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A critical review of the physiological importance and analysis of sperm movement in mammals*

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The identification of human sperm hyperactivated motility has potential importance in sperm function tests, as well as in quality control assays and in reproductive toxicology investigations. However, relatively little is known about this phenomenon and the variety of definitions used for hyperactivation has led to a great deal of confusion as to its occurrence and physiological relevance. This presentation is a critical review of a number of aspects of hyperactivated motility, including its identification and potential role(s) in mammalian fertilization. The initial sections of the review consider the mechanisms involved in the development and maintenance of mammalian sperm motility, and the structural and functional changes in spermatozoa which occur during transport through the female reproductive tract. The methods available for the quantification of aspects of sperm movement are also discussed, with an historical overview of sperm movement analysis.

Key words: CASA/flagellum/hyperactivation/kinematics/spermatozoa

Sperm ultrastructure

To understand the cellular events which are necessary for the development of sperm motility and specifically hyperactivated motility, one must first consider the fundamental structure of the spermatozoon. Although there are species-specific features, mammalian spermatozoa share the same basic sperm structure, i.e. a head and a tail, which is composed of a midpiece, principal piece and end piece. The principal function of the sperm head is to deliver a haploid set of chromosomes to the oocyte. The function of the flagellum is to provide cell motility to allow the spermatozoon to penetrate the boundaries of the female reproductive tract and the zona pellucida. The structural features of the head and tail of the spermatozoon reflect these functional roles.

The flagellum may be considered to be composed of four regions: the connecting piece, the midpiece, the principal piece and the terminal or end piece (Fawcett, 1965). The axoneme and outer dense fibres are also located within the flagellum (Figure 1). The axoneme is present for most of the length of the flagellum, terminating in singlet microtubules in the end piece. It is composed of two central microtubules connected by linkages (Pedersen, 1970), surrounded by nine microtubule doublets (the '9 + 2' pattern) (Fawcett, 1965). Each doublet consists of an A subunit forming a complete microtubule, and a B subunit which is C-shaped with its ends attached to the A subunit. A central sheath composed of a spiral of two fibres surrounds the two central microtubules (Pedersen, 1970). In animals with internal fertilization, auxiliary dense fibres (outer dense fibres) and a fibrous sheath surround the 9 + 2 axonemal structure. Attached to the A subunit of the microtubule doublets are the dynein arms (Afzelius, 1959; Gibbons and Grimstone, 1960; Gibbons, 1961). Dynein is a multisubunit ATPase complex (Gibbons, 1965) which translates chemical energy (ATP) into kinetic energy by allowing adjacent microtubule doublets to slide relative to one another, causing axonemal bending and hence flagellar movement. This occurs in an attachment–detachment cycle between the dynein arms and the adjacent doublet (Marchese-Ragona and Johnson, 1990).

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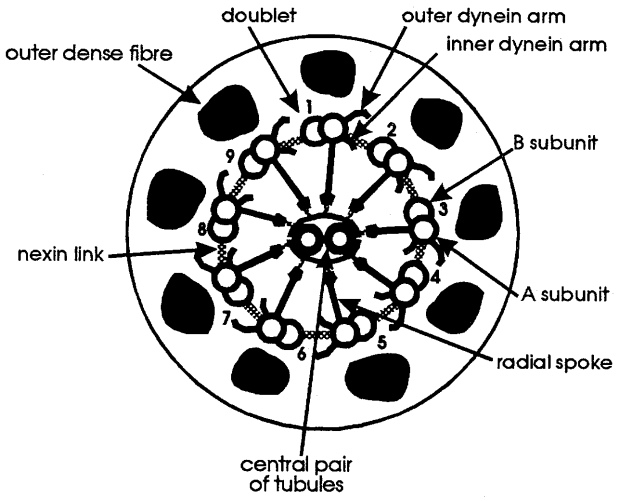

Figure 1. Transverse section of a mammalian sperm flagellum.

Adjacent microtubule doublets are connected by nexin links (Gibbons, 1965; Stephens, 1970) between the A and B subunits (Baccetti *et al.*, 1985). It has been suggested that these are elastic elements which allow regulation of the shear forces during doublet sliding, or that they assist in the retention of axonemal symmetry during sliding (Linck, 1979). It has been shown that nexin can be digested by elastase, causing an increase in the flagellar bend angle, and a concomitant decrease in flagellar beat frequency, suggesting that the nexin links are elastic elements involved in the regulation of the amplitude of flagellar bending (Brokaw, 1980). However, it has also been observed that the nexin links undergo cycles of displacement to permit microtubule sliding which would suggest that their role is not purely for the provision of elastic recoil. It has also been proposed that the links regulate the spacing of the microtubules to optimize dynein–tubulin interaction (Bozkurt and Woolley, 1993). The precise interaction between dynein and tubulin is still not clear, although it is known that the dynein-tubulin binding which allows active sliding involves the B subunit of the neighbouring microtubule doublet, and is influenced by ATP (Gagnon, 1995).

Radial spokes project from the A subunit of the microtubule doublets towards the central sheath (Afzelius, 1959; Gibbons and Grimstone, 1960; Hopkins, 1970). These spokes are composed of 17 proteins, 12 in the stalk region (attached to subfibre A of the microtubule doublet) and five in the globular head region which projects towards the central pair (Gagnon, 1995). Several of the radial spoke proteins, including five stalk proteins, are known to be phosphorylated, but the reason for this is not yet known (Curry and Rosenbaum, 1993). The radial spokes are known to attach and detach during the microtubule doublet sliding cycle.

The axoneme of a mammalian spermatozoon is surrounded by nine outer dense fibres which are attached to the distal end of the segmented columns in the connecting piece (Fawcett, 1975; Baccetti *et al.*, 1976). Each outer dense fibre is associated with an axonemal microtubule doublet, and numbered according to the doublet with which it is associated. These fibres each contain a cortex and a medulla and are composed of a keratin-like protein (Baccetti *et al.*, 1973). There are two groups of disulphide cross-linked polypeptide chains, with the disulphide cross-linking occurring during epididymal maturation (Olson and Sammons, 1980). The length of the outer dense fibres are variable relative to each other (Fawcett, 1965), but stereological analysis of human spermatozoa has shown that the order of termination of each dense fibre along the flagellum is constant (Serres *et al.*, 1983). They extend for up to 60% of the length of the principal piece of the flagellum, with two short fibres (3 and 8; 6 μm), three medium length fibres (2, 4 and 7; 17–21 μm) and four long fibres (5, 6 and 9; 31–32 μm and fibre 1; 35 μm). Compared with hamster spermatozoa, the outer dense fibres of human spermatozoa are relatively small in cross-section, which confers greater potential flexibility on the flagellum than is seen for other species (Phillips, 1972; Paddock and Woolley, 1980).

The exact role for outer dense fibres in sperm motility has not been determined conclusively. Early studies suggested that they were active motor elements in flagellar movement, but later studies have shown that this is unlikely. For example, in toad spermatozoa the axoneme and accessory fibre (which is analogous to the outer dense fibre) are separate structures connected by a membrane, and it was determined that the accessory fibre did not move independently of the axoneme, but rather had a role in stiffening the flagellum (Swan *et al.*, 1980). Further evidence for the suggestion of a stiffening role for the outer dense fibres is the observation that maximum flagellar curvature in sea urchin spermatozoa (which do not have outer dense fibres) occurs in the proximal region of the flagellum, while the flagellar curvature of mammalian spermatozoa in seminal plasma increases with propagation (Denehy, 1975), and the maximal curvature occurs in the region of termination of the outer dense fibres (Woolley, 1979). Using a mathematical approach, Rikmenspoel (1984) showed that the outer dense fibres were not actively involved in the generation of the forces necessary for motility in bull spermatozoa. The keratin-like protein confers elastic properties upon the outer dense fibres, and it is thought that they cause an elastic recoil of the axoneme

after microdoublet sliding (Phillips, 1972). It has been suggested that the asymmetry of the termination of the dense fibres may play a role in the progressive alteration of the plane of flagellar beating (Serres *et al.*, 1983), but asymmetry of flagellar beating has also been observed in species without outer dense fibres, and in others with outer dense fibres of equal lengths (Woolley, 1979).

The connecting piece is a short linking segment between the flagellum and the sperm head. It is composed of the segmented columns and a dense fibrous structure, the capitellum or capitulum (Fawcett, 1965). The sperm head and tail are connected via the capitellum and the basal plate which is at the caudal end of the nucleus. It has been suggested that the columns may be articulated, which would allow the neck region to bend without straining the link between the capitellum and the basal plate (Curry and Watson, 1995). The proximal centriole is embedded transversely within the segmented columns, perpendicular to the plane of the flagellum (i.e. the transverse plane of the central pair) and marks the origin of the central pair of axonemal microtubules. The outer dense fibres are attached to the distal end of the segmented columns.

The midpiece of the mammalian spermatozoon extends from the distal end of the connecting piece to the annulus, a structural element marking the junction between the midpiece and the principal piece. It contains a helical arrangement of mitochondria which generate energy used for flagellar movement (Curry and Watson, 1995). The inner mitochondrial membrane is the site of energy production, and the position of the mitochondria around the proximal portion of the axoneme suggests that they are necessary for the supply of ATP used for flagellar motility.

The principal piece of the flagellum extends from the annulus to the terminal piece, and is characterized by the presence of the fibrous sheath. The fibrous sheath is a cytoskeletal structure surrounding the axoneme and outer dense fibres. It is composed of two peripheral longitudinal columns in the plane of the central pair of microtubules, connected by more-or-less semicircular circumferential ribs that branch and anastomose (Fawcett, 1965). The two columns of the fibrous sheath have been shown to overlie, and be fused with, the two shortest outer dense fibres and continue an attachment with their associated microtubule doublets following the distal termination of these outer dense fibres. There is extensive disulphide bonding between the constituent proteins of the fibrous sheath (Oko, 1988; Brito *et al.*, 1989), making the structure extremely stable, and contributing to the hypothesis that the structure provides support to the flagellum in the control and restriction of flagellar movement, thereby assisting in sperm motility. The axonemal complex is attached to the sperm plasma membrane in the principal piece by the 'zipper', a double row of interdigitating oval-shaped intramembranous particles adjecent to outer dense fibre 1 (Friend and Fawcett, 1974; Enders *et al.*, 1983). The attachment of the axonemal complex to the plasma membrane allows greater efficiency of movement than would occur if the axoneme was beating within an unattached plasma membrane envelope (Koehler, 1983).

The terminal piece is the region beyond the distal end of the fibrous sheath. This region contains only the 9 + 2 axoneme covered by the plasma membrane. The axonemal elements are terminated successively, with the disappearance of the dynein arms, followed by the termination of the central pair of microtubules, the separation of the microtubule doublets and the successive disappearance of the B microtubule subunits (Woolley and Nickels, 1985).

The whole sperm cell is covered by a plasma membrane, but the acrosome, nucleus and mitochondria are each encapsulated by their respective membranes. The plasma membrane has definite structural subdivisions which are related to the cellular subcomponents. On the sperm head, freeze-etching studies of bull spermatozoa have shown that the plasma membrane can be divided into two main sections over the acrosome and the post-acrosomal region, with the equatorial segment forming a transitional region (Koehler, 1966). It has been observed that there is differential, regional binding of lectins (Nicolson and Yanagimachi, 1974) and monoclonal antibodies (Myles *et al.*, 1981) to the plasma membrane of mammalian spermatozoa, suggesting localized regulation of membrane components (Nicolson *et al.*, 1977). The distribution of lectin binding sites over the flagellum has been observed to be less regular than over the head region, leading to the proposal that there may be greater mobility of the glycoproteins in the flagellar plasma membrane (Koehler, 1983). It would follow then that these differences may be related to the specific functions of each region in terms of the ionic requirements and second messenger systems involved. The view that there may be specialized membrane components for specific regions of the spermatozoon is supported by observations of an arrangement of parallel striations, the basal cords, which appear to be highly stable membrane specializations, immediately anterior to the posterior ring (Holt, 1984). The stability of this membrane structure suggests that it acts to isolate contiguous membrane domains, preventing the loss of essential membrane components to a different region of the sperm cell.

Consideration of sperm ultrastructure would also argue for the existence of separate and distinct regions, with the posterior ring acting to segregate the cytoplasmic

compartments of the sperm head and flagellum. Further evidence for this view is provided by consideration of the difference in the metabolic requirements of the sperm head, which is relatively inactive prior to fertilization, and the flagellum, which is extremely active when the spermatozoon is motile.

Theories of flagellar movement

It is known that flagellar bending occurs as a result of asymmetric sliding of axonemal components; however, the exact mechanism has still not been elucidated completely. The energy for flagellar motility is derived from dynein-ATPase catalysed hydrolysis of ATP, which then drives microtubule sliding. The ATP is regenerated by the breakdown of glucose or fructose to lactate via glycolysis, and by mitochondrial oxidation of substrates via the citric acid cycle (Ford and Rees, 1990).

The sliding filament hypothesis for flagellar bending postulates that bending occurs as a result of active shearing forces between the microtubule doublets which lead to localized sliding movements generated through the interaction of the dynein arms with ATP. The binding of ATP to the dynein arms, and its hydrolysis, are thought to cause a cyclical change in the angle of the dynein arms, which is coordinated with the repeated making and breaking of their attachment to successive binding sites along the length of the adjacent microtubule doublet. This attachment–detachment cycle results in a sliding motion caused by the dynein arms 'walking' one microtubule doublet along its neighbour. The localized sliding movements of the axoneme then cause the generation of a bending moment (Gibbons, 1974). There is no 'memory' of the phase of the beat along the flagellum, since if the beat is lost for any reason, the flagellum is able to resume beating at a different phase to the beat just lost (Eshel *et al.*, 1992).

The microtubule doublets in the axoneme are numbered clockwise, with the number 1 doublet being the only doublet which is in the same plane as (i.e. parallel to) the central pair (Afzelius, 1959). It has been considered that the method of movement is sequential attachment of the dynein arms of doublet 1 to the B subunit of doublet 2, and the dynein arms of doublet 2 attaching to the B subunit of doublet 3 etc. Sale and Satir (1977) have shown that the movement of a doublet relative to its neighbour is that the lower numbered doublet slides headward on its higher numbered neighbour by the action of the dynein arms projecting from the doublet. In this way, the bend in the axoneme would be transferred helically, leading to the transmission of the bend along the flagellum. This model would predict that the flagellar wave, as viewed external to the cell, would be perfectly helical in nature. However, a helical pattern of wave transmission would not confer movement upon the spermatozoon, so this hypothesis has been considered to be a simplification (Kanous *et al.*, 1993). The flagellar wave has been described as a 'twisted plane' (a three-dimensional waveform consisting of essentially planar, twisted waves) in golden hamster spermatozoa (Woolley, 1977); as an elliptical helix in ram spermatozoa (Denehy *et al.*, 1975); and as elliptically conical in bull spermatozoa (Rikmenspoel, 1965). These observations would support the view that the simple pattern of transmission of axonemal bends from doublet to doublet is not completely accurate.

It has been observed in some species that there is an association between microtubule doublets 5 and 6 either physically or functionally, i.e. acting as a single unit rather than as two separate doublets (Afzelius, 1959, 1988; Gibbons and Fronk, 1972; Warner, 1974). Observations of sliding disintegration of reactivated demembranated rat and bull spermatozoa (Lindemann *et al.*, 1992; Kanous *et al.*, 1993) have suggested that doublets 3 and 8 are connected to the central pair of microtubules, since they were not extruded from the axoneme. It was also observed in these experiments that there were two groups of doublets (9,1,2 and 4,5–6,7) which were extruded together. In all cases, doublet group 9,2,1 were extruded first. It was observed that these groups of doublets were more like flattened ribbons than separate doublet units, and considered that the concerted sliding of a group of doublets rather than one doublet at a time would give a better empirical model to explain the observed flagellar beat patterns of mammalian spermatozoa.

An extension of the sliding filament theory is the 'Geometric Clutch' hypothesis which was developed initially to describe the movement of a 10 μm cilium and a simple flagellum (Lindemann, 1994a,b). More recently, this hypothesis has been extended to model flagellar movement in mammalian, specifically bull, spermatozoa (Lindemann, 1996). From computer simulations and empirical data it is proposed that, via the outer dense fibres, the connecting piece of the spermatozoon provides the necessary basal resistance to microtubular sliding and that the anchoring of the outer dense fibres in the connecting piece is necessary for bending torque generation and is a key element in normal dynein bridge switching.

