

A NEW METHOD OF MEASURING THE ACTIVITY OF SPERMATOOZOA

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INTRODUCTION

The problem of measuring the speeds at which spermatozoa swim presents many difficulties. If the suspension is sufficiently dilute, measurements can be made by the 'dark-ground track' method (Rothschild & Swann, 1949; Rothschild, 1951). The spermatozoa are illuminated with a dark-ground condenser, and a photographic plate is exposed for a known time above the suspension. As the head of the spermatozoon scatters more light than the tail, a track is formed on the photographic plate. The length of the track divided by the exposure time gives the speed of the spermatozoon. This method cannot be used when the sperm suspension is of normal density, as the spermatozoa in the field scatter too much light for photography. Unfortunately, the dilution necessary to achieve successful results is such that the spermatozoa may undergo physiological changes, often of a pathological nature, immediately after dilution. These changes constitute the well-known dilution effect. Apart from the dark-ground track method, attempts have been made to measure by eye the speeds at which individual spermatozoa swim. This naturally gives no information about the speeds at which representative spermatozoa swim, nor about the important property of a sperm suspension, its mean or average speed. Grave & Downing (1928) measured the time that *some* spermatozoa in a suspension took to swim along a capillary tube of known length. Such observations tell one little about the speeds at which spermatozoa swim—even the ones under observation; for it was assumed, without any justification, that the spermatozoa swam straight along the capillary tubes. The problem of measuring sperm speeds has proved so intractable that many workers concerned with this subject have contented themselves with examining a suspension under the microscope and assigning marks to it according to its activity, assessed by eye. The reader may ask why one cannot take a ciné-film of a sperm suspension and subsequently project the film frame by frame, plotting the positions of individual spermatozoa. The lines connecting the consecutive positions of the spermatozoa can clearly be used to calculate their speeds and therefore the mean speed of the suspension. There are two objections to this method, though it has been used (Rothschild, 1950). First, it is extremely time-consuming; secondly, unless high film speeds are used, it may not be possible to identify one particular spermatozoon on successive frames. This point is illustrated in Fig. 1. Errors due to this uncertainty will falsify estimates of

mean speed and distort the distribution of sperm speeds, if this is of interest. It also creates serious problems for the person plotting the positions of the spermatozoa, as difficult decisions have continually to be made. The uncertainty can of course be obviated by using sufficiently high film speeds, but this increases the labour of plotting and the cost of the experiment.

The object of this paper is to describe a new and quantitative method of estimating the mean speed of a sperm suspension of any density, the estimate having a known precision.* The method is based on the principle known as probability-after-effect, which is described in some detail in Chandrasehkar's review, 'Stochastic problems in physics and astronomy' (1943). Little attention has hitherto

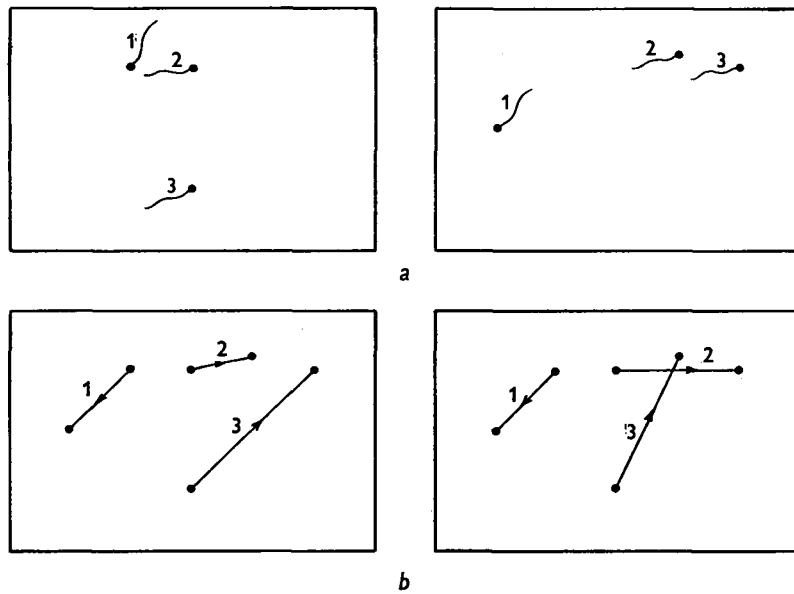


Fig. 1. *a*, positions of three spermatozoa on consecutive cinematograph frames. *b*, alternative routes by which two of the three spermatozoa could get from their positions on the first frame to their positions on the second. There is no chance of a mistake in the case of sperm 1 as it is swimming in the opposite direction to sperms 2 and 3.

been paid to the precision of the method, without which it is of limited interest. The principle of probability-after-effect can be roughly described as follows. Suppose we examine two suspensions of particles or organisms, *A* and *B*, and that the particles in *A* are 'frozen' so that they do not move. The average speed of the particles in *A* is therefore zero. The particles in *B* are not 'frozen' and move about. If a small region in *A* is observed at times t_1 and t_2 , the number of particles in that region will be the same. This will not be the case in a small region in *B*, because some particles which were outside the region at t_1 may be inside at t_2 , while some which were inside the region at t_1 may be outside at t_2 . The numbers

* A note on the method has been published in *Nature* (Rothschild, 1953).

entering and leaving the region will not necessarily be the same. We can say that in the case of system *A*, the number of particles in the region at t_1 is completely 'correlated' with the number in the region at t_2 . In the case of system *B*, the number of particles in the region at t_1 will be *partly* correlated with the number in the region at t_2 , because particles have entered and left the region in the time interval (t_1, t_2) . If the time interval between observations were made sufficiently long, the correlation between the numbers would be zero. The fluctuations with time in the number of particles in the region under consideration is clearly some function of the speeds at which the particles move (actually the mean speed of the particles). Consequently, the mean speed should in principle be determinable from the observed time fluctuations in the number of particles.

