METHODS OF MEASURING SWIMMING SPEED OF SPERMATOZOA

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Summary. Three basic approaches for determining the mean swimming speed of a suspension of microorganisms were compared, using bull and ram spermatozoa. Number fluctuation counting was performed automatically on a Quantimet 720 image analysing computer, the mean speed being obtained using ‘probability after’ statistics. The other two approaches were photomicrographic: number flux counting was performed on single photomicrographs; on the same photomicrographs, the mean speed was estimated from measurement of ‘whole’ and ‘half’ track lengths. These results were compared with each other and with the Quantimet results. The ‘probability after’ method was also compared, on additional samples, with cine-photomicrographic tracking. The mean speeds predicted by the ‘probability after’ method compared favourably with the other methods (range 68 $\mu\text{m}/\text{sec}$ to 162 $\mu\text{m}/\text{sec}$). The results also suggested that, on single photomicrographs, measurement of ‘half’ track lengths or number flux counting were generally preferable to measurement of whole track lengths.

INTRODUCTION

There are two basic approaches for estimating the mean swimming speed of a sperm suspension: (1) the speeds of a statistically significant number of spermatozoa can be individually measured and the mean taken, or (2) a more gross property of the entire suspension can be measured, and translated into mean speed by application of kinetic theory and ‘probability after’ statistics. The first approach has the advantage of always providing information about the entire distribution of speeds as well as the mean. Its most common application to date has been with cine or single time-exposure photomicrography, both rather time-consuming procedures. A notable exception has been the approach of Harvey (1960) in which spermatozoa were tracked visually with the aid of a grid. Methods based on this approach are restricted to relatively low concentrations so that individual tracks are sufficiently distinguishable. We have in-

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vestigated the bias against fast swimmers in this method and describe a way of reducing it in single time-exposure photomicrography.

A different tracking approach is the photoelectric method (Rikmenspoel, 1957; van Duijn & Rikmenspoel, 1960), results from which compared well with results from cine-microphotography. Unfortunately, relatively high dilutions (at least $\times 40$ for bull semen) are required in an optically very clear diluent (such as clarified egg yolk-citrate), and simultaneous recording from several apertures reduces measurement time to roughly 4 min.

A relatively simple statistical approach to the measurement of mean swimming speed itself is number flux counting. This method was originally proposed by Baker *et al.* (1957), and was properly formulated and checked for very dilute suspensions of a protozoan by Ojakian & Katz (1973). The concentration of spermatozoa again had to be quite low.

Another statistical approach makes use of the 'probability after effect' (von Smoluchowski, 1916; Chandrasekhar, 1943) in the fluctuations in the number of organisms that occupy a given area or volume at discrete time intervals. The applicability of the 'probability after' effect, which also requires randomness, in determining mean swimming speed of a population of spermatozoa was suggested by Rothschild (1953), who applied it to suspensions of bull spermatozoa using cine-photomicrography, a laborious procedure. In the study reported below this approach has been used and a means has been developed of accurately applying it automatically by use of an image analysing computer.

MATERIALS AND METHODS

Ejaculated bull and ram semen was collected with an artificial vagina. All samples were used within 8 hr of collection. A dilution was performed with dialysed skim milk (Harrison & White, 1972), or phosphate-buffered saline; 35.5 mM-glucose was sometimes added. The temperature was maintained at either 30°C or 37°C and the rate of dilution was varied to provide a range of surface densities and swimming speeds over which to study the methods of measurement.

It is optically convenient (though not computationally necessary) to utilize a thin slide preparation. Accordingly, a 5 μ l droplet of sperm suspension was placed on a preheated slide, and covered with a 1½ grade coverslip (22 $\times$ 22 mm). The resulting suspension was approximately 10 μ m deep, thus constraining the spermatozoa to move in effectively two dimensions only. The slide was placed on the controlled heated stage of the microscope; and 2 min allowed for equilibrium to be attained before commencement of measurements.

Number fluctuation counting

If the distribution of swimming velocities in a suspension of microorganisms is spatially and temporally random, then information about fluctuations in the numbers of organisms in a given area or areas can be used to determine the mean swimming speed of the suspension. The applicability of this 'probability after' approach is clearly influenced by the presence of non-motile spermatozoa

in a suspension, and also by departures from normal movement such as 'bent tail' and circular swimming. Any assessment of the approach must account for these effects.