Using *Ciona* spermatozoa as a model, it has been proposed that the inner dynein arms act to maintain the bend curvature, while the outer dynein arms perform work against viscous resistances (Brokaw, 1996). This hypothesis was proposed because it was found that large changes in the amplitude and wavelength of bend

propagation occurred with little change in the bend initiation parameters other than frequency. In human spermatozoa, it has been found that spermatozoa lacking outer dynein arms were still motile, although the velocity of movement of the sperm head and the flagellar beat frequency were lower than ('approximately half') those of normal spermatozoa (Jouannet *et al.*, 1983). Similarly, the removal of the outer dynein arms of demembranated spermatozoa by treatment with 0.5 M KCl resulted in a reduction in flagellar beat frequency to half that of control demembranated spermatozoa (Gibbons, 1974).

Biochemical regulation of axonemal function

Calcium is an important regulatory element in flagellar beating, with high concentrations causing suppression of sperm movement (Mohri, 1993). In intact human spermatozoa, a decrease in the concentration of intracellular Ca^{2+} was found to reduce the degree of flagellar curvature. A concentration of $\geq 10^{-6}$ M Ca^{2+} in the external medium was required to sustain high amplitude flagellar bends (Serres *et al.*, 1991). Calmodulin, or calmodulin-binding proteins are present in sperm flagella adjacent to or associated with axonemal components, suggesting a role in signal transduction in the regulation of flagellar movement by calcium (Tash, 1990).

Cyclic adenosine monophosphate (cAMP) is necessary for the initiation and activation of sperm movement in mammals and other animals. Adenylate cyclase is activated by bicarbonate ions during epididymal maturation and at ejaculation (Morisawa and Morisawa, 1990; Mohri, 1993). This stimulates the production of cAMP, leading to the activation of a cAMP-dependent protein kinase, and thence to the phosphorylation of protein(s) which participate in the conversion of axonemal sliding to flagellar bending. Protein kinase C has been proposed as a regulator of human sperm motility (Rotem *et al.*, 1990). Calcium uptake has been suggested to trigger changes in cAMP levels in bovine epididymal spermatozoa (Hoskins *et al.*, 1983).

Viscosity

Increased viscosity of the external medium results in a decreased amplitude of the flagellar wave (Mohri, 1993). In *Ciona* sperm flagella, the reduction in the amplitude of the propagating bends is associated with a reduction in the bend amplitude during bend initiation (Brokaw, 1996).

Analysis of sperm motility

Several methods have been introduced to estimate the velocity of spermatozoa in a semen sample or in a sperm preparation. The methods used became more sophisticated with improvements in image analysis and computing. Descriptions of the various methods and the results available from each are discussed below.

Passage counting

This is the least complex method and involves microscopic observation of the number of spermatozoa crossing a defined line or area on the field of view in a given time period. The mean velocity of spermatozoa in a sample can then be calculated from this value using a nomogram which also includes the sperm concentration. This value has been referred to as 'sperm motile efficiency' (Ishii *et al.*, 1977). These analyses have been made directly from observations down the microscope (Hynie, 1962; Barták, 1973) or with timed exposure photomicrographs (Janick and MacLeod, 1970). The values obtained are averaged for the whole sample, and so give only a single, rudimentary kinematic value.

Turbidimetry and nephelometry

These procedures involve the measurement of the rate of sperm migration out of semen into artificial medium, calculated as a function of the increase in optical density of the artificial medium. The proportion of rapidly-moving spermatozoa and their average velocity can be estimated using this method (Sokoloski *et al.*, 1977; Atherton *et al.*, 1978; Levin *et al.*, 1980, 1981, 1984; Morton and Sagadraca, 1981; Halangk and Bohnensack, 1986). A sperm motility index determined using this technique was significantly correlated with the proportion of progressively motile spermatozoa (Atherton *et al.*, 1979). This test can be carried out in optical cuvettes or in capillaries, and forms the basis for a commercial instrument, the Sperm Quality Analyser (SQA; United Medical Systems Inc, Santa Ana, CA, USA).

Laser doppler velocimetry (LDV)

The principle of LDV is that a population of motile spermatozoa will scatter a monochromatic laser beam thereby, according to the Doppler effect, giving a spectrum of light frequencies dependent upon parameters of sperm movement (Jouannet *et al.*, 1977). The parameters determined by LDV are the number and proportion of motile spermatozoa, the velocity distribution and the three-dimensional instantaneous ('characteristic') velocity (Dubois *et al.*, 1975; Naylor *et al.*, 1982; Craig *et al.*, 1982; Woolford and Harvey, 1982; Rigler and Thyberg, 1984; Pusch, 1985; Earnshaw *et al.*, 1985). Variations of this technique which have been used in the evaluation of sperm

motility are Photon Correlation Spectroscopy, which is a fully digital technique (Frost and Cummins, 1981) and twin-beam laser velocimetry (Wilson and Harvey, 1983; Wilson *et al.*, 1987). The advantage of the latter method is that the translational and rotational velocity values are not combined. The disadvantage of these techniques, as for the turbidimetry techniques, is that there is no visualization of sperm movement and therefore, they are indirect estimates of motility parameters (Boyers *et al.*, 1989).

Timed-exposure photomicrography (TEP)

For TEP methods, spermatozoa are visualized microscopically using dark field optics and movement is recorded by exposure of a single frame of photographic film for a set length of time (typically 1 s). Track analysis is made using the negatives of these photomicrographs, giving a black track on a white background (Janick and MacLeod, 1970). A number of track measurements are possible using TEP, allowing the estimation of the proportion of subpopulations of spermatozoa in a preparation (Overstreet *et al.*, 1979), and objective evaluation of differences in sperm movement due to culture conditions (Milligan *et al.*, 1978) or to fertility status (Milligan *et al.*, 1980). Quantitative evaluation of the movement characteristics of individual tracks is also possible using TEP (Aitken *et al.*, 1983), using manual and semi-automated analysis systems (Mortimer, 1986). The movement characteristics: curvilinear and straight-line velocities, amplitude of lateral head displacement and linearity (see 'Kinematic analysis methods', below); as well as the proportions of motile and progressively motile spermatozoa have been determined using TEP (Aitken *et al.*, 1983; Mortimer, 1986). A variation on the TEP technique was introduced in which the TEP film strip was analysed by optical diffractometry, giving a diffractogram, the spectrum of which differed according to the proportion and count of motile spermatozoa in the picture (Guerin *et al.*, 1988). This method was quite specialized, and was not widely adopted.

A colour TEP method was devised, in which the microscope field was illuminated with a green light for one second, followed by a red light flash then a blue light flash. Motile tracks appeared as green lines, with red and blue dots at the end (allowing determination of the start and end points of each trajectory), and immotile spermatozoa appeared as white dots on a black background, since they had been exposed to all three lights while in the same place (Lysikiewicz and Enhorning, 1983). While it was predicted by the authors that this method would prove to be useful for clinical analysis, the development of computer-aided sperm analysis shifted the focus of attention.

Multiple exposure photomicrography (MEP)

Similarly to TEP, MEP uses a still camera to record images of spermatozoa in a microscope field. However, rather than a continuous image, a light flash is used, giving six images of the field/s. Images of motile spermatozoa appear as 'six-ringed chains' the length and shape of which describe the distance travelled by each spermatozoon in 1 s, while immotile spermatozoa are overexposed (Makler, 1978; Makler and Blumenfeld, 1980). Analysis of these photomicrographs could be made using manual or semi-automated methods (Makler *et al.*, 1980, 1984), giving sperm concentration, the proportion of motile spermatozoa, individual and average sperm speed (straight-line velocity, see below), and the frequency distribution of sperm speed (Makler, 1978; Kamidono *et al.*, 1983). The major disadvantage of this system is the number of images recorded per second (six) which is too low to allow calculation of other kinematic values, such as curvilinear velocity, amplitude of lateral head displacement and beat/cross frequency (see below). Another disadvantage is that darkfield illumination must be used, and this requires that a thin film of fluid be used, meaning that deep preparations cannot be used.

Microcinematography ('Cine')

As for the previous photographic methods, micro-cinematography allows visualization of sperm movement, but in this case, cinematographic film is used. The number of 'pictures' per second which can be recorded with cine can range from <20 to several hundred, allowing the option of detailed analysis of both sperm head and tail movement. Generally, analysis of cine films requires manual reconstruction, involving tracing the consecutive positions of a particular point on the spermatozoon, or of the flagellum, or the whole cell, in consecutive frames. All of the kinematic values derived for sperm head and flagellar movement can be determined using the analysis of cine images (e.g. David *et al.*, 1981; Serres *et al.*, 1984; Freund and Oliveira, 1987). Cine has also been used in the estimation of the proportion of motile spermatozoa and their relative velocity using passage counting (see above), in conjunction with frame-by-frame reconstructions to determine motility patterns (van Duijn, Jr *et al.*, 1971). The disadvantages of cine are the cost of the film and film processing, and the time required for processing. Also, there are no commercially-available systems for the automated analysis of cine, although some have been developed. One system relied upon digitization of video images of projected cine films, and could determine curvilinear velocity, mean velocity for all spermatozoa in a

preparation, and the proportion of motile spermatozoa (Amann and Hammerstedt, 1980). Another was much more sophisticated, allowing the determination of instantaneous velocity, curvilinear velocity, straight-line velocity, wavelength of the head trajectory, linearity and the frequency of lateral displacement of the head (Schoevaert-Brossault, 1984). Cine and TEP methods were used in the validation of the first automated sperm analyser (Katz and Dott, 1975).

Videomicrography

As a result of the disadvantages of cine, particularly the time and expense involved, videomicrography became widely used for the analysis of sperm movement. Initially, videotapes of motile spermatozoa were replayed and the net displacement of a spermatozoon determined by the distance travelled over one second, determined with reference to a concentric circle pattern on an acetate overlay placed on the monitor screen (Katz and Overstreet, 1981; Jenks *et al.*, 1982; Sokol *et al.*, 1988). The proportion of motile and progressively motile spermatozoa and the mean swimming speed for a sperm population were determined in this way. Semi-automated analysis methods were then developed for the analysis of video images of spermatozoa. The sophistication of these systems varied, with some capable of providing a progressiveness ratio (similar to linearity) (Samuels and Van der Horst, 1986) and others giving all of the common centroid-derived kinematic values (defined below) (Jenks *et al.*, 1982; Tessler and Olds-Clarke, 1985; Mortimer *et al.*, 1988a).

A video picture is composed of an array of picture elements or pixels. When light strikes the charge-coupled device (CCD) array of a video camera the relevant pixel is activated, and produces a voltage proportional to the intensity of the light. These voltages are then encoded as numbers in the digitization process, and a video picture is formed. If only black and white are used, then the digitization is binary, if shades of grey, or colours are used, the digitization process is more complex (Boyers *et al.*, 1989). When the camera scans an image, it scans the even rows of the array, then the odd rows, giving two 'fields'. These fields are then combined, or interlaced, to form a 'frame'. The number of frames/s, or frame-rate, depends upon the video camera and video recorder. In some countries (e.g. Australia, UK.), the PAL system is used, giving 25 frames or 50 fields/s for analysis. In other countries (e.g. Canada, USA, Japan), the NTSC system is used, giving 30 frames or 60 fields/s. It has been recommended that 60 images/s (Hz) be used for the analysis of human sperm head movement, since a more accurate trajectory can be reconstructed at this image sampling frequency than at the lower image sampling frequencies (Owen and Katz, 1993).

Videomicrography has several (mostly economic) advantages over microcinematography for the analysis of sperm movement characteristics, since videotape does not require processing after recording, and images are available for analysis immediately upon completion of recording. The relatively low image sampling frequencies of commonly-available video systems can be a disadvantage, however, as it is not possible to obtain a clear image of the flagellum. However, the use of mechanically shuttered video cameras, which restrict the image acquisition time to 1/500 s, for example, has allowed some laboratories to combine video imaging with flagellar analysis. Alternatively, video cameras and recorders with high image sampling frequencies (200 Hz) are available, although these represent a significant investment. Although sperm motility is a consequence of flagellar movement it is easier to image the sperm head than the flagellum, especially at lower image sampling frequencies and so most studies using video analysis are of sperm head movement, although there are some notable exceptions (such as Burkman, 1984; Hoshi *et al.*, 1988; Morales *et al.*, 1988).

Computerized digital image analysis using CASA

Computerized digital image analysis became a practical possibility with the development of progressively faster microcomputers and video frame-grabber boards (Glazzard *et al.*, 1983), resulting in the introduction of real-time sperm motility analysers. These are referred to collectively as computer-aided sperm analysis (CASA) instruments (Boyers *et al.*, 1989), and at present, most analyse sperm head movement either from videotapes or in real time. The advantages of CASA over manual or semi-automated track analysis are that there is no requirement for manual trajectory reconstruction, and that each trajectory can be analysed in a fraction of the time used for manual reconstruction and kinematic analysis.

Identification of spermatozoa by CASA

The advent of CASA instruments made population studies of sperm kinematics a practical possibility, since the movement characteristics of literally hundreds of spermatozoa could be determined in minutes. However, the use of purely automated systems required several compromises in the methods used for trajectory analysis, since only mathematical methods of analysis could be used (discussed in detail, below). Also, the use of digital image processing required a number of specific considerations, since the instrument itself had to identify each spermatozoon in a microscope field and then reconstruct a

trajectory for each one. Generally, to be identified as a spermatozoon, the image of the sperm head must be within preset parameters for length and width and/or be of a threshold brightness (Mortimer, 1994).

Sperm movement analysis

It has been determined that the most successful image analysis of sperm movement is made using negative high (NH) phase contrast or dark ground optics, in both cases the sperm head appears as a bright object on a dark background (Yeung and Nieschlag, 1993). The method used for the identification and tracking of a spermatozoon varies between CASA instruments. For example, some systems (e.g. CellSoft) identify the sperm head as a contiguous series of pixels, and the calculated 'centre' of this group of pixels is the 'centroid' (Berns and Berns, 1982); while the CellTrak system (MotionAnalysis) uses edge detection, whereby only the pixels which define the perimeter of an object are digitized, and the 'centroid' of the sperm head is calculated from these coordinates (Boyers *et al.*, 1989). The Hamilton Thorne CASA instruments identify the point of the spermatozoon to follow by locating the brightest region of each bright image (Yeung *et al.*, 1992). For human spermatozoa this is approximately the centre of the sperm head, but for rat spermatozoa, for example, it has been reported to track a point on the proximal region of the midpiece (Yeung *et al.*, 1992). Only one commercial CASA system [Strömberg-Mika Cell Motility Analyser (CMA)] checks each image identified as a sperm head for the presence of a tail (Neuwinger *et al.*, 1990). Other CASA instruments available include Sperm Motility Quantifier (SMQ) (Kaskar *et al.*, 1994); Hobson Sperm Tracker (Green *et al.*, 1995); and SpeedSperm (Le Lannou *et al.*, 1992). Other CASA instruments which analyse both head and flagellar movement have been developed (Stephens and Hoskins, 1990), but only one is commercially available at present (Jeulin *et al.*, 1996). Each of these systems has unique features which are suited to different research applications. For comparison of results between CASA instruments and between laboratories, it has been noted that careful attention must be given to the relative regions of the sperm head tracked for trajectory reconstruction, to the method used for identification of spermatozoa, to the parameter settings used, and to the algorithms used in the calculation of movement characteristics, as different results may be obtained for the same semen sample (Knuth *et al.*, 1987; Mortimer and Mortimer, 1988; Davis and Katz, 1992, 1993; Davis *et al.*, 1992b; Yeung and Nieschlag, 1993; Holt *et al.*, 1994).

Potential errors in CASA measurement

There are some problems with CASA which are common to all instruments. These include the discrimination between debris and spermatozoa, especially clumped or agglutinated spermatozoa, and aspects of trajectory reconstruction. The distinction between spermatozoa and debris is particularly important for semen analysis, when CASA is used for the determination of sperm concentration as well as sperm motility. Identification of debris or non-sperm cells as spermatozoa gives an artificially high sperm concentration and a correspondingly low estimation of the proportion of motile spermatozoa (Vantman *et al.*, 1988). Attempts have been made to address this problem by the use of fluorescent markers to identify spermatozoa and/or the assessment of the concentration of motile spermatozoa in a preparation, rather than determination of the concentration and proportion of motile spermatozoa separately (Zinaman *et al.*, 1996; Farrell *et al.*, 1996b). When the images of clumped or agglutinated spermatozoa are digitized, individual sperm heads cannot be identified and the whole group is identified as a piece of debris and so rejected from analysis on the grounds of size. When this occurs, the opposite effect to that observed with the inclusion of debris in the analysis occurs. That is, the concentration of spermatozoa is underestimated if clumped spermatozoa are not included in the analysis, and the proportion of motile spermatozoa will be overestimated. The presence of non-sperm cells, debris and clumped spermatozoa is uncommon in swim-up or density gradient-selected sperm populations, so this problem is not a significant issue in the kinematic assessment of capacitating sperm populations.

