie in a preferred plane (P_1 , P_2 , etc.). A waveform of this type is thought to underlie the undulations seen in frozen-substituted hamster sperm flagella. The drawing shows each successive plane twisted through an idealized angle of 40° , but this is probably a simplification (see text).

Whichever mechanism operates, the suppression of twisting in nonrotating spermatozoa will need to be explained. The first possibility has been tentatively suggested by Gibbons (11) from his studies of demembrated sea urchin sperm flagella which had been quickly arrested by abstracting ATP from the medium ("rigor waves"). The characteristic twist between bends was reported to be between 40° and 60° and was said to alternate in direction along the flagellum. However, as Gibbons says, it is difficult to reconcile active axonemal twisting with the reportedly planar waveform of the flagellum. On the other hand a twisted-plane wave form, containing no twist in the axoneme itself, would, if it were artefactually flattened in processing, closely mimic Gibbons' waveforms.

Thus, it may be possible to interpret Gibbons' results without postulating twisting of the flagellum. The alternative idea, that the plane of action might twist without a twisting of the axoneme, was proposed provisionally for rat spermatozoa (23) when it seemed that, in the few cells studied, distal segments of the flagellum, which were not twisted, were moving in a plane which was oblique in relation to plane P_1 . Pursuing this interpretation for hamster spermatozoa, the mean angles between the three planes ($P_1 \dots P_2 = 46.6 \pm 6.0^\circ$;

$P_1 \dots P_3 = 79.3 \pm 6.5^\circ$) are similar to the angles between the peripheral doublets 1 and 2, and 1 and 3, which, if we assume the axoneme to be exactly cylindrical, will be 40° and 80° . If no twisting of the axoneme occurs, this similarity suggests that if plane P_1 is topographically dictated by doublet 1, plane P_2 may be dictated by doublet 2 and so on. This speculation is attractive because its implication, if correct, would be that the nine peripheral doublets, which seem to be structurally and chemically equivalent, are equivalent also in their function. There is, however, an obvious problem in accepting this: the mean angle between planes P_1 and P_2 is just significantly greater than 40° , i.e., $360^\circ \div 9$. The importance of this discrepancy cannot be assessed at present. In expecting the angle to be exactly 40° one is assuming that (a) the nine dense fibers, which are of unequal thickness and length, do not complicate the waveform, and that (b) the actively bending axoneme retains an exactly circular cross-sectional profile such that the angle subtended at its center by any two doublets is exactly 40° . Neither assumption can be made with confidence. In view of these uncertainties, the author does not at present reject the interpretation that successive doublets (no. 1, 2, and 3) in some way influence the successive planes of bending (P_1 , P_2 , and P_3)

but considers it worth retaining as a working hypothesis. Thus Fig. 20*d*, in which the successive planes are shown each displaced through 40°, is in this respect hypothetical.

From the current investigation the proposed interpretation of the three-dimensional wave forms is that bending cycles undergo a clockwise twist in their plane of action as they are succeeded by new bending cycles. The hydrodynamic consequences of this twisted plane model have yet to be considered. As a first simple prediction it would seem that more than 90% of rotating hamster spermatozoa should rotate (i.e., roll) in the same direction, which would be opposite in sense to the direction of twisting, that is, anticlockwise if viewed from in front of the sperm. Unfortunately it is technically very difficult to test this prediction (7). The envelope formed by a twisted plane flagellum has the form, approximately, of a screw surface (Fig. 20*c* and *d*) and it will be of interest to calculate the propulsive efficiency of such undulations, so that comparisons may be made with planar and helical wave forms.

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