The image analysing computer (Quantimet 720: Imanco Systems, Melbourn, Cambridgeshire) scans the field of a microscope using phase-contrast optics, and is capable of counting the number of spermatozoa in a specified rectangular area of the field at specified successive instants in time. Suitable adjustment of the sensitivity of the computer allows for counting either the total number of spermatozoa or the number of immotile spermatozoa. The counts of the total number of spermatozoa were displayed in digital form on the television screen of the Quantimet, and either spoken into a tape recorder or punched directly into a small digital computer by an observer.

In most experiments, we programmed the Quantimet to perform counts in a square of side = $200\text{ }\mu\text{m}$ (l) in the suspension. The microscopic magnification was $\times 63$ so that this square represented approximately $1/9$ of the total field scanned by the Quantimet. The fixed time interval between counts was 1.466 sec (t). Approximately 100 counts were made in each test, thereby ensuring a coefficient of variation generally less than 30% (see Equations 1 and 2). Counts were made of the instantaneous total number of spermatozoa in the square, and the instantaneous number of motile spermatozoa was approximated by subtracting an effective mean number of motionless spermatozoa for the run. This latter number was obtained by performing automatic counts immediately before and after the run, and combining them with visual counts made on the Quantimet television screen during the run. Since the number of motionless spermatozoa in the square itself fluctuated during the run (immotile spermatozoa being pushed into and out of the square by motile ones), an error was thus introduced into the procedure. Strictly speaking the area occupied by the immotile spermatozoa should be subtracted from the total area of the square. The slight alterations in predicted mean speed thus introduced were generally insignificant.

According to Rothschild's basic derivation of the 'probability after' method, the mean speed of the suspension is computed from the formula

$$\bar{v} = -\frac{\pi l}{4t} \cdot \log_e \left(1 - \frac{\bar{\delta}^2}{2\bar{n}} \right) \quad (1)$$

in which l is the side of a square in the field within which the counts are made, t is the time interval between successive counts, $\bar{n}$ is the mean number of spermatozoa in the square based on all counts, and $\bar{\delta}^2$ is the mean square difference of successive counts. Equation 1 properly applies only to a suspension of randomly moving organisms enjoying 100% motility. The presence of numbers of immotile organisms introduces a non-random component into the movements of the suspension as a whole with a consequent error in computations based on total numbers of spermatozoa. If it can be assumed that the immotile spermatozoa do not disrupt any randomness inherent in the movement of the motile cells, then Equation 1 determines the mean speed of such moving cells, provided counts are made of numbers of motile spermatozoa only. The standard error, S , associated with Equation 1 is obtained from:

$$S^2 = \frac{\bar{V}^2}{k(x \cdot \log_e x)^2} \left[\frac{(1-x)^2(3+x)}{1+x} + \frac{x(1-x)}{\bar{n}} \right] \quad (2)$$

where $x = \exp\left(-\frac{4\bar{V}t}{\pi l}\right)$, the correlation of successive counts, and k is the total number of counts. The same considerations of total versus active spermatozoa as above apply here.

The expression for the correlation can be used to formulate a test of temporal suspension randomness in the counting square, the notion being that in such a case the numbers in that square fluctuate in a Poisson-wise way if counted at uncorrelated instants in time. The χ^2 statistics based on this hypothesis is then simply:

$$\chi^2 = \frac{1}{\bar{n}} \sum_{i>1}^{k'} (n_i - \bar{n}')^2 \quad (3)$$

where k' is the number of counts made at intervals t' such that x is small, say 0.05; $\bar{n}'$ is the mean of such counts.

Photomicrographic tracking

Cine and single time-exposure photomicrography were employed to determine sperm swimming speeds. Cine films were taken with a 16 mm Bolex Paillard camera operating at 24 frames/second. Continuous quartz iodide illumination was applied through a neutral filter to phase-contrast optics. The film was Ilford FP4. The developed films were projected frame-by-frame on a Vanguard cine-film analyser, and the track of each measured spermatozoon was determined by marking the successive positions of the junction between the head and midpiece for approximately 2 sec. In some cases the head pitched significantly from side to side. When the pitching constituted only a small lateral component to primarily forward motion, the effective track was taken as projecting through the median of the motions. The length of each track was measured using a high quality map measurer, each measurement being accurate to $\pm 1 \mu\text{m}$ in the field. The swimming speed of each spermatozoon was then computed as the ratio of track length and time elapsed as determined from the number of frames utilized. The individual measurement error was consequently never greater than 2%. At least twenty tracks were measured for each determination of mean speed.