After the sperm head has been defined, the next problem is trajectory reconstruction. When manual trajectory reconstruction is performed, it is simple to follow the same spermatozoon from one image to the next. However, for the automated tracking of a spermatozoon, an area of maximum probability, or 'search radius', must be defined (Schoevaert-Brossault, 1984). This is the circular area around the current image of the sperm head in which the subsequent image is likely to be found. The area used is defined by the maximum velocity value set by the user (calculated as maximum velocity ÷ image sampling frequency) and included in the setup of the CASA instrument. The higher the maximum velocity value, the greater the search radius. With increasing sperm concentrations this can become a problem, as the areas of maximum probability for two or more spermatozoa can overlap, increasing the possibility of sperm experiencing 'actual' or 'perceived' collisions. Some CASA instruments truncate trajectories in which this occurs, with the result

that the sperm track may be lost from the analysis if too few track points had been followed prior to the 'collision'. The alternative approach which has been used is to follow both spermatozoa through the 'collision' and 'reanastomose' the trajectories. It has been shown that this approach can result in significant errors in kinematic values if the wrong track segments are joined (Mortimer and Mortimer, 1989), but one system (CMA) has used vector analysis to reduce the chance of this occurrence.

Clinical relevance of CASA

The potential for CASA in clinical applications is very high. Studies have shown that there are significant relationships between aspects of sperm movement and cervical mucus penetration, as determined using a cervical mucus substitute (Mortimer *et al.*, 1990), and fertilization *in vitro* (Holt *et al.*, 1985; Chan *et al.*, 1989; Sukcharoen *et al.*, 1995a, 1996) and *in vivo* (Barratt *et al.*, 1993; Irvine *et al.*, 1994; Macleod and Irvine, 1995). CASA instruments have also been used for the kinematic analysis of capacitating sperm populations to identify the proportion exhibiting hyperactivated motility (discussed in detail, below). It should be noted, however, that some CASA-based studies of sperm kinematics have considered mean kinematic values for populations, rather than defining kinematic values for the motility pattern(s) of interest and then determining the relevant proportion of spermatozoa in the study population. The latter approach was recommended in a recent Consensus Workshop (ESHRE Andrology Special Interest Group, 1996), since the former approach can interfere with meaningful statistical analysis of results (Gladen *et al.*, 1991).

Kinematic analysis methods

Kinematics are 'time-varying geometric aspects of motion that are distinct from calculations of mass and force' (Drobnis *et al.*, 1988a). Methods for the quantification of aspects of both flagellar and head movement of spermatozoa have been developed. While most clinical emphasis is on kinematic evaluation of sperm head movement, it must be remembered that flagellar movement defines motility, and hence should be considered in the development and evaluation of kinematic descriptions of sperm head movement.

Flagellar movement analysis

The trajectories of free-swimming spermatozoa are dictated by the flagellar beat shape. Thus, different flagellar beat patterns will give rise to different trajectories (Suarez *et al.*, 1983) (Figure 2). Early studies of flagellar movement used hydrodynamic principles to determine the movement properties of 'elements' of a flagellum (e.g. Gray and Hancock, 1955; Gray, 1955, 1958; Denehy, 1975). In these studies, the movement of points on the flagellum relative to a longitudinal axis were studied, and flagellar beat amplitude, wavelength, frequency and wave velocity were determined. Wave velocity was determined by calculation of the phase differences of consecutive points along the flagellum. Alternative methods for flagellar analysis have since been proposed, and these have been used in many of the recent flagellar movement studies. These values are summarized below:

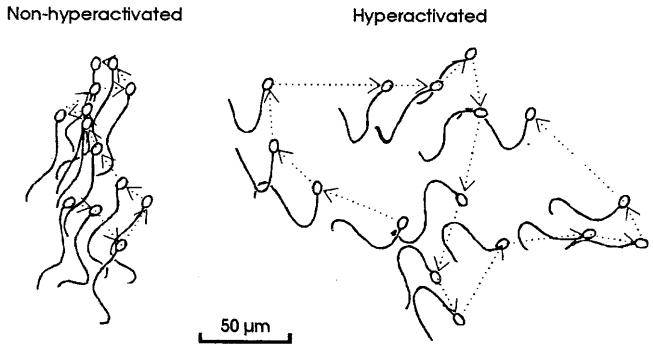

Figure 2. Comparison of hyperactivated and non-hyperactivated sperm tracks, illustrating the influence of flagellar beat pattern upon the sperm head's trajectory.

Wavelength of flagellar beat

This is the distance between consecutive peaks along the flagellum (Katz *et al.*, 1978b; Ishijima *et al.*, 1986). This distance is usually calculated as twice the half wavelength, that is the distance between a peak and trough (Figure 3).

Velocity of flagellar wave propagation

The wave peak is derived using the midpoint method (Goldstein, 1976) (Figure 3), and the distance travelled along the flagellum by the wave peak is determined by measuring the increase in the distance from the neck to the midpoint in successive images (Gibbons and Gibbons, 1980; Serres *et al.*, 1984, 1991; Schoevaert *et al.*, 1988; Aoki *et al.*, 1994).

Flagellar beat frequency

This is determined in a similar fashion to the earlier studies, i.e. calculation of the number of flagellar beats per second (Katz *et al.*, 1978b, 1986; Katz and Overstreet, 1979; Serres *et al.*, 1984, 1991; Burkman, 1984; Ishijima *et al.*, 1986).

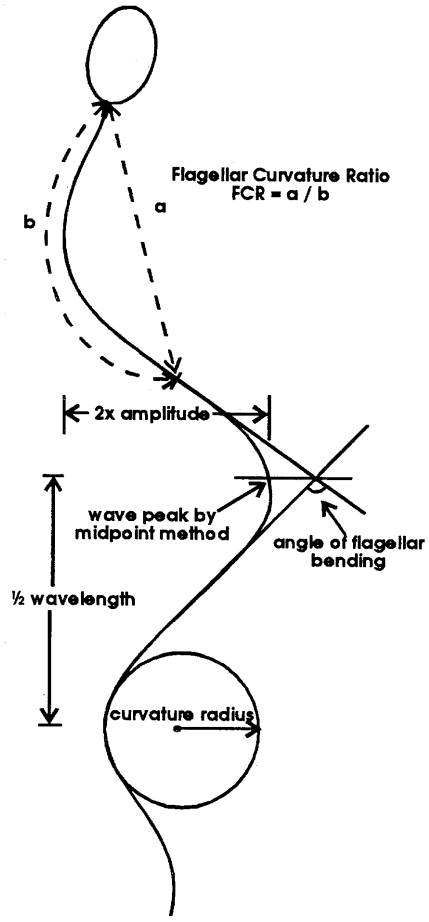

Figure 3. Common methods of flagellar analysis.

Flagellar beat amplitude

This is calculated from the maximum width of the flagellar beat envelope (Burkman, 1984) or as the maximum displacement of the flagellum relative to an arbitrary centre line (Katz and Overstreet, 1979).

Since it is not possible to visualize the entire length of the flagellum, causing problems in the calculation of flagellar amplitude, an alternative measure, the flagellar curvature ratio (FCR) was developed (Suarez *et al.*, 1983). It is determined for the image in which the flagellum reaches its maximum curvature, and is defined as 'the straight-line distance from the head/midpiece junction to the first inflection point of the tail, divided by the curvilinear distance between these two points as measured along the tail' (Suarez *et al.*, 1983) (Figure 3). This ratio is inversely related to the degree of flagellar curvature, with the ratio approaching 1.0 for a straight flagellum, and approaching zero for a tightly curved flagellum. A later study suggested that FCR be expressed as the inverse ratio, with the minimum value of 1.0 for a straight flagellum, and no maximum value, to allow for greater differentiation between the degree of flagellar bending (Katz *et al.*, 1986), but this has not been generally adopted, leading to both ratios being referred to as FCR. To avoid confusion, the FCR results have been discussed in this review in terms of increased or decreased curvature, without consideration of the ratios themselves.

Curvature radius

This is an expression of the amount of flagellar bending, with a low curvature radius indicating a tightly-bent flagellum (Schoevaert *et al.*, 1988). The curvature radius may be estimated by fitting circles of known radius into images of flagellar bends (Drobnis *et al.*, 1988b) (Figure 3).

Angle of flagellar bending

This value is the supplementary angle to that formed by the intersection of the two tangents to the flagellar wave (Goldstein, 1976), and hence will be high for a tightly-bent flagellum (Gibbons and Gibbons, 1980; Serres *et al.*, 1984; Aoki *et al.*, 1994) (Figure 3).

Other flagellar kinematic values are: beat efficiency, the ratio of either head movement velocity or velocity of wave propagation to flagellar beat frequency (Serres *et al.*, 1984, 1991); kinetic efficiency, the ratio of VSL to the product of flagellar beat frequency and sperm length (Katz and Overstreet, 1979); and angular velocity of flagellar wave development, the rate of curvature formation as a function of time (Serres *et al.*, 1984).

Centroid movement analysis

Although earlier studies used the head-midpiece junction as the point of reference on the spermatozoon (e.g. Suarez *et al.*, 1983), the advent of video imaging and CASA has meant that this point is more difficult to identify than it was using manual and semi-automated track analysis methods, and the sperm head centroid is now used. The established kinematic values calculated for human sperm centroid trajectories are outlined below. These values were developed by a number of investigators, and have been referred to by a number of different names. In 1988, two consensus meetings were held (at the American Society of Andrology meeting in Houston, Texas, in March, and the Federation CECOS meeting in Montpellier, France, in April), in which the terminology to be used for kinematic centroid analysis was standardized (Mortimer, 1990).

Velocity

Three velocity values are used commonly for the description of the movement of the human sperm centroid. These are the curvilinear velocity (VCL), the straight-line velocity (VSL) and the average-path velocity (VAP)

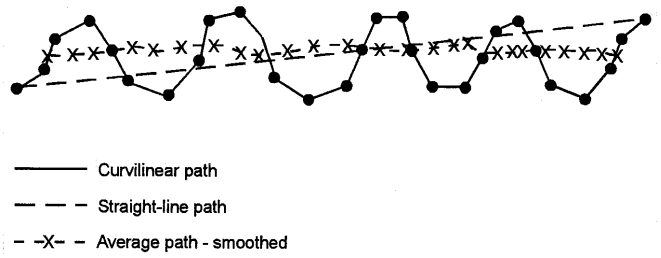

Figure 4. Paths used in the calculation of the velocity of the sperm head. The length of each respective path is corrected for distance/time to give the velocity, e.g. the distance travelled along the curvilinear path/s is the curvilinear velocity (VCL), etc.

(Boyers *et al.*, 1989). Each of these velocities describes a different aspect of the progression of the spermatozoon (Figure 4).

The curvilinear path is a two-dimensional projection of the actual three-dimensional trajectory. Curvilinear velocity is the distance travelled by the spermatozoon along its curvilinear path/s and is calculated by finding the sum of the distances along the trajectory then correcting for time. Previously, VCL has been termed V_c (Katz and Yanagimachi, 1980; Mortimer, 1986; Neill and Olds-Clarke, 1987; Stephens *et al.*, 1988); V_h (sperm head velocity: Suarez *et al.*, 1983; Schoevaert-Brossault, 1984); and V_T (total swimming speed: Katz and Overstreet, 1979).

The VSL is determined by finding the straight-line distance between the first and last points of the trajectory and correcting for time. This value then gives the net space gain within the period. Past terminology for VSL included: V_n (net velocity or net displacement: Tessler and Olds-Clarke, 1985; Neill and Olds-Clarke, 1987; Stephens *et al.*, 1988); Vp (progressive speed or swimming speed: Katz and Overstreet, 1979; Katz and Yanagimachi, 1980; Schoevaert-Brossault, 1984; Ishijima *et al.*, 1986; Mortimer, 1986); and v (forward velocity: Denehy, 1975). Both VCL and VSL can be derived by either manual or mathematical means, i.e. by direct measurement or by finding the sum of distances between points.

The average path velocity gives an indication of the length of the general trajectory of the spermatozoon. It has been referred to previously as V_a (position-averaged velocity: Suarez *et al.*, 1983; Tessler and Olds-Clarke, 1985; Neill and Olds-Clarke, 1987; Stephens *et al.*, 1988). VAP is calculated by finding the length of the average path and correcting for time. The average path may be determined manually or mathematically. The average path may be derived manually by visual interpolation, drawing an average path onto the curvilinear path (Figure 5a). This is the 'gold standard' method for derivation of the average path (Mortimer and Mortimer, 1990). Alternatively, the average path may be derived geometrically, by drawing a line from an apex to the consecutive apex on the other side of the path all the way along the trajectory. The midpoint of each line is found and the average path is the line joining the midpoints (Figure 5b). To determine an average path by purely mathematical means, the average is taken of the track's (x,y) coordinates. The average (x,y) value may be found using rectangular, fixed-point smoothing or by adaptive smoothing. Rectangular smoothing, or Tukey windows, uses the average of a fixed number of (x,y) coordinates to give the 'smoothed' (x,y) coordinates for a point (Davis *et al.*, 1992a). For example, for five-point smoothing, the average is taken of the (x,y) coordinates of the preceding two track points, the point being 'smoothed' and the following two track points. The value obtained is the smoothed value corresponding to the actual track point (point number 3 in this case). When all of the track points have been smoothed, the average path can be constructed by joining all of the smoothed points, then finding the total distance covered by the smoothed path per unit time (Figure 5c). The track points can also be 'weighted' to emphasize the position of the point being smoothed (Davis *et al.*, 1992a). The problem with this method of calculation of the average path is that the points at the ends of the curvilinear path cannot be smoothed, i.e. for five-point rectangular smoothing, five-point-averaged values cannot be calculated for the first and last two track points. To circumvent this, some investigators have 'reflected' the ends of the curvilinear path , thereby apparently extending it, and allowing averaging of the 'true' track points (Katz and Davis, 1987) (Figure 5d); others have used 'window smoothing', progressively altering the number of points included in the running average at each end of the track, with the same first and last track points on both the curvilinear and average paths (Mortimer and Swan, 1995a) (Figure 5c).

The rectangular smoothing method of calculation of the average path is quite satisfactory for regular trajectories, but can be inaccurate for irregular trajectories. For example, if there are a number of track points clustered in one area, the average path will be pulled towards these points, causing it to be longer than the 'true' average path. Similarly, if there are few track points in an area of the track, the distance between the true track points and their smoothed points will be artificially high, leading to inaccuracies in the calculation of the amplitude of lateral head displacement. Also, the amount of smoothing required to determine an accurate average path is sensitive to the image sampling frequency used in the trajectory reconstruction (Davis *et al.*, 1992a). Higher image sampling frequencies result in trajectories with more small deviations than found in tracks reconstructed at lower image sampling frequencies (Mortimer *et al.*, 1988b). It is possible for tracks to be over- or under-smoothed. Over-smoothing

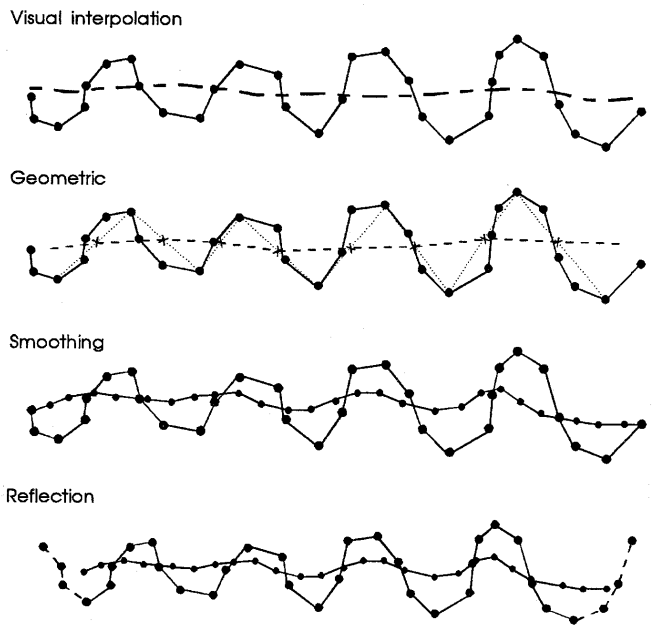

Figure 5. Methods used in the derivation of the average path for calculation of average path velocity (VAP). Methods (**a**) and (**b**) are commonly used in manual and semi-automated trajectory analysis, while methods (**c**) and (**d**), which use mathematical 'smoothing', are commonly used by computer-assisted sperm analysis (CASA) instruments. The difference between methods (**c**) and (**d**) relates to the determination of the smoothed values of the average path at the extremes of the trajectory. The average path is also used in the determination of beat/cross frequency and may be used for the determination of the amplitude of lateral head displacement.

occurs when too many track points are averaged to find the smoothed points. A very straight average path is produced by over-smoothing, since nuances in the trajectory are overrun. Under-smoothing occurs when a low number of track points are used for smoothing, and small deviations in the actual path can influence the average path. The under-smoothed trajectory is longer than the 'true' average path (Davis *et al.*, 1992a). To counter this occurrence, adaptive smoothing programs have been written for use with CASA instruments. Adaptive smoothing takes into account the relative spread of track points, and will increase the degree of smoothing when track points are clustered together, and decrease the degree of smoothing when track points are scattered more widely.

Velocity ratios

To describe the trajectory further, three velocity ratio values have been developed. These are linearity (LIN), a comparison of the straight-line and curvilinear paths, straightness (STR), a comparison of the straight-line and average paths, and wobble (WOB) a comparison of the average and curvilinear paths (Boyers *et al.*, 1989).

Linearity is an expression of the relationship between the two-dimensional projection of the three-dimensional path taken by the spermatozoon (i.e. curvilinear path) and its net space gain, calculated as (VSL/VCL) × 100. A circling trajectory would have low linearity, because the curvilinear path (i.e. the circumference of the circle) would be much higher than the net space gain (i.e. the distance between the first and last points of the trajectory). A high linearity trajectory is one where the curvilinear path has a relatively low amplitude of lateral head displacement (see below), and the general direction of movement is the same as that of the straight-line path. Previously, this value was calculated as the 'progressiveness ratio', with a maximum value of one, rather than 100 as for LIN (Katz and Overstreet, 1979; Katz and Yanagimachi, 1980; Schoevaert-Brossault, 1984; Tessler and Olds-Clarke, 1985; Samuels and van der Horst, 1986; Mortimer, 1986; Neill and Olds-Clarke, 1987; Stephens *et al.*, 1988).

Straightness gives an indication of the relationship between the net space gain and the general trajectory of the spermatozoon, calculated as (VSL/VAP) × 100. A trajectory with evenly spaced track points and a low amplitude would have a high STR value, since the average path would approximate the straight-line path. A circling track would be expected to have a low STR because the average path is the average of the curvilinear path, so the STR would be higher than the LIN, but still low. The previous name for STR was the 'linear index', and it had a maximum value of one (Tessler and Olds-Clarke, 1985; Neill and Olds-Clarke, 1987; Stephens *et al.*, 1988).

Wobble is the expression of the relationship between the average and curvilinear paths, calculated as (VAP/VCL) × 100. WOB would be low for a track with a wide trajectory (high ALH, see below), but high for a circling track, since the curvilinear and average paths would be similar. In the past, WOB has been referred to as the 'curvilinear progressiveness ratio (PR_C)' (Tessler and Olds-Clarke, 1985; Neill and Olds-Clarke, 1987; Stephens *et al.*, 1988).

Amplitude of lateral head displacement

The amplitude of lateral head displacement (ALH) is used as an approximation of the flagellar beat envelope. It is not a true amplitude, in that it does not measure the perpendicular distance between the peak of a wave and the point of inflection of the curve, but rather gives the distance between the 'peak' and 'trough' of the centroid's path. The relationship between ALH and flagellar beat envelope has been studied for seminal human spermatozoa (David *et al.*, 1981; Serres *et al.*, 1984), although no studies have investigated this relationship for capacitating spermatozoa.

The seminal studies showed that ALH was: related to the width of the beat envelope, not related to the amplitude 20 μm along the tail, and related to the evolution of flagellar bending. There are several methods of calculation of ALH, and two types of ALH reported. ALHmax is the maximum ALH found along the trajectory, while ALHmean is the mean of all or some of the ALH values along the trajectory. The ALH can be determined manually, using geometric methods, or by mathematical calculation using the (*x*,*y*) coordinates of each track point (Figure 6). The manual methods are: (i) midpoint method. A line is drawn between two consecutive peaks, and its midpoint identified. The ALH is defined as the distance between the midpoint and the trough between the two peaks (Figure 6a); (ii) perpendicular distance method. Parallel lines are drawn between adjacent peaks and troughs, and the perpendicular distance between the lines is measured to give the ALH (Figure 6b); (iii) perpendicular geometric method. A line is drawn between consecutive peaks and a perpendicular is dropped from the trough between the peaks to the line joining them. The length of the perpendicular is the ALH for that beat. The geometric and midpoint values can be the same for very regular trajectories, but for irregular tracks, the values can be quite different (Mortimer, 1994) (Figure 6c).

To determine the ALH by mathematical methods, risers are used (Boyers *et al.*, 1989). This method requires the derivation of an average path by smoothing. The distance from the true track coordinates to their corresponding smoothed coordinates is termed a riser. A peak is considered to occur when there is a local maximal riser value. The ALH is calculated as double the riser height (Figure 6d). For a regular trajectory, the riser would be a perpendicular from the true point on the curvilinear path to its smoothed point on the average path.

For any of these methods to give the 'true' ALH, a regular trajectory is required. When an irregular trajectory is analysed, the assumptions made in the development of the ALH calculations are not met, and less accurate results may be obtained. This is of particular relevance when analysing the kinematic values of capacitating spermatozoa, since irregular trajectories are not uncommon under these circumstances. The image sampling frequency will also influence the values obtained for ALH, since the profile of a sperm trajectory will change dramatically (Mortimer *et al.*, 1988b). When higher image sampling frequencies are used, the trajectory becomes less smooth, with many small peaks and troughs. This can confound the ALH values determined by riser calculation, as many small 'peaks' will be identified, instead of the true apices which are related to the flagellar movement. Also, it is possible that peaks would be missed using the riser method, if the average path was pulled very close to a peak by virtue of a large number of track points in the area, then the riser value will be smaller than if the average path was not pulled towards the apex. Previously, ALH was referred to as A_h (David *et al.*, 1981; Serres *et al.*, 1984; Schoevaert-Brossault, 1984; Mortimer, 1986).

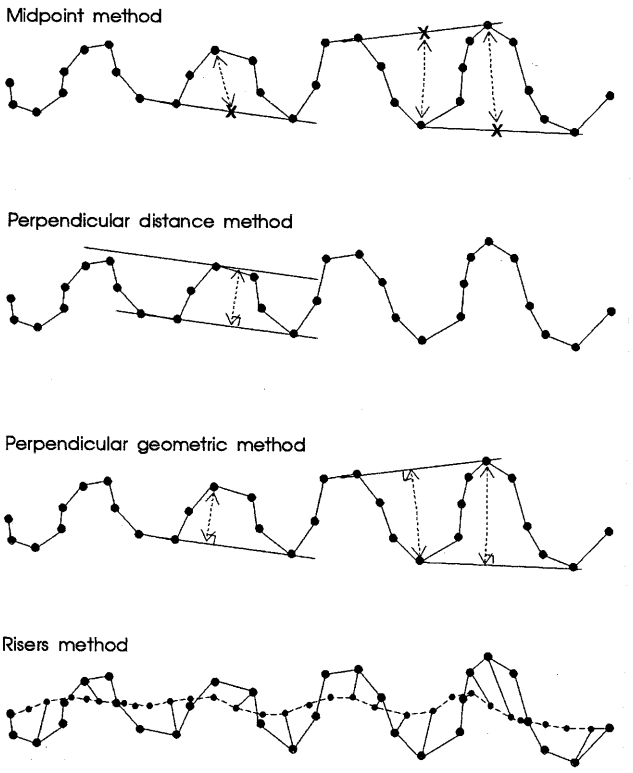

Figure 6. Methods used in the derivation of amplitude of lateral head displacement (ALH). The first three methods (**a**,**b**,**c**) are used commonly in manual trajectory analysis, the fourth (**d**) is used commonly by computer-assisted sperm analysis (CASA) instruments, and is relative to the mathematically 'smoothed' average path. Because, by convention, the ALH is measured as the full width of the centroid trace, the riser values are doubled to give the ALH for each peak.

Beat/cross frequency

Beat/cross frequency (BCF) was developed to give an indication of the flagellar beat frequency, since each apex is the result of a change in flagellar beat, and each time the curvilinear path crosses the average path is the result of a new flagellar beat, assuming that the spermatozoon rotates with each beat initiation. Previously, BCF has been referred to as N_h (Schoevaert-Brossault, 1984) and N (Serres *et al.*, 1984). It is simple to determine BCF manually, since it is just a matter of counting the number of times the curvilinear path crosses the average path per second (Mortimer and Mortimer, 1990). For mathematically-derived results, the BCF is calculated as the number of local maxima in the

risers–1, since it is presumed that the first local maximum precedes the first curvilinear/average path cross (Mortimer and Mortimer, 1990). Since BCF is a frequency, it is sensitive to changes in the image sampling frequency, and has been found to increase with increasing frame rate (Mortimer *et al.*, 1988b). Also, the presence of irregular trajectories can confound the mathematical calculation of BCF in the same way as for ALH - the presence of small differences in the riser values can lead to an apparent apex even when this is just a 'blip' on the trajectory. Another consideration with BCF calculations may be the Nyquist number (Walker, 1988). The Nyquist number is equal to half the image sampling frequency, and to ensure that a representative wave profile is obtained, the frequency of the event measured must be less than the Nyquist number. If the frequency of the measured event is above the Nyquist number, then it is possible that events will be missed, and the profile of the signal processed will be changed, causing aliasing (Walker, 1988). While it has been reported that aliasing is a common problem associated with BCF results, using computer simulated trajectories (Owen and Katz, 1993), the Nyquist number has not been calculated for capacitating human spermatozoa, so it is not known which image sampling frequency would give the most reliable results (Davis and Siemers, 1995).

Other measures

Two derived kinematic values for the determination of hyperactivated motility are Dance (= VCL × ALHmean) and Dancemean (DNCmean = VCL/VSL × ALHmean) (Robertson *et al.*, 1988). Dancemean is determined by some CASA instruments, but since not all calculate ALHmean (e.g. Hamilton Thorne instruments), it is not universally reported.

Mean angular displacement (MAD) is a measure of the trajectory curvature, defined as 'the time average of absolute values of the instantaneous turning angle of the head along its curvilinear trajectory' (Boyers *et al.*, 1989). In essence, it is the mean of all of the angles of direction change along the trajectory. Similar values to MAD are the absolute and algebraic angles (Stephens and Hoskins, 1990). The absolute angle is the mean of all turning angles relative to the average path, irrespective of the direction of movement change, while the algebraic angle is the mean of all turning angles relative to the average path, but positive and negative values are assigned in respect of the direction of movement change. A similar method of angle measurement was described in an earlier study (Schoevaert-Brossault, 1984).

The consideration of the centroid path as a signal rather than as a two-dimensional projection of a three-dimensional trajectory, has led to the development of several new kinematic measures: HAR (frequency of the fundamental harmonic), MAG (magnitude of the fundamental harmonic) and PBW (power-bandwidth of the signal, the area of the fundamental harmonic) (Davis *et al.*, 1992a); also HLO and HHI (lowest and highest head oscillation harmonics), HMX (maximum amplitude head oscillation harmonic), HBS (basic or fundamental head oscillation harmonic, equal to BCF/2) and H_Y (harmonic amplitudes) (Boyers *et al.*, 1989). These are expressions of the track harmonics, computed using fast Fourier transformation, but they have not been included in the 'mainstream' of kinematic values perhaps because their relevance to actual trajectories and to flagellar movement patterns have not yet been determined.

Since the complexity of the centroid path varies between spermatozoa, calculation of the fractal dimension of each trajectory has been suggested. The concept of fractals was developed by Mandelbrot (1983). A curve or trajectory is a fractal if more details are observable the more closely it is inspected. For a sperm trajectory, it has been shown that as the image sampling frequency increases, more track details are observable (Mortimer *et al.*, 1988b), so the basic requirement for it to be a fractal is met. Another requirement for a fractal is that it has a fractal dimension (Mandelbrot, 1983). The fractal dimension is an expression of the degree to which a line fills a plane. If a line is straight, with no deviations, its fractal dimension will be 1.0, since it is only in the first dimension (length). However, if the line is meandering, then it starts to cover more of the plane, and introduces the concept of breadth. Since the line itself still has a single dimension, it cannot be described as a two-dimensional plane, so the fractal dimension of the line is between one and two, since it takes up more space than a single dimension, but cannot have a second dimension since it is only a line. Similarly, if the line begins to fold back on itself, for example, when a trajectory is recursive, a concept of depth can be introduced (even though the one-dimensional line is on the page) allowing the line to have a fractal dimension above two (Katz and George, 1985). Therefore, it may be considered that the fractal dimension of a curve indicates its complexity. A curve with a low fractal dimension would be regular and predictable, whereas a curve with a high fractal dimension would have irregularly spaced changes in direction, apparently at random. The concept of a curve which contains random direction changes has been defined in mathematics as a 'random walk' (Katz, 1988). It has been shown recently that the fractal dimensions of hyperactivated human sperm trajectories are 'random walks', and that it is possible to distinguish between hyperactivated and non-

hyperactivated spermatozoa on the basis of their fractal dimensions (Mortimer *et al.*, 1996)

Sperm function and physiology

The preceding sections have considered the mechanisms involved in the development and maintenance of mammalian sperm motility, and the methods available for the quantification of aspects of sperm movement. Before hyperactivated motility can be considered in detail and in relation to reproduction, however, it is important to understand the physiology of the changes in sperm structure and function which occur during transport through the female reproductive tract prior to fertilization.

Male reproductive tract

Spermatozoa are produced in the testis then transported through the caput and corpus regions of the epididymis and stored in the proximal cauda epididymis. The spermatozoa mature during epididymal transit and storage, acquiring functional competence due to a series of morphological, biochemical and physiological changes. The most obvious maturational change in spermatozoa is the acquisition of the ability to move when they come into contact with seminal plasma or with physiological media. Other changes which occur during epididymal maturation of spermatozoa are alterations to the plasma membrane. These changes include alterations in the distribution of intramembranous particles, increased net negative surface charge, adsorption of antigens, glycoproteins and sialic acid, incorporation of cholesterol, as well as reduction in surface sulphydryl groups (reviewed in Yanagimachi, 1981). The alterations in the sperm plasma membrane occur sequentially along the epididymis, as the chemical composition of epididymal fluids is region-specific (Yanagimachi, 1994). Also in the epididymis, chromatin condensation and stabilization of the spermatozoon occurs, and the acrosome acquires its final shape (reviewed in Mortimer, 1994). At ejaculation, spermatozoa are transported from storage in the caudae epididymides and are mixed with prostatic fluid and seminal vesicle fluid before passage along the penile urethra. The first fraction of the ejaculate contains most of the spermatozoa, suspended in epididymal and prostatic fluid, subsequent fractions contain both prostatic and vesicular fluid (Mortimer, 1994).

Female reproductive tract

The site of deposition of semen differs between mammalian species, with some species having uterine deposition and others pericervical deposition. In the human, spermatozoa are deposited in the vagina, near the cervical os, during intercourse. Spermatozoa must swim through the cervical mucus, traverse the uterus, enter the oviduct and reach its ampullary portion where fertilization occurs. The sperm motility patterns associated with each of these regions differ, and the spermatozoa are also modified ('capacitated', see below) during transport through the female reproductive tract. The motility patterns differ due to differences in the physical and chemical composition of the micro-environments encountered by the spermatozoa.

Cervix

Cervical mucus is a visco-elastic substance in the lumen of the cervix. Goblet cells in the cervical epithelium secrete mucin granules which swell and become transformed, forming a hydrogel matrix of interconnected macromolecules with low viscosity mucus plasma in their interstices (Katz *et al.*, 1989). The interstices have been calculated to be of the order of 1 μm for bovine cervical mucus, which is smaller than a spermatozoon (Tam *et al.*, 1982), prompting the conclusion that spermatozoa must interact directly with the macromolecular portions of the mucus, rather than swimming through the interstices (Overstreet and Katz, 1990). There is considerable variability in the viscosity of cervical mucus, perhaps due to differences in the mucus secretory process; or to changes wrought by the vanguard spermatozoon, the first spermatozoon to penetrate the mucus column (Overstreet and Katz, 1990). Cervical mucus is receptive to spermatozoa in the pre-ovulatory and immediate post-ovulatory period, with mucus secretion under endocrine control (Kremer and Jager, 1988). The proliferation of watery mucus is controlled by oestrogen, which is highest in the immediate pre-ovulatory phase (World Health Organization, 1992). Increased estrogen levels stimulate the luteinizing hormone (LH) surge, which in turn stimulates ovulation. Following ovulation, serum concentrations of oestradiol decline and progesterone concentrations increase with a concomitant shift in cervical mucus secretion and a decline in mucus receptivity to spermatozoa. Therefore, the receptivity of mucus to spermatozoa is highest when there is a chance of fertilization.