Single time-exposure photomicrographs were taken with an Exakta 35 mm single lens reflex camera under dark-ground illumination. The camera shutter was automatically operated by a solenoid controlled by an electronic trigger, thereby ensuring an accurate exposure time of 2 sec for each photograph. The film was Ilford FP4, and the total magnification on the finished prints was $\times 385$. The tracks on a print were due to the light scattered by the midpiece of each progressing spermatozoon. The width of a track reflected the amount of pitching of the midpiece. The shapes of the beginning and end of a track were characteristic, and allowed determination of swimming speed with a measuring error not exceeding 2%.

In many instances the tracks on a print considerably overlapped and intertwined, making identification of discrete, entire tracks difficult. Consequently an additional procedure was followed. The tracks emanating from, but not beginning or ending at, the border of the print were measured, provided they obviously did not re-enter the border. This amounted to drawing an imaginary line at random and defining track lengths as measured from that line. The mean swimming speed of the sample was then computed as the mean 'half' track length divided by twice the 2-sec exposure. If all half tracks emanating from both sides of such a line were measurable, the mean speed so computed would be the same as that determined from the total lengths of the same tracks. The practical advantage of this approach was that only one end of a track needed to be discernible for that track to be usable. In dense prints, this approach was very useful; and the bias against the faster swimmers, whose tracks are more likely to be obscured, was reduced. The distribution of sample half track speeds did not need to be the same as that of 'whole' track speeds, and may be expected, in principle, to have greater variance.

Photomicrographic number flux counting

The same single photographic prints described above were used to measure the number of spermatozoa crossing a line segment of given length during the known 2-sec time exposure. This segment was provided by placing a transparent triangle of side 24.3 cm on the 24 × 29 cm print. The number of tracks crossing the edge of the ruler thus represented the number of spermatozoa crossing the line segment from both sides. In practice, four such counts were made for each print, the ruler being successively positioned to cover the entire field in the print though otherwise at random. The mean swimming speed as applied here in the number flux method was computed from the formula of Ojakian & Katz (1973):

$$\bar{V} = \frac{\pi}{2} \frac{N}{L t_f \bar{n}} \quad (4)$$

in which N is the total number of spermatozoa crossing a line of length L during time t_f , and $\bar{n}$ is the surface density of swimming organisms. Whenever possible, $\bar{n}$ was determined directly from the photograph. For very dense prints this was not possible, and $\bar{n}$ was estimated by taking the value measured by the Quantimet just before the photograph was taken.

RESULTS

Comparison of 'whole' and 'half' tracks

The mean speed for the half track method on all photographs was 114 $\mu\text{m}/\text{sec}$ and for the whole track 104 $\mu\text{m}/\text{sec}$ ($t = 2.648$; d.f. 118; $0.02 > P > 0.01$).

In the total for the 60 single time-exposure photomicrographs, the standard deviation of the mean speed for the half track measurement always exceeded that of the whole track method. A comparison of variances within individual photographs proved significant overall (F test; $P \leq 0.01$); it was insignificant in only eighteen cases. In 49 cases, the mean speed as determined from half tracks

exceeded that from whole tracks (Sign test, $P < 0.01$). The two predictions of mean speed were, however, strongly correlated ($r = 0.877$; $P < 0.001$).

These results are consistent with the notion that the half track method tends to reduce the bias against the fastest swimmers. This bias can be studied by examining the relationship between the ratio $\bar{V}_H/\bar{V}_W$ of half and whole track means, and an appropriate measure of the density of the tracks on the photograph. This density is related to swimming speed and concentration. Accordingly, regressions were performed of $\bar{V}_H/\bar{V}_W$ and $\bar{V}_H + \bar{V}_W/2$, together or singly on surface density, and on the standardized measure of number flux (N). Anticipating a forthcoming result, we may expect N to be significantly proportional to the product of surface density and mean speed. In order to minimize statistical discrepancies between the half and whole track approaches, only the eighteen cases in which the variances proved insignificant were considered. The correlations with mean speed and number flux were weak but not insignificant, $r = 0.377$ ($P < 0.05$) and $r = 0.227$ ($P < 0.15$), respectively. The regression of $\bar{V}_H/\bar{V}_W$ upon surface density of motile spermatozoa had a slope of 0.023 and an intercept of 0.909 ($r = 0.562$; $P < 0.005$). Thus the surface density of spermatozoa is more likely to influence the bias against faster swimmers than either the mean speed or number flux.