During cervical mucus penetration, spermatozoa experience high shear forces from contact with the macromolecular matrix. Flagellar movement modifications in cervical mucus are an increased beat frequency, and a decreased beat efficiency (Katz *et al.*, 1978a), with the development of a 'figure of 8' beat pattern in the distal part of the flagellum (Katz and Overstreet, 1980). The

centroid motility pattern of human seminal spermatozoa which has been correlated with the ability to penetrate cervical mucus is VAP >25μm/s and ALH >4.5 μm, that is spermatozoa must be highly progressively motile with significant lateral head movement (Serres *et al.*, 1984; Aitken *et al.*, 1985; Feneux *et al.*, 1985; Mortimer *et al.*, 1986). Motile spermatozoa with antibodies bound to their surface are unable to penetrate cervical mucus, either *in vivo* or *in vitro* (Hansen and Hjort, 1979; Alexander, 1984; Bronson *et al.*, 1984; Clarke, 1988; Kremer and Jager, 1988). Therefore, cervical mucus is thought to act as a barrier, resulting in the selection of 'competent' spermatozoa for passage into the uterus (Mortimer *et al.*, 1982; Katz *et al.*, 1990). During epididymal maturation and during contact with seminal plasma [although the extent of contact between spermatozoa and seminal plasma *in vivo* has not been determined (Dott *et al.*, 1979)], molecules are adsorbed onto the sperm head plasma membrane. Another postulated effect of cervical mucus is the removal of some of these molecules in one of the steps of 'capacitation', discussed in detail, below.

Uterus

Following their passage through cervical mucus, spermatozoa swim from the internal cervical os into the uterus. There is no conclusive experimental evidence to explain how spermatozoa traverse the uterus, but it has been proposed that the primary transport mechanism is via myometrial contractions. These contractions are random and uncoordinated and probably result in a uniform distribution of spermatozoa throughout the uterus (for reviews see Mortimer, 1983; Yanagimachi, 1994). While a 'rapid transport' mechanism for the transfer of spermatozoa through the reproductive tract in rabbits has been identified (Overstreet and Katz, 1990), the relevance of these observations to humans, and to fertilization, since these spermatozoa are dead or moribund upon reaching the oviduct, has been questioned (Hunter, 1987; Mortimer, 1995).

Oviduct

The spermatozoa must then enter the oviduct via the uterotubal junction. This junction contains mucus (Jansen, 1978; Jansen and Bajpai, 1982) and may act as another barrier to spermatozoa with poor motility. It has been postulated that a sperm reservoir exists in the isthmic portion of the oviduct (Hunter, 1987). Spermatozoa have been observed to bind to the epithelial cells of the isthmus in a number of mammalian species (Hunter, 1996). The isthmus has a direct supply of blood enriched with ovarian hormones via the uterotubal artery (Hunter *et al.*, 1983), so the changes in hormone concentrations associated with the follicular, ovulatory and luteal phases would be experienced by spermatozoa stored there. The implications of this are discussed below. Upon ovulation, it is postulated that a few spermatozoa at a time are released into the ampulla of the oviduct, where fertilization occurs (for review see Hunter, 1996; Harrison, 1996). Contractile movement of the oviduct, beating of the epithelial cilia and sperm motility all contribute to transport of the spermatozoa from the isthmus to the ampulla (Yanagimachi, 1994). The oviductal and ciliary movements direct the fluid in the oviductal lumen. Spermatozoa are constrained to swim against currents (Rothschild, 1962; Roberts, 1970), so if a directed current from the ampulla towards the isthmus occurred, spermatozoa would be orientated towards the ampulla.

Capacitation

Mammalian spermatozoa cannot fertilize oocytes immediately upon ejaculation nor upon retrieval from the epididymis. Despite these spermatozoa having the functional competence conferred by activation, a series of metabolic and physiological changes must occur before they acquire the ability to penetrate the zona pellucida and bind to the oocyte. In nature, these changes occur during transit through the female reproductive tract (Austin, 1951; Chang, 1951), and are collectively termed 'capacitation'. The original definition was the observation that a 'sperm must undergo some form of physiological change or capacitation before it is capable of penetrating the egg' (Austin, 1952). This imprecise definition led to some controversy over the exact nature of capacitation, with opposing views as to whether this meant that a capacitated spermatozoon was in a state in which it could begin the steps involved in zona binding and penetration, or whether it had already undergone them (Chang, 1984). The current, accepted definition for capacitation is the former view, i.e. it is a process which provides otherwise mature spermatozoa 'with the reaction pathways necessary to undergo the exocytotic acrosome reaction in response to an inducing signal from the zona pellucida' (Storey, 1995).

A number of modifications to the sperm plasma membrane occur during epididymal transit, including an increased net negative surface charge due to uptake of sialoglycoproteins, sulphoglycerolipids and sterol sulphates (Langlais and Roberts, 1985); active glycosylation of surface components and incorporation of cholesterol into the plasma membrane (Yanagimachi, 1994). It has been postulated that these changes occur to stabilize the spermatozoon, preventing it from capacitating in the male reproductive tract, since the completion of

capacitation marks the beginning of membrane destabilization events which eventually lead to cell death (Harrison, 1996). Thus, one of the functions of capacitation is the removal of these residues, although capacitation involves more than one step (Yanagimachi, 1981). It is not possible to visualize the capacitation-related changes in the sperm plasma membrane, so it not possible to assay for capacitation itself, although it may be monitored through changes in chlortetracycline binding patterns on the sperm head (Ward and Storey, 1984; Mattioli *et al.*, 1996; Pérez *et al.*, 1996), or by changes in lectin binding sites (Gordon *et al.*, 1975; Singer *et al.*, 1985). The changes which occur during capacitation include (Yanagimachi, 1981; Farooqui, 1983): (i) removal of molecules adsorbed onto or incorporated into the sperm plasma membrane during epididymal transit (Voglmayr and Sawyer, Jr, 1986); (ii) reduction in the net negative charge of the sperm surface, e.g. by removal of sialic acid residues (Langlais and Roberts, 1985); (iii) increased rate of respiration, as measured by increased oxygen uptake and increased glycolytic activity (Hamner and Williams, 1963; Mounib and Chang, 1964); (iv) efflux of cholesterol (Davis *et al.*, 1980; Davis, 1982), which increases the membrane fluidity. Serum albumin mediates cholesterol loss *in vitro* (Go and Wolf, 1985; Ravnik *et al.*, 1993); (v) redistribution of intramembranous particles, leaving sterol-depleted areas over the acrosomal region (guinea pig spermatozoa: Langlais and Roberts, 1985); (vi) changes in lectin binding sites; and in chlortetracycline binding patterns (this is due to changes in Ca^{2+} distribution); (vii) changes in osmotic properties of the sperm plasma membrane and in membrane permeability; and (viii) accumulation of Ca^{2+}.

Capacitation may be reversibly inhibited, indicating that there are no major structural changes associated with it (Bedford, 1972). Capacitation inhibitors include 'decapacitation factor' (Chang, 1957) or cholesterol (Cross, 1996) from seminal plasma; factors removed from the plasma membrane during capacitation, such as sterol sulphates (Langlais and Roberts, 1985) and sialoglycoprotein (Farooqui, 1983); and the female hormones associated with the luteal phase and/or pregnancy, i.e. progesterone, high concentrations of LH or human chorionic gonadotrophin (HCG), or the absence of oestrogens (Farooqui, 1983). The female reproductive tract contains sterol sulphatase and neuraminidase, which digests sialoglycoprotein (Farooqui, 1983; Voglmayr and Sawyer Jr, 1986), so in mammals with uterine insemination, such as rodents, capacitation may begin in the uterus. In mammals with pericervical insemination, e.g. human, capacitation may begin with the removal of some sperm surface components during passage through cervical mucus, due to the high shear forces to which the spermatozoa are exposed (Katz *et al.*, 1989). However, in all mammals sequential exposure of spermatozoa to the uterus and oviduct leads to more efficient capacitation (for review see Bedford, 1972). Acceptance of the existence of a sperm storage site in the isthmus of some mammals has led to the hypothesis that the spermatozoa are held in a quiescent, 'semi-capacitated' state until ovulation, when small numbers of spermatozoa finish capacitation sequentially and are released from storage, providing the oocyte(s) with a succession of competent spermatozoa (for review see Hunter, 1987; Harrison, 1996).

It is also possible to induce capacitation *in vitro*, using appropriate culture media and conditions. Early studies of the induction of sperm capacitation *in vitro* used fairly simple salt solutions supplemented with blood serum or follicular fluid (for review see Yanagimachi, 1994). Many of the studies of the metabolic and ionic requirements for sperm capacitation have been performed *in vitro*. The ability to capacitate spermatozoa *in vitro* is an integral step in successful in-vitro fertilization (IVF) and other assisted reproductive technology procedures. Concomitants of capacitation are hyperactivated motility and the ability of the spermatozoon to undergo the acrosome reaction (Bedford, 1983). Because hyperactivation is caused by changes in the flagellar beat pattern, capacitation probably involves alterations in the physical and chemical properties of the tail plasma membrane as well as the head plasma membrane (Yanagimachi, 1988). Hyperactivated motility is discussed in detail, below.

The acrosome reaction is a pre-requisite for mammalian fertilization. It involves localized fusions of the plasma membrane and the outer acrosomal membrane over the anterior portion of the sperm head. The acrosome reaction of the fertilizing spermatozoon occurs on the surface of the zona pellucida and is generally believed to be stimulated by ZP3, a glycoprotein component of the zona pellucida (reviewed by Wassarman, 1995). Following completion of the acrosome reaction, the spermatozoon penetrates the zona pellucida. Opposing mechanisms of zona penetration have been postulated: either that it is a purely chemical process with the acrosomal enzymes digesting the glycoproteins of the zona pellucida, requiring only moderate flagellar movement (the 'binding–release' hypothesis); or that it is a purely mechanical process reliant upon vigorous flagellar movement to force the sperm head through the zona pellucida (reviewed by Yanagimachi, 1994). In either case, it is important that the fertilizing spermatozoon be tightly bound by the zona pellucida prior to zona penetration as the flagellum is still beating at that time and so otherwise could pull the sperm head free from the zona pellucida (Baltz *et al.*, 1988). After successful

penetration of the zona pellucida the spermatozoon enters the perivitelline space, comes into contact with the oocyte and the post-acrosomal region of the sperm head binds to the oolemma. Flagellar motility ceases at this time, and fusion is initiated between the oolemma and the equatorial segment of the spermatozoon. The whole spermatozoon is then engulfed by the oocyte. The nucleus of the sperm head decondenses to form the male pronucleus. This can then fuse with the female pronucleus which was formed following the resumption of oocyte meiosis triggered by sperm–oolemma contact. The fertilized oocyte is referred to as a zygote, and as an embryo following the first cleavage.

Hyperactivation

Definition and description of hyperactivated motility

Hyperactivated motility was first described in golden hamster spermatozoa undergoing capacitation *in vitro* (Yanagimachi, 1970). The high amplitude flagellar beats and vigorous movement were described as 'activation'. In the same report it was noted that similar movement patterns had been observed *in situ* through the walls of the oviductal ampulla of golden hamsters, indicating a potential physiological role in fertilization. This motility pattern was renamed hyperactivation (Yanagimachi, 1981) to reduce the incidence of confusion with the alternative meaning of activation, whereby immature spermatozoa in the male tract acquire motility upon contact with seminal plasma or culture medium.

Hyperactivation has since been observed *in vitro* for all eutherian spermatozoa studied, including rabbit (Johnson *et al.*, 1981), guinea pig (Yanagimachi and Mahi, 1976), rhesus monkey (Boatman and Bavister, 1984), chimpanzee (Gould *et al.*, 1988), mouse (Fraser, 1977), dolphin (Fleming *et al.*, 1981), bat (Lambert, 1981), dog (Mahi and Yanagimachi, 1978), rat (Shalgi and Phillips, 1988), bull (Singh *et al.*, 1983; Blottner *et al.*, 1989), ram (Cummins, 1982), boar (Blottner *et al.*, 1989), lion and tiger (Blottner *et al.*, 1989), and human (Mortimer *et al.*, 1984; Burkman, 1984); and an equivalent type of motility pattern may exist in marsupial spermatozoa (Taggart, 1994). There are a number of similarities in the hyperactivated movement patterns of mammalian spermatozoa, although interspecies differences have also been discerned. For example, rabbit spermatozoa seem to cycle between hyperactivated and non-hyperactivated motility patterns (Johnson *et al.*, 1981), while guinea pig and hamster spermatozoa have different phases of hyperactivated motility, with periods of high-amplitude whiplash-like flagellar movements interspersed with periods with different flagellar bending patterns (Katz and Yanagimachi, 1980). This was observed through the walls of the ampullary region of the oviduct in in-situ preparations. Hamster spermatozoa have been observed to have a transitional phase of hyperactivation, which is a progressive hyperactivated motility phase in which there is 'moderately acute flagellar bending punctuated periodically by abrupt turning of the head' (Suarez, 1988). Detailed examination of the flagellar beat patterns of hyperactivated rabbit spermatozoa revealed significant differences relative to seminal spermatozoa (Suarez *et al.*, 1983). The hyperactivated spermatozoa recovered from the ampulla were observed to swim in circular trajectories, due to the development of asymmetric flagellar beats within a plane, while the ejaculated spermatozoa had linear trajectories, due to symmetrical flagellar beat patterns. The changes in the motility patterns of hyperactivated spermatozoa have been shown to be due to a change in flagellar bending, rather than to a change in the viscous drag associated with acrosome loss, as hyperactivated motility has been observed in headless guinea pig and hamster spermatozoa (Katz *et al.*, 1978b).

Objective definitions of hyperactivated motility

In the early studies of hyperactivated motility, the flagellar movement patterns were given descriptive terms such as 'high amplitude whiplash' (Fleming *et al.*, 1981), and 'whiplash' (Johnson *et al.*, 1981; Cummins, 1982). Another descriptive term 'dancing' was coined to describe the pattern of sperm head displacement of hyperactivated mouse spermatozoa in timed-exposure photographs (Tessler *et al.*, 1981). Mouse spermatozoa exhibiting a 'crawling' pattern of movement in the uterus and oviduct may also have been hyperactivated (Phillips, 1972). To reduce the confusion that such qualitative descriptions of hyperactivation engendered, attempts were made to quantify the flagellar motility patterns associated with hyperactivation. However, estimations of the wavelengths and amplitudes of flagellar beats were complicated in the analysis of hyperactivated spermatozoa because it was not possible to keep the whole flagellum in focus. An alternative descriptive term for flagellar bending was devised: the flagellar curvature ratio (FCR) (defined above). Hyperactivation was associated with decreased flagellar beat frequency and increased flagellar curvature in the hamster (Katz *et al.*, 1986).

Since the movement of the sperm head–midpiece junction is affected by the flagellar beat angle and degree of flagellar bending, and because CASA instruments analyse head movement rather than flagellar movement, attempts have been made to define hyperactivated motility in terms of

head rather than flagellar movement characteristics. Also, it was determined that the mean sperm head envelope width and the maximum flagellar bend angle could be used to differentiate between hyperactivated and non-hyperactivated mouse spermatozoa with the hyperactivated spermatozoa having higher values. This definition gave a total error of 4% in classification of tracks as hyperactivated or non-hyperactivated (Cooper, 1984). The first definition of hyperactivated motility based solely upon head movement characteristics was developed for mouse spermatozoa (Neill and Olds-Clarke, 1987). For 30 Hz analysis of the trajectory of the head-midpiece junction, hyperactivated spermatozoa were defined as having WOB <56% and VCL >169 μm/s. Later studies of different mouse strains gave different hyperactivation definitions: LIN <34% and VCL >170 μm/s; and LIN <42% and VCL >125 μm/s (Olds-Clarke, 1989). Definitions for hyperactivated motililty have also been developed for rabbit spermatozoa: WOB ≤69% and VCL ≥55 μm/s when analysed at 30 Hz (Young and Bodt, 1994). The common factor in all of these studies of the sperm head kinematics of hyperactivated spermatozoa is the high VCL and ALH and the low linearity, or wobble, of the trajectories. These properties are common to hyperactivated spermatozoa of all the species studied, and reflect the similarity in the flagellar movement patterns which cause hyperactivation. That is, the high amplitude flagellar bends associated with increased flexibility of the proximal midpiece.