Comparison of number flux counting with half and whole tracking

Regressions were performed of mean speeds predicted from number flux

TEXT-FIG. 1. Regression of mean speed of spermatozoa determined from flux counts ($\bar{V}$ flux) against the mean speed from half tracks ($\bar{V}_H$) ($\bar{V}$ flux = $0.485 + 1.018 \bar{V}_H$; $r = 0.854$, $P < 0.001$) and from whole tracks ($\bar{V}_W$) ($\bar{V}$ flux = $38.577 + 0.691 \bar{V}_W$; $r = 0.774$, $P < 0.001$).

counting on the photographs, and the half and whole track results (Text-fig. 1). The comparison with half tracking was particularly good, with a slope of 1.018 and an intercept of 0.485 ($r = 0.954$; $P < 0.001$). The comparison with whole tracks was well correlated ($r = 0.774$; $P < 0.001$), but the regression line itself (slope 0.691; intercept 38.577) may be accounted for by the absence of populations of very slow swimming spermatozoa.

Number fluctuation counting ('probability after' effect) compared with half tracking and cinemicrography

In order to span a relatively wide range of suspension surface densities and swimming speeds, the mean speeds determined from automatic number fluctuation counting on Quantimet were compared with half track results from single photomicrographs and whole track results from cine photomicrographs. The latter, while more time-consuming, afforded a considerable increase in surface density, from roughly $5 \times 10^4/\text{cm}^2$ to $3 \times 10^5/\text{cm}^2$. The range of mean speeds determined on cine photomicrographs was 68 to $162 \mu\text{m}/\text{sec}$. Each counting run on the Quantimet required $2\frac{1}{2}$ min, its results being compared with photomicrographic data obtained within the following 30 sec. The comparisons indicate that the accuracy of the automatic Quantimet approach, as applied,

TEXT-FIG. 2. Regression of mean speed of spermatozoa determined from the Quantimet counts ($\bar{V}_Q$) against mean speed from flux counts ($\bar{V}_F$) ($r = 0.725$, $P < 0.005$)

was strongly influenced by the surface density of active swimmers. The results when there were ≥ 15 spermatozoa in the field ($\bar{n}$) were in reasonably good agreement with the tracking results ($r = 0.725$; $P < 0.005$; S.E. 11%). By comparison, for $\bar{n} < 15$ there was less correlation ($r = 0.636$; $P < 0.005$; S.E. 25%). For the former, the photomicrographic results was always within the precision of the 'probability after' result (Text-fig. 2). Among the acceptable results, i.e. $\bar{n} > 15$, there was little correlation between $\bar{V}_Q/\bar{V}_F$ ($\bar{V}_Q$ being the result determined on the Quantimet and $\bar{V}_F$ the photomicrographic result) and percentage motility ($r = 0.125$; $P < 0.7$), or mean swimming speed $\bar{V}_F$ ($r = -0.346$; $P < 0.2$). The accuracy of $\bar{V}_Q$ appeared to be unaffected by the percentage of motile spermatozoa or the mean swimming speed ($\bar{V}_Q/\bar{V}_F$ on % motile: $r = 0.125$, $P < 0.7$; $\bar{V}_A/\bar{V}_F$ on $\bar{V}_F$: $r = 0.746$, $P < 0.2$). The accuracy, defined as $[1 - \bar{V}_Q/\bar{V}_F]$, when the χ^2 test of suspension randomness had a significance of $\leq 10\%$ did not differ from that at all other levels ($t = 1.118$; d.f. 16; $0.3 > P > 0.2$), but does not indicate that accuracy was independent of suspension randomness.

DISCUSSION

The results of our study provide three basic suggestions regarding the measurement of mean swimming speed: (1) on single photomicrographs, measurement of half track lengths is generally preferable to measurement of whole tracks; (2) number flux counting provides an accurate estimate; (3) number fluctuation counting, which can be performed automatically, also provides an accurate estimate.

Analysis of single photomicrographs has been reconsidered here since in the absence of sophisticated measuring equipment such an approach remains the simplest quantitative means of determining average swimming speed. In some species, e.g. man, the whole semen may be sufficiently dilute to preclude any need to resort to half track measurement, or indeed number flux counting on the photographs. However, with more concentrated and/or faster swimming spermatozoa, measurement of whole tracks simply may not be practicable. Even if it is, it will tend to underestimate both the mean speed and the variance of the distribution of swimming speeds in the suspension, the latter primarily because of the bias against one tail of the distribution. Half tracking, on the other hand, while reducing this bias will inherently tend to overestimate the variance of the distribution.

With thick photomicrographs, half tracking becomes unreliable, but number flux counting as described above may still be possible if used with an alternative measurement of concentration or surface density. The mathematical derivation of the formulae used in this method (Ojakian & Katz, 1973) applies in fact to two different situations. First, the microorganisms can be swimming in straight line trajectories in random directions, and in such dilute suspension that no collisions or interactions of any kind occur between organisms. This was the case considered by Ojakian & Katz. Secondly, movement can be a diffusion-like process resembling random walk, implying significant interactions amongst spermatozoa. Most laboratory suspensions of spermatozoa fall between these

two extremes, probably more resembling the latter. Nonetheless, the accuracy of our results together with those of Ojakian & Katz recommends this approach, if possible by direct observation (e.g. Bartak, 1971), or else with photomicrographs.

The mathematical derivation of the Equation 1 for mean speed from the probability after effect (Rothschild, 1953), actually incorporates the two-dimensional number flux result. It follows that, with suitable sampling procedures, the accuracy of the latter should correlate well with that of the former. The results presented here, which were obtained automatically with the image analysing computer, represent sampling conditions at the limit of applicability of the method. This is due primarily to the relatively large time interval ($t = 1.466$ sec) between successive counts of the number of spermatozoa in the square. The 'probability after' method is based upon a strong correlation between successive counts. The expression for this correlation is $\exp(-4\bar{V}t/\pi l)$, which for the above t , over $l = 200 \mu\text{m}$, and say $\bar{V} = 100 \mu\text{m/sec}$, yields a relatively low value of 0.343. The method also requires that all spermatozoa within the square have essentially the same instantaneous probability of leaving it, hence increases in l are restricted. Under these conditions, approximately 100 counts (requiring roughly $2\frac{1}{2}$ min) are necessary to maintain the theoretical precision of the method at a tolerable level (see Equation 2). During such a time, numbers of immotile spermatozoa are likely to be conveyed into and out of the counting square, the Quantimet is unable to distinguish between the passive movers and the actively swimming spermatozoa in the mode in which its counting accuracy is greatest, and consequently the mean swimming speed is likely to be underestimated, as was often the case. Sperm agglutination in the suspension exacerbates this problem, not only increasing fluctuations in the number of immotile spermatozoa in the square, but also limiting access to the square, i.e. decreasing the effective value of l . In addition, suspension ageing, which can be quite significant during $2\frac{1}{2}$ min on a slide, is a potential problem. These factors, combined with the small but finite lack of counting precision of the Quantimet (when applied to suspensions containing motile spermatozoa), are the likely cause of the greater accuracy for $\bar{n} \geq 15$. The lower limit, $\bar{n} = 15$, is however not necessarily unique, and represents the best limiting value within our data.

As noted earlier, if suspension movements are random the numbers counted in the square at equal and uncorrelated time intervals should fluctuate in a Poisson manner. The χ^2 test (Equation 3) of temporal randomness in the square should in principle have been accompanied by an instantaneous test of spatial randomness within subsquares of the $200 \mu\text{m}$ square. Only if the suspension movements were strictly random, and there were no immotile spermatozoa, would the two tests be equivalent. Such a test was performed on the cine films by Rothschild, but is not possible on our computer. The fact that the significance level of our χ^2 test did not correlate well with the accuracy of the method reflects the insufficiency of the test. The degree of suspension randomness does indeed influence the applicability of 'probability after' statistics. However, the very good accuracy of the results of number flux counting and the reasonably good accuracy of the results of number fluctuation counting suggest that lack of

suspension randomness was not a significant problem in our experiments. An exception would be a suspension comprised primarily of circularly swimming spermatozoa.

In spite of all the above shortcomings, the accuracy of our automatic number fluctuation counting, with a standard error of only 11%, is quite tolerable. We are presently engaged in attempts to decrease it. Once accomplished, ultimately using a direct interface between the image analysing computer and a digital computer, and with detection errors minimized, a more accurate determination of mean speed should require only a few seconds. An excellent ability to determine instantaneously both total surface density and the number of motile spermatozoa, has already been demonstrated for the Quantimet (Dott, 1975). Thus, mean speed, total number density, and percentage motility, three primary physical parameters that characterize a suspension, could be simultaneously and automatically determined.

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