Biochemical requirements for hyperactivated motility

Hyperactivation is a flagellar phenomenon, even though it is often measured by changes in the movement of the sperm head. The difference between the flagellar beat patterns of hyperactivated and non-hyperactivated spermatozoa is caused by changes in the degree of bending of the axoneme, as well as changes in the propagation of the flagellar beats (Katz *et al.*, 1978b; Katz *et al.*, 1986; Mortimer *et al.*, 1997). Energy is required for the axoneme to bend, and for the bend to be propagated along the flagellum. The ionic and metabolic requirements for the propulsion of seminal spermatozoa have already been discussed, but the nature of hyperactivated motility suggests that there may be specific requirements which are distinct from those for progressive motility.

It is generally accepted that hyperactivation is a calcium-dependent phenomenon, with evidence accumulated from a number of species, including human (Yanagimachi, 1988, 1994). In hamster spermatozoa, it has been shown that hyperactivated motility was related to the calcium ion concentration in a dose-dependent manner (Yanagimachi, 1982). Similarly, in mouse spermatozoa, hyperactivation did not occur in the absence of Ca^{2+}, but removal of the Ca^{2+} by chelation after the onset of hyperactivated motility did not reduce the proportion of hyperactivated spermatozoa over 3 h, suggesting that a 'priming' by calcium was necessary (Cooper, 1984). Precocious hyperactivation has been induced in mouse spermatozoa by incubation of fresh caudal spermatozoa in medium containing the calcium ionophore A23187 and bovine serum albumin (BSA) (Suarez *et al.*, 1987). Spermatozoa became hyperactivated 2 min after addition of BSA, and retained this motility for 10 min, before reverting to the movement patterns of fresh epididymal spermatozoa. Incubation of mouse spermatozoa in solutions containing lactate has been shown to delay hyperactivation, with the authors presuming this effect to be due to chelation of free calcium ions (Neill and Olds-Clarke, 1988).

Biochemical studies of hamster sperm hyperactivation revealed that capacitation of caudal spermatozoa was accompanied by increased cAMP concentrations, and that this increase preceded the onset of hyperactivated motility (White and Aitken, 1989). When no exogenous calcium was present, the cAMP concentrations were lower than in the control spermatozoa, and the incidence of hyperactivation was reduced. When a calmodulin antagonist was used, hyperactivation declined, even though cAMP concentrations were unaffected, and conversely, addition of a phosphodiesterase inhibitor increased cAMP concentrations to those associated with hyperactivation, but none occurred. The authors concluded that hyperactivated motility involved a rise in cAMP concentrations which was controlled by the influx of exogenous Ca^{2+}. The involvement of calcium and cAMP in the development of hyperactivated motility was implicated in another study, in guinea pig spermatozoa (Mújica *et al.*, 1994). In this study, procaine-treated spermatozoa were found to become hyperactivated in the absence of exogeneous calcium, when glucose was present in the culture medium. The concentrations of both ATP and cAMP were found to be significantly higher in hyperactivated spermatozoa. The authors concluded that hyperactivated motility required 'cAMP-dependent phosphorylations of specific sperm protein(s)', as well as regulation of intracellular calcium at the plasma membrane. Procaine is a local anaesthetic, and these chemicals are known to cause fluidization of membranes. The authors postulated that the removal of the requirement for exogeneous Ca^{2+} in the presence of procaine was due to relocation of the calcium ions within the plasma membrane; however, in consideration of the definite

membrane regions of spermatozoa described above, it is difficult to understand this reasoning. An alternative explanation for this observation may be that the procaine mimicked the action of Ca^{2+}. This was the conclusion in studies of the effect of ethanol upon intact and reactivated demembranated ram and oyster spermatozoa in which it was found that the asymmetrical flagellar beat pattern observed was due to a direct effect of ethanol upon the axoneme, rather than to calcium (Molinia and Swan, 1991; Vishwarath *et al.*, 1992).

Bicarbonate ions are necessary for hyperactivated motility in mouse spermatozoa (Neill and Olds-Clarke, 1987). Incubation of spermatozoa in the absence of HCO_3^- but in the presence of BSA inhibited hyperactivation, but the converse did not. This effect was also observed in hamster spermatozoa (Stauss *et al.*, 1995). In human spermatozoa, hyperactivated motility *in vitro* has been found to require 25 mM bicarbonate (Anderson *et al.*, 1989). Bicarbonate ions are involved in the activation of adenylate cyclase, so these results provide further evidence that hyperactivation is dependent upon increased intracellular cAMP concentrations.

The most convincing evidence for the requirement of calcium ions for hyperactivated motility has come from studies of indo-1 emission patterns in intact hamster spermatozoa (Suarez *et al.*, 1993; Suarez and Dai, 1995). The concentration of calcium ions was increased in the head and midpiece of hyperactivated spermatozoa, and even more so in acrosome-reacted spermatozoa. It was also observed that the concentration of calcium ions was greater in the flagellum during hyperactivated motility (Suarez and Dai, 1995). Also, the concentration of calcium ions oscillated with the formation of each flagellar bend, both principal and reverse bends. The greatest correlation between the frequency of calcium concentration oscillations and flagellar beat frequency was detected in the proximal flagellar midpiece (Suarez *et al.*, 1993). This study showed that calcium ions entered both the head and tail of spermatozoa and oscillated in both regions during flagellar bending.

Progesterone has been shown to stimulate hyperactivated motility in human spermatozoa (Mbizvo *et al.*, 1990b); however, it has been shown that the action of progesterone on spermatozoa is to increase the intracellular calcium concentration (Blackmore *et al.*, 1990; Oehninger *et al.*, 1994), so it possible that this is the manner in which progesterone's effect is mediated. Further evidence for this is in the observations that the progesterone inhibitor mifepristone (RU486) inhibited both human sperm hyperactivation (Yang *et al.*, 1994) and penetration of zona-free hamster oocytes (Yang *et al.*, 1996). However, opposing results were obtained by other investigators (Uhler *et al.*, 1992; Emiliozzi *et al.*, 1996). Progesterone's effect on spermatozoa is non-genomic (Blackmore, 1993; Revelli *et al.*, 1994) and it is thought to act on a membrane receptor for γ-aminobutyric acid (Blackmore *et al.*, 1994; Roldan *et al.*, 1994) which can be activated by other steroidal compounds which do not have 'progestational effects' (Alexander *et al.*, 1996).

In some species, hyperactivation must be stimulated *in vitro* by artificially increasing the intracellular cAMP concentrations. In rhesus spermatozoa, this has been achieved by the addition of caffeine or theophylline, both phosphodiesterase inhibitors, in the presence or absence of dibutyryl cAMP, a membrane-permeable analogue of cAMP (Boatman and Bavister, 1984). It was found that dibutyryl cGMP could not be substituted for dibutyryl cAMP, implicating a rise in cAMP, specifically, for the development of hyperactivated motility. Artifically increasing cAMP concentrations by the addition of caffeine to mouse spermatozoa also increased the proportion exhibiting hyperactivated motility (Fraser, 1979). In human spermatozoa, the addition of pentoxifylline (another phosphodiesterase inhibitor) has been shown to increase the proportion of hyperactivated spermatozoa in a preparation (Kay *et al.*, 1993). The increase in hyperactivation in preparations of frozen–thawed human spermatozoa treated with pentoxifylline has been shown to be a significant predictor of pregnancy in a donor insemination programme (Johnston *et al.*, 1994).

Glucose is also required for hyperactivated motility. It has been found that hyperactivated mouse spermatozoa in solutions containing both D-glucose and lactate had higher velocities than those in solutions containing either substrate alone. Addition of glucose to a calcium-primed system resulted in the immediate onset of hyperactivated motility (Fraser and Quinn, 1981). It was also observed that the medium components which were necessary for fertilization were those required for both hyperactivated motility and acrosomal exocytosis. Another study of the metabolic requirements of mouse sperm hyperactivation also found a specific requirement for D- rather than L-glucose, and that while pyruvate and lactate allowed the maintenance of a non-hyperactivated motility pattern with velocities similar to those achieved with glucose, neither could support hyperactivated motility (Cooper, 1984). These results implicate a specific role for glucose in hyperactivation. Inhibition of glucose metabolism at the phosphohexoseisomerase step of glycolysis also inhibited hyperactivation, supporting the observation that the action of glucose in supporting hyperactivation, as well as

motility itself (Ford and Rees, 1990), is via the glycolytic pathway.

Potassium ions have also been found to be necessary for the development of hyperactivated motility in mouse spermatozoa (Fraser, 1983). Very low or very high concentrations of K^+ resulted in significant decreases in the proportion of hyperactivated spermatozoa, but transfer of these populations into moderate concentrations of K^+ restored hyperactivation levels to those observed in the controls. The similar results to those observed with glucose prompted the proposition that the potassium effect was mediated via glycolysis. Similarly, rabbit spermatozoa recovered from the isthmic portion of the oviduct have been observed to have significantly reduced amplitude of flagellar beats, and increased potassium concentrations were postulated as the cause (Johnson and Overstreet, 1982).

Taurine and hypotaurine and epinephrine have also been found to mediate hyperactivated motility in hamster spermatozoa *in vitro* (Leibfried and Bavister, 1982; Suarez *et al.*, 1984), while pH does not have a significant effect (Yanagimachi, 1970). Low temperatures have been found to inhibit hyperactivation (Mahi and Yanagimachi, 1973), although hyperactivation has been observed in human spermatozoa at room temperature (Mack *et al.*, 1989).

Hyperactivation has been linked with the superoxide anion, both in culture medium (de Lamirande and Gagnon, 1993a,b; de Lamirande *et al.*, 1993) and in semen (de Lamirande and Gagnon, 1993c). The appearance of hyperactivated motility in semen was linked to low superoxide scavenging capacity of the seminal plasma. The action of the superoxide anion was postulated to be direct or indirect activation of an NADPH oxidase in the sperm plasma membrane. The physiological relevance of this observation has not yet been elucidated.

Hamster spermatozoa demembranated with Triton X-100 were observed to express hyperactivated flagellar beat patterns in medium containing ATP, cAMP and Mg^{2+} (Mohri and Yanagimachi, 1980), leading to the conclusion that spermatozoa are intrinsically able to become hyperactivated, and that a mechanism exists to prevent its occurrence before it is required (Yanagimachi, 1981). In another study, hamster spermatozoa recovered from the caput region of the epididymis showed movement patterns similar to those of hyperactivation (although movement was very weak), while spermatozoa recovered from the caudal epididymis exhibited the normal, activated movement pattern (Suarez, 1988). That hyperactivated motility is then (re-)acquired in the female tract in association with capacitation has led to the hypothesis that hyperactivated motility is inhibited during epididymal maturation, perhaps by the oxidation of sulphydryl groups in the flagellum (Suarez and Pollard, 1990). However, observations on the biochemical requirements for hyperactivation have shown that while similar conditions are required for hyperactivation as for capacitation and the acrosome reaction, they are not tightly coupled. It has been shown that transient, precocious hyperactivated motility can be induced by the addition of calcium ionophore then bovine serum albumin to uncapacitated caudal mouse spermatozoa. After a short hyperactivated phase, the spermatozoa reverted to pre-hyperactivated motility patterns and then underwent the capacitation process normally, with both the treatment and control groups expressing hyperactivated motility at the same time (Suarez *et al.*, 1987). Also, the inhibition of hyperactivation observed in the absence of bicarbonate, but not in the absence of BSA, indicated that these processes were not inextricably linked, as capacitation in mouse spermatozoa requires both bicarbonate ions and BSA (Neill and Olds-Clarke, 1987). Further evidence of a distinction between capacitation and hyperactivation for mouse spermatozoa was the observation that hyperactivation could occur in the presence of diluted epididymal fluid, i.e. under decapacitating conditions (Fraser, 1984). It is likely that the processes involved in capacitation and hyperactivation share biochemical and microenvironmental requirements, but this would be expected if both are to occur prior to the acrosome reaction (Suarez and Pollard, 1990) and in similar regions of the female tract *in vivo*.

In-vivo/in-situ studies of sperm hyperactivation

Establishment of the site of the onset of hyperactivation is important in the understanding of its physiological relevance. Such studies have not been possible in the human to date, so all information in this regard has been derived from comparative studies.

Cervical mucus

The high viscosity of the cervical mucus, and the requirement for spermatozoa to be highly progressively motile for successful mucus penetration (discussed above), suggest that hyperactivated motility would not occur in this region. If it did, the spermatozoa would not traverse the cervical mucus successfully, and so would be lost from the female tract.

Uterus and utero-tubal junction (UTJ)

In rabbits, it has been reported that some spermatozoa recovered from the uterus 1.5 h after mating showed

evidence of hyperactivated motility, although the observations were not published in their entirety (Suarez *et al.*, 1983). Hyperactivated flagellar bending has also been observed in both the uterus and isthmus of excised mouse tracts 1–2 h post-coitus (Suarez and Osman, 1987). However, it has been shown in hamsters that a linear pattern of motility is required for passage through the UTJ, as when capacitated (or hyperactivated) and uncapacitated spermatozoa were placed in either uterus, significantly fewer spermatozoa were recovered from the oviducts of the uteri inseminated with capacitated spermatozoa (Shalgi *et al.*, 1992). A similar result was observed in the mouse (Olds-Clarke and Sego, 1992).

Oviduct

Rabbit spermatozoa recovered from the isthmic portion of the oviduct have very weak flagellar movements, but become hyperactivated almost immediately upon dilution in either culture medium (Johnson *et al.*, 1981) or in non-ionic isotonic medium (Burkman *et al.*, 1984). This effect has been attributed to changes in external potassium concentrations (Johnson and Overstreet, 1982), and/or to the presence of a mucus substance in the isthmus which would increase the viscosity of the isthmic fluid (Suarez *et al.*, 1991) The low motility and relatively high concentrations of spermatozoa in the isthmic portion of the oviduct have led to the proposition that this is a storage site for spermatozoa, with ovulation prompting the continuous release of small numbers of capacitated spermatozoa to the site of fertilization, the ampulla of the oviduct (Harrison, 1996; Hunter, 1996). Observations of transilluminated mouse oviducts have shown significantly more free-swimming spermatozoa (with more sharply bent flagella than the bound spermatozoa) in the ampulla than the isthmus, suggesting that hyperactivated motility may have a role in moving the spermatozoa away from the isthmic reservoir (Smith and Yanagimachi, 1991; Demott and Suarez, 1992). Hamster spermatozoa observed in the ampullae of excised oviducts have shown the characteristic 'whiplash' movement of hyperactivation, similar to those observed in in-vitro preparations (Yanagimachi, 1970; Katz and Yanagimachi, 1980). When the motility patterns were assessed in relation to the oviductal epithelium, it was observed that the hyperactivated spermatozoa could swim rapidly along the surfaces of the epithelium, achieving swimming speeds approaching 500 μm/s. When spermatozoa were not closely apposed to the epithelial surfaces, they were observed to 'undulate' with no net space gain, or to 'dart' across the lumen (Katz and Yanagimachi, 1980). Rat spermatozoa have also been observed to exhibit hyperactivated motility shortly after entering the oviductal ampulla of naturally-cycling rats (Shalgi and Phillips, 1988).

Changes in the flagellar beat patterns associated with hyperactivated motility have been observed in a number of species. The most obvious biphasic motility patterns have been observed in rabbit spermatozoa (Cooper *et al.*, 1979; Johnson *et al.*, 1981)which have both progressive and non-progressive phases of hyperactivated motility, both distinct from the movement pattern of non-hyperactivated spermatozoa. Calculation of the power output of hyperactivated spermatozoa has shown no significant difference between the two hyperactivated movement patterns, although both had 20× greater power outputs than non-hyperactivated spermatozoa (Johnson *et al.*, 1981). Analysis of the movement patterns of hamster spermatozoa in the oviduct has also revealed changes in the flagellar beat patterns of hyperactivated spermatozoa, but the authors noted that the differences were not as pronounced as those in rabbit spermatozoa (Katz and Yanagimachi, 1980).

Physiological relevance of hyperactivated motility

The observations that this motility pattern occurs at or near the site of fertilization have led to the development of a number of hypotheses as to the physiological relevance of hyperactivation.

A mechanism to reduce the chance of entrapment in the crypts of the oviduct

Hyperactivated rabbit spermatozoa recovered from the ampulla showed frequent changes in their direction of movement (Suarez *et al.*, 1983). It was proposed that this could stop the loss of spermatozoa from the site of fertilization as well as provide an opportunity for a 'search pattern' for the cumulus; also, that by changing direction often, spermatozoa would be less likely to be trapped in the folds and crypts of the ampullary epithelium compared with straight-swimming non-hyperactivated spermatozoa which would spiral into the crypts and then be unable to escape. In this experiment, ejaculated spermatozoa were observed to glide along glass surfaces without changing direction. Observations of the interaction of human spermatozoa with epithelial cell cultures of both isthmic and ampullary portions of the human oviduct have shown that spermatozoa will attach to the oviductal epithelium (Pacey *et al.*, 1995b). In another study, it was observed that some of the spermatozoa which detached from the endosalpinx appeared to be hyperactivated, prompting the suggestion that this movement pattern was necessary for detachment, but the results were not conclusive, with some

detaching spermatozoa showing non-hyperactivated movement patterns (Pacey *et al.*, 1995a).

Search pattern for the oocyte

Since fertilization occurs in the relatively large volume of the ampulla, a mechanism to ensure contact of spermatozoa with the cumulus oophorus would increase the chance of fertilization. The three-dimensional, tumbling movement pattern of hyperactivated spermatozoa has been proposed as this mechanism, firstly by reducing the chance of wastage of highly selected, and therefore 'good', spermatozoa by slowing their passage to the oviductal fimbria and thence the peritoneal cavity; and secondly by increasing the total area traversed by each spermatozoon, increasing the chance of contact with the cumulus (Katz *et al.*, 1978b; Suarez *et al.*, 1983).

The mechanism by which spermatozoa are retained in one region due to a particular movement pattern induced by the chemistry of the local environment is termed 'chemokinesis' (Cosson, 1990). Chemokinesis is distinct from chemotaxis in that chemotaxis causes the spermatozoa to make directed turns up a concentration gradient (Rothschild, 1962), i.e. there is a 'selective orientation of the sperm in line with the attractant source' (Cosson, 1990). Thus, while chemotaxis involves a change in direction, chemokinesis involves a change in movement pattern. Some recent studies have proposed that human spermatozoa are attracted to the oocyte by chemotaxis, and that the chemotactic agent stimulates hyperactivated motility, thereby causing chemokinesis (Ralt *et al.*, 1994; Cohen-Dayag *et al.*, 1994). However, these studies were only of the effects of follicular fluid, and there was no conclusive evidence for chemotaxis since spermatozoa were not shown to change their direction of movement in response to the stimulus. Also, the assessment of motility patterns was not performed according to recently-published guidelines for the analysis of human sperm hyperactivation (ESHRE Andrology Special Interest Group, 1996; see below), so the identification of chemokinesis due to the development of hyperactivated motility could not be considered to be conclusive. Finally, the method used in these studies has been criticized as not proving that any chemotaxis observed was not secondary to hyperactivation and oviductal movement *in vivo* (Yanagimachi, 1994).

Stirring of fluid

Hyperactivated hamster spermatozoa have been shown to stir 'substantially more fluid' than non-hyperactivated spermatozoa, even though their power outputs were similar (Katz and Dott, 1975). It has been suggested that *in vivo*, the stirring caused by the high amplitude flagellar movement patterns of hyperactivated spermatozoa would increase the homogeneity of the chemical content of the ampullary fluid (Katz *et al.*, 1978b). This would act to maximize the exchange of metabolites and/or stimulatory factors necessary for the high power-output flagellar beat patterns required for cumulus penetration and fertilization (Katz *et al.*, 1989).

Conferring ability to traverse the cumulus matrix

In hamsters, only hyperactivated spermatozoa are able to enter the cumulus oophorus, although the flagellar beat patterns are modified within the cumulus matrix, with a decrease in both beat frequency and flagellar curvature (Suarez *et al.*, 1984; Cummins and Yanagimachi, 1986; Katz *et al.*, 1986). This effect was also noted in solutions of similar viscosity to the cumulus matrix, with a concomitant decrease in the flagellar wavelength and average path velocity. In highly viscous solutions, hyperactivated spermatozoa retained their progressive motility (97 compared with 7% for activated spermatozoa), and developed greater flagellar thrust (Suarez *et al.*, 1991). The change in flagellar movement patterns upon entry into cumulus therefore may be simply a response to the viscosity of the medium, since upon dilution of the viscous solution the spermatozoa returned to the three-dimensional hyperactivated motility pattern (Suarez *et al.*, 1991), and similarly when the cumulus matrix was compressed *in vitro*, expelled spermatozoa were observed to return to the same hyperactivated motility pattern as previously before re-entry into the cumulus (Cummins and Yanagimachi, 1982; Drobnis *et al.*, 1988a). Since fertilization cannot occur *in vivo* if the spermatozoa do not traverse the cumulus matrix, the mechanical advantages conferred by hyperactivated motility would argue strongly for the physiological relevance of this movement pattern. In a study of human spermatozoa incubated with solubilized cumulus fractions, a different movement pattern to that of hyperactivation was also discerned, but this study considered head movement patterns rather than flagellar beat patterns. The cumulus-related motility was characterized by 'very rapid, linear and progressive movement with high (BCF) and low (ALH)' (Tesarik *et al.*, 1990). Similarly, human sperm hyperactivation was induced by cumulus-conditioned medium, but not by granulosa-cell conditioned medium (Fetterolf *et al.*, 1994). The active substance in the development of this movement pattern was not determined, and so further studies are required for its elucidation. It has been determined that mammalian spermatozoa have a protein with hyaluronidase activity (PH-20) present on their plasma

membranes (Gmachl *et al.*, 1993; Gacesa *et al.*, 1994; Overstreet *et al.*, 1995). Purified PH-20 has been found to solubilize cumulus, and spermatozoa with the PH-20 sites blocked by anti-PH-20 antibodies were unable to penetrate cumulus (which has a hyaluronic acid matrix) (Lin *et al.*, 1994). These results suggest that more than just hyperactivated motility is required for penetration of the cumulus oophorus.

Power generation for penetration of the zona

When the spermatozoon contacts the zona pellucida, a simple bond is formed, then tight binding occurs after the completion of the acrosome reaction. The flagellum is still beating at this time, so the strength of the initial bonds must be sufficient to prevent detachment of the spermatozoon from the zona pellucida, while allowing the optimal, tangential, orientation of the sperm head for penetration following the acrosome reaction (Baltz *et al.*, 1988). The passage of the spermatozoon through the zona has been proposed as either a purely chemical process, i.e. by proteolytic digestion by acrosin (Austin, 1975); or alternatively, as a purely mechanical process (Green, 1978). However, it has been noted that the role of hyperactivated motility in zona penetration had not been considered in either of these hypotheses (Green and Purves, 1984).

The maximum force generated by free-swimming spermatozoa has been calculated to be $<3 \times 10^{-4}$ μN, using the minimum amount of suction required to hold a spermatozoon in a micropipette, while the sperm–zona bond is estimated to have a strength of $\sim 4 \times 10^{-4}$ μN (Baltz *et al.*, 1988). These calculations illustrated that free-swimming, non-hyperactivated spermatozoa would be unlikely to generate sufficient force to break the sperm–zona bonds and to penetrate the zona pellucida. Investigations of the power output of hyperactivated spermatozoa have shown significant increases compared with non-hyperactivated spermatozoa in the rabbit (Johnson *et al.*, 1981). In the guinea pig and hamster, the power output increased continuously with the flagellar beat amplitude, accompanied by an increase in progressive swimming speed, although the swimming speed declined at very high beat amplitudes (Katz *et al.*, 1978b). The observed inefficiency of high amplitude flagellar beats in the directional propulsion of free-swimming spermatozoa in simple media, and their calculated ability to provide the maximum thrust to a stationary spermatozoon support the contention of a contribution of hyperactivated motility to zona penetration.

Observation of zona attachment and penetration by hamster spermatozoa has revealed a bimodal sperm motility pattern, with high amplitude, low frequency 'lever' strokes (in which the flagellar bend was not propagated) interspersed with low amplitude, high frequency, propagated, sinusoidal flagellar waves (Drobnis *et al.*, 1988b). This movement pattern was calculated to provide a force of up to 2.7×10^{-2} μN, which is up to two orders of magnitude higher than that calculated using more simple flagellar beat patterns (Baltz *et al.*, 1988). A role for the cumulus matrix in zona penetration has also been suggested, with a reduction in the distal flagellar movement, and an increased beat amplitude in the proximal midpiece observed in spermatozoa attached to cumulus-intact oocytes (Drobnis *et al.*, 1988b). This effect allows the development of higher net forces and torques, thereby conferring an advantage in zona penetration. However, even though zona penetration was higher for cumulus-intact oocytes, it still occurred in cumulus-free oocytes, indicating that sufficient force to penetrate the zona can occur in its absence.

Also, a study using a laser optical trap has shown that different forces are generated by different flagellar movement patterns of human spermatozoa (Westphal *et al.*, 1993). The laser power at which the spermatozoon could escape the optical trap was considered to be proportional to the force generated by flagellar movement. Spermatozoa with linear motility were found to have much lower force than hyperactivated spermatozoa, with spermatozoa showing the cumulus-related motility pattern having even higher relative force than those which were hyperactivated. The power generated by the different flagellar movement patterns can be related to the work the spermatozoon must do in each micro-environment, with more force generated by the movement pattern associated with the highly viscous cumulus region.

Further, direct evidence for the role of hyperactivation in zona penetration has been provided in a recent study in the hamster (Stauss *et al.*, 1995). Following attachment of spermatozoa to the zonae of cumulus-free oocytes, hyperactivation was quenched by the addition of either verapamil, a calcium channel blocker, or cadmium, which inhibits axonemal bending. Only one out of 84 of the test oocytes were penetrated, compared with 25/40 in the control treatment, indicating the importance of flagellar motility, and particularly hyperactivated motility, in the penetration of the zona pellucida. It is therefore unlikely that zona penetration is simply a chemical process of digestion of the zona matrix by enzymes released from the fertilizing spermatozoon during the acrosome reaction. This contention is supported by the recent observation in the mouse that acrosin was not necessary for fertilization (Baba *et al.*, 1994). It is likely that hyperactivation is an

integral part of more than just one of the processes involved in sperm transport through the female tract, and in sperm–egg interactions. Therefore, it may be supposed that failure of hyperactivation would be associated with failure of fertilization, both *in vivo* and *in vitro*.

Human sperm hyperactivation

Much of the work published on human sperm hyperactivation has dealt with the practical aspects of definition of hyperactivated motility in human spermatozoa, and on its analysis and relevance to infertility diagnosis and treatment. In contrast to many of the studies on animal sperm hyperactivation, much of the work that has been published on human sperm hyperactivation has dealt with analysis of head or centroid movement, rather than with flagellar movement patterns. It was considered that hyperactivation may not occur in human spermatozoa, since they did not show a 'typical activated movement pattern' (Yanagimachi, 1981), but a description of changes in human sperm movement patterns following swim-up into synthetic culture medium suggested that this was not the case (Mortimer *et al.*, 1983). This study did not consider flagellar movement, only changes in Vp (now called VSL) and Ah (now called ALH), so hyperactivation could not be identified specifically. The authors also observed that the acquisition of the ''activated' state of motility need not necessarily equate with attaining the fully capacitated state', as has been suggested for other mammalian spermatozoa (see above).

The first detailed description of both flagellar and head movement patterns of hyperactivated human spermatozoa used analysis of 15 Hz video recordings of sperm movement in 200 μm deep chambers (Burkman, 1984). Sperm were prepared by swim-up from a washed pellet into Ham's F-10 medium containing fetal cord serum. Three motility patterns associated with hyperactivation were described, 'thrashing', 'wide amplitude' and 'star-spin'. All of these patterns were characterized by flagellar movements which were distinct from those observed in semen, with rapid, successive flexions of the flagellum with concomitant changes in the plane of the flagellar beat ('thrashing') or with wide flagellar beat amplitudes and moderate flagellar beat frequencies ('wide amplitude' and 'star-spin'). It was observed that spermatozoa exhibiting the star-spin pattern of motility could revert to 'vigorous, progressive swimming' in a manner similar to that observed with rabbit spermatozoa, although no objective data were presented. This observation was repeated in another study in which it was postulated that the distinctive 'whiplash' pattern of hyperactivated flagellar movement could be due to a delay in wave propagation. This hypothesis was not tested, however, as the image sampling frequency (10 Hz) was judged to be too low for meaningful analysis of flagellar wave development (Mortimer *et al.*, 1984). Morales *et al.* (1988) used a higher image sampling frequency for the analysis of capacitating human sperm populations (60 Hz). They observed a subpopulation of spermatozoa with increased proximal flagellar bending and amplitude and decreased flagellar beat frequencies which were presumed to be hyperactivated. However, the authors did not derive centroid movement characteristics for these spermatozoa, as their objective was to compare the movement patterns of spermatozoa with different head morphologies from fertile and infertile donors. Hoshi *et al.* (1988) also examined capacitating human spermatozoa, using a 10 μm deep Makler chamber and a 200 Hz video system to record flagellar movement. They found that the proportion of spermatozoa with particular motility patterns changed with incubation time, with a group of non-progressive spermatozoa with erratic flagellar movement observed initially after swim-up, then a progression through two movement patterns with decreased lateral head movement and more ordered patterns of flagellar beating, to the 'whiplash' pattern of motility which appeared 2–3 h after incubation in Biggers–Whitten–Whittingham (BWW) medium. This pattern was characterized by long wavelength flagellar beats and large lateral displacements of the head. It was observed that there was still a lag time between the onset of this flagellar beat pattern and the penetration of zona-free hamster oocytes, prompting the authors to conclude that 'hyperactivation may occur considerably before the acrosome reaction takes place'. However, again, there was no detailed analysis of the head movement patterns which accompanied the flagellar movement patterns.

Centroid-based definitions of hyperactivation

Unlike animal studies, most of the investigations on human sperm hyperactivation have relied upon analysis of the trajectory of the sperm head, rather than flagellar beat patterns. This has led to some confusion in the literature as to the 'true' hyperactivated pattern of movement of human spermatozoa, since head movement results from flagellar movement rather than defining it. The head trajectories of capacitating human spermatozoa can be roughly placed into three categories, although some authors have subdivided these. The forward progressive or non-hyperactivated trajectories are generally very straight, with very low ALH values and relatively low VCL and relatively high VSL

values. In contrast, progressive hyperactivated tracks have high VCL and ALH with low LIN and moderate VSL values, indicating some net space gain. These tracks can sometimes be observed to describe large circles, due to the asymmetric flagellar beat pattern (Burkman, 1990). The third group are the star-spin or non-progressive hyperactivated tracks. These tracks are similar to the progressive hyperactivated tracks, in that they have high VCL and ALH values and low LIN, but the VSL values are extremely low, indicating a low net space gain, despite a high level of flagellar activity (Robertson *et al.*, 1988; Mortimer and Mortimer, 1990). These differences in centroid trajectories have been the basis for most of the studies of human sperm hyperactivation. Differences in the centroid-based definitions of hyperactivated motility have occurred due to differences in the sperm preparation methods, videomicrography conditions and movement analysis techniques. The published centroid-based definitions for human sperm hyperactivation are discussed in detail below, with a summary presented in Table I.

The first detailed set of centroid-based kinematic definitions of human sperm hyperactivation were published in 1988 (Robertson *et al.*, 1988; Table I). These definitions were derived using a CellSoft CASA instrument, and hyperactivation was defined in terms of VCL, LIN and a derived parameter, Dancemean (DNCmean), defined as (VCL/VSL) × ALHmean. Two types of hyperactivated motility were defined in this study, 'transition' (corresponding to progressive, hyperactivated) and 'star-spin' (corresponding to non-progressive, hyperactivated), with those spermatozoa which did not fit into either of these categories defined as forward progressive, or non-hyperactivated. It was postulated that the 'transition' phase spermatozoa were exhibiting motility patterns intermediate to hyperactivation. Flagellar movement patterns were not considered in this study.

Table I. Kinematic definitions for hyperactivation

Reference	Frame rate	Motility pattern	Kinematic definitions
Robertson *et al.*, 1988	30 Hz	Non-HA	Outside hyperactivation ranges
		Trans	VCL >80; 19 <LIN ≤34; DNCmean ≥17
		Star	VCL> 80 ; LIN≤19; DNCmean ≥17
Mortimer and Mortimer, 1990	30 Hz	Non-HA	VSL ≥40; LIN ≥60; ALHmean <5
		Trans	VCL ≥100; VSL ≥30; LIN <60; STR ≥60; ALHmean ≥5
		Star	VCL ≥100; VSL<30; LIN <60; STR <60; ALHmean ≥5
		All HA	VCL ≥100; LIN <60; ALHmean ≥5
Grunert *et al.*, 1990		All HA	VSL <46.4; VCL >91.5; LIN <33.1; ALH >9.9; Dance >951; DNCmean >35.2
Burkman, 1991	30 Hz	Circling	VCL ≥100; LIN 36–65; ALHmax ≥5; VSL ≥60
		Thrash	VCL ≥100; LIN ≤35; ALHmax ≥8.0; VSL 16–39
		Helical	VCL ≥80; LIN 20–60; ALHmax ≥8.0; VSL 40–59
		Star	VCL ≥80; LIN <20; ALHmax ≥8.0; VSL ≤15
		All HA	VCL ≥100; LIN ≤65; ALHmax ≥7.5
Pilikian *et al.,*1991	25 Hz	All HA	VCL >80; LIN <60; ALHmax ≥5.0
Zhu *et al.*, 1994	25 Hz	All HA	VCL ≥90; ALH ≥5.0; LIN ≤80
Griveau and Le Lannou, 1994	40 Hz	NonHA	VCL <100
		Trans.	VCL >100; LIN >40; ALH >4.5
		Star	VCL >100; LIN <40; ALH >4.5
Sukcharoen *et al.*, 1995a	25 Hz	Star	VCL >90; LIN <20; DNCmean >45.8
		All HA	VCL >80; ALHmax ≥6; DNCmean >14.6
Green *et al.*1995	25 Hz	All HA	VCL >70; ALH >7.5; LIN <30
Mortimer and Swan, 1995a	60 Hz	All HA	VCL ≥180; LIN ≤45; WOB <50; ALHmax >10.0
Farrell *et al.*, 1996a	60 Hz	All HA	STR <30; LIN <15

VCL = curvilinear velocity; VSL = straight-line velocity; LIN = linearity; STR = straightness; ALH = amplitude of lateral head displacement; DNCmean = dance mean; ALHmean = mean ALH; ALHmax = maximum ALH; WOB = wobble.
Units: VCL,VSL: μm/s; LIN, STR: %; ALH, DNCmean: μm.
Non-HA = non-hyperactivated; Trans = transition; Star = star-spin; All HA = all hyperactivated

The next set of centroid-based definitions of hyperactivated motility were published in 1990 (Mortimer and Mortimer, 1990; Table I). As for the previous study, three movement patterns were defined, however this study used a first principles approach in the definition of hyperactivation, with observation, but not analysis, of the flagellar movement patterns. To differentiate between hyperactivated and non-hyperactivated tracks, a combination of VCL, LIN and ALHmean values were used, while the subclassification of hyperactivated tracks into transition and star-spin categories used VCL, VSL, LIN, STR and ALHmean. The use of a first-principles approach meant that there was no bias in the results due to a particular CASA instrument, as the 'gold-standard' methods were used (except for the derivation of the average path and ALH values of the star-spin tracks).

The other centroid-based kinematic definitions for human sperm hyperactivation which considered flagellar movement were those of Burkman (1991), Zhu *et al.* (1994) and Sukcharoen *et al.* (1995a) (Table I). Burkman (1991) observed both head and flagellar movement and defined four categories of hyperactivated movement: circling, thrash, helical and star-spin. These categories were defined in terms of VCL, LIN, ALHmax and VSL, with 'all hyperactivated' defined using VCL, LIN and ALHmax only. The ability of a single spermatozoon to exhibit a variety of movement patterns was observed with six different movement patterns observed over a 4 s period, but this was not extended into a full study. The kinematic definitions were derived using a CASA instrument (Hamilton-Thorn 2030 v7.0), and the 'all hyperactivated' definition has been used by many researchers subsequently. Zhu *et al.* (1994) and Sukcharoen *et al.* (1995a) derived their centroid-based definitions of hyperactivation using 25, rather than 30 Hz, trajectories. In both cases, the flagellar movement patterns described by Robertson *et al.*(1988) and Mortimer and Mortimer (1990) were used to categorize the cells. Using their kinematic definitions of hyperactivated motility, Sukcharoen *et al.*, (1995a) determined that the proportion of hyperactivated spermatozoa after 3 h incubation could account for up to 50% of the variance in fertilization rates in a human IVF programme. These results indicated a likely link between hyperactivation and fertilization in humans, as was observed in animal studies.

The effect of the image sampling frequency upon the classification of spermatozoa as hyperactivated has been investigated by analysis of the same sperm preparations by different CASA instruments, although not by a first-principles approach. It was determined that 30 Hz kinematic definitons for hyperactivation could not be applied to trajectories reconstructed at 60 Hz, as different sperm subpopulations were identified (Morris *et al.*, 1996). These results emphasized the importance of determining the image sampling frequency used for hyperactivation analysis, and then using definitions appropriate for that frequency.

Sperm preparation and culture conditions

As noted earlier, much of the published work on the evolution of hyperactivated motility in human spermatozoa has dealt with aspects of preparation and culture of capacitating sperm populations, and there are several which appear to be critical. The method used to isolate the sperm population is important, since if care is not taken it is possible to promote the production of reactive oxygen species ('free radicals') which can irreversibly damage spermatozoa by causing plasma membrane lipid peroxidation (Aitken and Clarkson, 1988; Mortimer, 1991). The sperm preparation methods which reduce the risk of free radical production are centrifugation through a density gradient medium, or direct swim-up from semen (reviewed by Mortimer, 1994). Because of the risk of artefacts caused by the production of free oxygen radicals, studies involving sperm preparation by centrifugal sperm washing must be viewed with caution since the pathway by which free radicals affect sperm movement is not clear. A further confounding aspect to the direct comparison of studies on human sperm hyperactivation has been that, in the past, some studies did not harvest the motile sperm population after swim-up but left it over the washed pellet and only sampled aliquots from the upper layer at each time point (e.g. Mack *et al.*, 1988; Robertson *et al.*, 1988), or alternatively, resuspended the entire washed pellet, with no selection of the motile spermatozoa (Centola *et al.*, 1995). In other studies, the supernatant was harvested into fresh tubes at the end of the swim-up incubation, allowing re-mixing of the entire suspension before sampling at each time point (e.g. Mortimer *et al.*, 1984; Hoshi *et al.*, 1988; Mbizvo *et al.*, 1990a). Clearly, the latter approach is preferable to preclude detrimental influences from the pelleted spermatozoa in the lower layer, and to ensure that a sample is taken of the entire swim-up population, not just the proportion that happened to be in the upper portion of the swim-up preparation tube at the time of sampling. Also, it is difficult to determine the physiological relevance of studies which use an unselected sperm population, since motility is an important mechanism for sperm selection within the female tract (discussed above).

The culture media that have been used most commonly for sperm hyperactivation studies are those which are known to support capacitation and IVF, media such as BWW (Mortimer *et al.*, 1984; Hoshi *et al.*, 1988; Morales *et al.*, 1988), Earle's balanced salt solution (EBSS) (Zhu *et al.*, 1992; Green *et al.*, 1995; Sukcharoen *et al.*, 1995b), T6 (Mortimer and Mortimer, 1990), Ham's F10 (Burkman, 1984; Mack *et al.*, 1989; Hurowitz *et al.*, 1995), Quinn's HTF (Pang *et al.*, 1993; Rose and Scott, 1994; Swanson *et al.*, 1995) and Ménézo's B2 (Mortimer *et al.*, 1984; Westphal *et al.*, 1993; Griveau and Le Lannou, 1994). Although some hyperactivated motility has been reported in all of these media, it is still unclear exactly which, if any, components apart from HCO_3^-, Ca^{2+} and glucose are necessary; for example, some animal studies have indicated that increased pH and osmolarity may promote hyperactivation (for review see Yanagimachi, 1994). In a preliminary study, it was found that the incidence of human sperm hyperactivation increased with increasing calcium concentrations (up to 5 mM) and with increasing bicarbonate concentrations (up to 25 mM). The presence of HEPES reduced the incidence of hyperactivated motility, even when 30 mM bicarbonate was also present (Anderson *et al.*, 1989). A cautionary note has recently been raised regarding the use of Ham's F-10 as a culture medium, particularly when used with centrifugation of diluted semen, as it contains iron, and therefore promotes higher free radical production than other culture media (Gomez and Aitken, 1996).

It appears that the requirement for protein supplementation in the culture medium used for the study of hyperactivation is not so much for the development of hyperactivated motility as for the prevention of the 'sticking-to-glass' phenomenon. Spermatozoa which are stuck to glass by their tails may appear to be hyperactivated because of thrashing movements of the head with no net space gain, leading to artificially high estimations of the proportion of apparently hyperactivated spermatozoa in a preparation. Initially, there was no general agreement as to the minimum protein concentration which should be used, with published methods having used: 3.0, 3.45, 4.6, 10.0 and 30.0 mg human serum albumin (HSA)/ml; 5.0 and 7.5% fetal cord serum; 3.0 mg BSA/ml and 3.8, 7.5 and 15.0% maternal serum. In a study of the relative effects of protein concentration upon hyperactivated motility it was found that human sperm hyperactivation was maximal in 0.3% HSA (Mack *et al.*, 1989). However, the authors did not exclude the possibility of the inclusion in the kinematic analysis of spermatozoa which were stuck to the glass surfaces.

Analysis conditions

While most studies have found that a temperature of 37°C is critical to the development of mammalian sperm hyperactivation (Mahi and Yanagimachi, 1973), another has suggested that room temperature promotes higher levels of hyperactivation and attributed this to a possible temperature-dependent calcium flux, although investigations by these authors were subsequently made at 37°C (Mack *et al.*, 1989).

The most commonly used chamber depths for the analysis of human sperm hyperactivation have been 10 μm (Makler chamber; e.g Hoshi *et al.*, 1988; Chan *et al.*, 1990), 20 μm (Microcells or Cell-VU chambers: Lewis *et al.*, 1994), ~32 μm (Chartpak chambers: Mack *et al.*, 1988; Robertson *et al.*, 1988), 50 μm (Microcells, or Microslide flat glass capillary tubes: e.g. Le Lannou *et al.*, 1992; Lewis *et al.*, 1994), 100 μm (haemocytometers and Microslides: e.g. Le Lannou *et al.*, 1992; Sukcharoen *et al.*, 1995a) and 200 μm (Microslides: Burkman, 1984; Mortimer *et al.*, 1984; Yang *et al.*, 1994). It is believed that the shallower preparations (20–30 μm) may constrain flagellar movement sufficiently to prevent the development of star-spin hyperactivated motility while allowing transitional hyperactivated motility, while the very shallow chambers (i.e. 10 μm) may prevent the expression of hyperactivated motility almost completely (Grunert *et al.*, 1990; Le Lannou *et al.*, 1992).

Guidelines for the study of human sperm hyperactivation

Because hyperactivation is a flagellar phenomenon, the studies which have considered flagellar movement patterns in the definition of tracks as hyperactivated or non-hyperactivated (Mortimer and Mortimer, 1990; Burkman, 1991; Zhu *et al.*, 1994) should be considered to take precedence over those that only considered centroid movement patterns (e.g. Robertson *et al.*, 1988; Green *et al.*, 1995). One of the major problems in the study of human sperm hyperactivation has been the confusion over what is meant by 'hyperactivation', and much of this confusion has probably arisen because of the paucity of studies which have considered both flagellar and centroid movement, with only those spermatozoa showing a high amplitude flagellar beat pattern being included in the 'hyperactivated' group for centroid analysis. Another source of confusion has been that investigators have applied the hyperactivation definitions in studies which have used different image sampling frequencies for CASA, and/or different sperm preparation and culture conditions

from those used in the original analysis. In an attempt to standardize the study of human sperm hyperactivation, two consensus documents have been published recently in which experimental guidelines have been set down (Mortimer *et al.*, 1995; ESHRE Andrology Special Interest Group, 1996). In the recommendations, which cover the sperm preparation method, culture and analysis conditions, it has been suggested that 'some published studies may, in retrospect ... be considered flawed' (ESHRE Andrology Special Interest Group, 1996), if the methods used were found to have been sub-optimal or even inappropriate for sperm preparation and analysis (i.e. different to those in the consensus guidelines). The recommendations require that: (i) the spermatozoa should not be isolated from liquefied semen in a way that will adversely affect sperm function. Hence selection of motile spermatozoa by density gradient centrifugation, or by direct swim-up from semen would be the preferred methods (Mortimer, 1991; World Health Organization, 1992); (ii) the culture medium used should be capable of supporting capacitation *in vitro*, and must contain at least 25 mM HCO_3^- and mM quantities of calcium and glucose, known to be necessary for the development of hyperactivated motility in mouse spermatozoa (Fraser and Quinn, 1981) and human spermatozoa (Anderson *et al.*, 1989); (iii) for human spermatozoa, analyses must be performed at 37°C; (iv) a minimum chamber depth of 30 μm is essential, because it has been shown that shallower chambers constrain flagellar movement (Le Lannou *et al.*, 1992). Deeper chambers would be less likely to constrain flagellar movement, but it is more difficult to keep the spermatozoon in focus for the time required to perform the analysis; (v) both the image sampling frequency and the smoothing algorithms used by the CASA instrument must be taken into account, with validation of the definitions to be used for hyperactivation necessary for each CASA instrument.

In the light of these guidelines, there are very few studies which can be considered, in retrospect, to be 'acceptable' relative to the experimental procedures used. Several of the studies in which hyperactivation definitions were derived (Robertson *et al.*, 1988; Mortimer and Mortimer, 1990; Burkman, 1991; Green *et al.*, 1995; Mortimer and Swan, 1995a) used serum as the protein supplement, with albumin concentrations ≤4.5 mg HSA/ml. Although albumin is known to be necessary for spermatozoa to undergo capacitation, much of the rationale behind the concentration of albumin suggested in the guidelines was to ensure that the sticking-to-glass phenomenon was avoided, as if the sperm tails are stuck to the microscope slide, the head movements can mimic those of a hyperactivated spermatozoon. However, when serum, rather than just albumin is used, there are other molecules present, such as lipids, which also reduce the possibility of sticking-to-glass. Therefore, while these studies do not meet current requirements, the use of serum and collodion coating of glass surfaces (Chapeau and Gagnon, 1987), would have minimized the risk of the sticking-to-glass phenomenon (e.g. Mortimer and Mortimer, 1990). Also, in three of those studies (Mortimer and Mortimer, 1990; Burkman, 1991; Mortimer and Swan, 1995a), only free-swimming spermatozoa were studied, and flagellar movement was considered, although not measured in detail, in the classification of spermatozoa as hyperactivated or non-hyperactivated. Other studies in this group (Table I) used inappropriate sperm preparation methods (Pilikian *et al.*, 1991), or used chambers which were too shallow for hyperactivation analysis (e.g. Zhu *et al.*, 1994; Farrell *et al.*, 1996a).

In summary, the analysis and preparation methods that have been used in past studies of human sperm hyperactivation have proved to be suboptimal in most cases when subjected to retrospective assessment. It is clear that the information that is available at present in regard to the definition of hyperactivated motility of human spermatozoa are is robust, and many studies have used definitions which were derived using different preparation and analysis methods. With the current development of a new generation of CASA instruments which use higher image sampling frequencies for image acquisition, and the new guidelines for the assessment of human sperm hyperactivation, it would seem an appropriate juncture to define human sperm hyperactivation in a manner which considers all of the current guidelines, as well as the new, higher sampling frequencies. Before further work on human sperm hyperactivation is embarked upon, it would seem prudent to ensure that the results obtained would be 'transportable' between laboratories. The need for the development of a robust definition for hyperactivation has been noted by several authors, resulting in the consensus meetings. The development of a robust definition for hyperactivation would also allow meaningful clinical studies to be made, giving information which could then be used in the assessment of the prognostic ability of hyperactivation in a sperm function test. Since hyperactivation is a flagellar phenomenon, it is also important that any new centroid-based definitions be related to particular features of flagellar movement, to reduce the risk of artefacts.

Research on hyperactivated motility in human spermatozoa

The studies of hyperactivated motility of human spermatozoa can be roughly divided into three groups: attempts to characterize the motility pattern(s) to allow

investigation of hyperactivated motility in relation to physiological events, such as capacitation and fertilization; prospective analyses of the ability of hyperactivation to predict IVF or pregnancy outcome (described in Mortimer, 1997); and analysis of the biochemistry of capacitation and fertilization, using hyperactivation as a marker (e.g. Aanesen *et al.*, 1995). While these studies have clear relevance to both science and medicine, it is obvious that without robust definitions of hyperactivation any conclusions drawn from these studies may not be universally applicable, i.e. independent of the kinematic analysis method. Thus, before these studies can be made with confidence, basic studies of the movement patterns of hyperactivated human spermatozoa must be made. These studies would include the development of centroid-based kinematic definitions of hyperactivation which reflect defined aspects of flagellar movement.

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