When estimates of mean speed are made by the probability-after-effect method of analysis, the experimental procedure is to place a suspension of spermatozoa on a microscope slide and photograph it at given time intervals. The number of spermatozoa in a region of known area is then counted, on each photograph. The mean speed of the suspension can then be estimated by means of a few simple calculations.

THEORY

Random movements of spermatozoa. In its simplest form, the theory requires that the directions of movement of the spermatozoa should be random. If this is so, the spatial distribution of spermatozoa in the suspension will also be random. It can be shown (Appendix, 1) that in such circumstances, the number of spermatozoa in a small region within the suspension will fluctuate Poisson-wise, provided that the sperm counts are made at sufficiently long time intervals (see later). In other words,

$$\Pr\{x\} = \frac{1}{x!} \bar{n}^x e^{-\bar{n}} \quad (x=0, 1, 2, 3, \dots), \quad (1)$$

where $\Pr\{x\}$ is the probability of there being x spermatozoa in the region and \bar{n} is the average number of spermatozoa in the region.

Probability-after-effect. Let the number of spermatozoa, n , in a region of known area be n_1, n_2, n_3, \dots in successive photographs taken at equal time intervals t_1, t_2, t_3, \dots . Let $|n_2 - n_1| = \delta_1, |n_3 - n_2| = \delta_2, \dots$ and $t_{i+1} - t_i = \tau$. Then it can be shown, Appendix, 2, that

$$E(\delta^2) = 2E(n) (1 - e^{-\lambda\tau}), \quad (2)$$

where $E(\delta^2)$ = the expected value of δ^2 , $E(n)$ = the expected value of n , and λ is a parameter which includes the average or mean speed, \bar{c} , of the sperm suspension.

In practice, equ. (2) becomes

$$\bar{\delta}^2 = 2\bar{n}(1 - e^{-\lambda\tau}), \quad (2.1)$$

where $\bar{\delta}^2$ = the estimated average value of δ^2 , \bar{n} = the estimated average value of n , and λ includes the estimated value of \bar{c} . By use of the concept of collision

frequency (Rothschild, 1952), it can be shown (Appendix, 4), that if the region under examination is a rectangle with sides x' and y' , a square with side x' , or a circle of radius r ,

$$\left. \begin{aligned} \lambda &= \frac{2\bar{c}(x' + y')}{\pi x' y'} \\ \lambda &= \frac{4\bar{c}}{\pi x'} \\ \lambda &= \frac{2\bar{c}}{\pi r} \end{aligned} \right\} \quad (3)$$

These relationships are derived in Appendix, 3.

Correlation. In the section entitled 'Random movements of spermatozoa', reference was made to the numbers of spermatozoa in a small region being distributed Poisson-wise, *provided counts are made at sufficiently long time intervals*. Suppose that consecutive counts have a short time interval between them so that the spermatozoa move a small distance in that time interval. Assume, for example, that at t_1 , the number of spermatozoa in a square with side 100μ is counted, and at t_2 , $1/24$ sec. later, the number in the same square is counted again. Assuming a mean sperm speed of $120\mu/\text{sec.}$, each spermatozoon will on the average move 5μ in $1/24$ sec. As a result, few of the spermatozoa within the square at t_1 will have swum out by t_2 , and few will have swum into the square in the interval (t_1, t_2) . In such a case the numbers will not fluctuate Poisson-wise, and the joint distribution of consecutive observations will be a bivariate correlated Poisson distribution in which

$$\text{corr}(n_1, n_2) = e^{-\lambda\tau}, \quad (4)$$

where $\text{corr}(n_1, n_2)$ is the correlation coefficient of n_1 and n_2 in this system. The derivation of equ. (4) is given in Appendix, 5. This concept is important as we shall be concerned with the best value of $\text{corr}(n_1, n_2)$, i.e. the value which maximizes the precision of estimates of \bar{c} .

Precision of estimates of \bar{c} . Denoting $e^{-\lambda\tau}$ by x and the number of sperm counts made on different frames by k , the variance of \bar{c} , $V(\bar{c})$, is given by

$$V(\bar{c}) = \frac{\bar{c}^2}{k\bar{n}(x \log_e x)^2} \left\{ \frac{\bar{n}(1-x)^2(3+x)}{(1+x)} + x(1-x) \right\}. \quad (5)$$

The derivation of equ. (5) is very complicated, and so as not to overweight this paper with mathematics, it will be published elsewhere.* This equation enables one to calculate the value of τ , the time interval between photographs, which gives maximum precision for any particular k or \bar{n} in the region under examination. As regards the latter, if the area of the region is reduced, the average number of

* In deriving equ. (5), the probability-generating function of n_2 given n_1 ,

$$\exp - \{n(1-s)(1-x)\} \{1 - (1-s)x\}^{n_1},$$

is used. From it, the relationship $E(n_2 | n_1) = n(1-x) + n_1x$ follows (see Appendix, 5).

spermatozoa in it will also be reduced, and vice versa. Fig. 2 shows a set of curves derived from equ. (5). If this equation is written

$$V(\bar{c}) \doteq \frac{\bar{c}^2}{k} \{f(\bar{n}, x)\}, \tag{5.1}$$

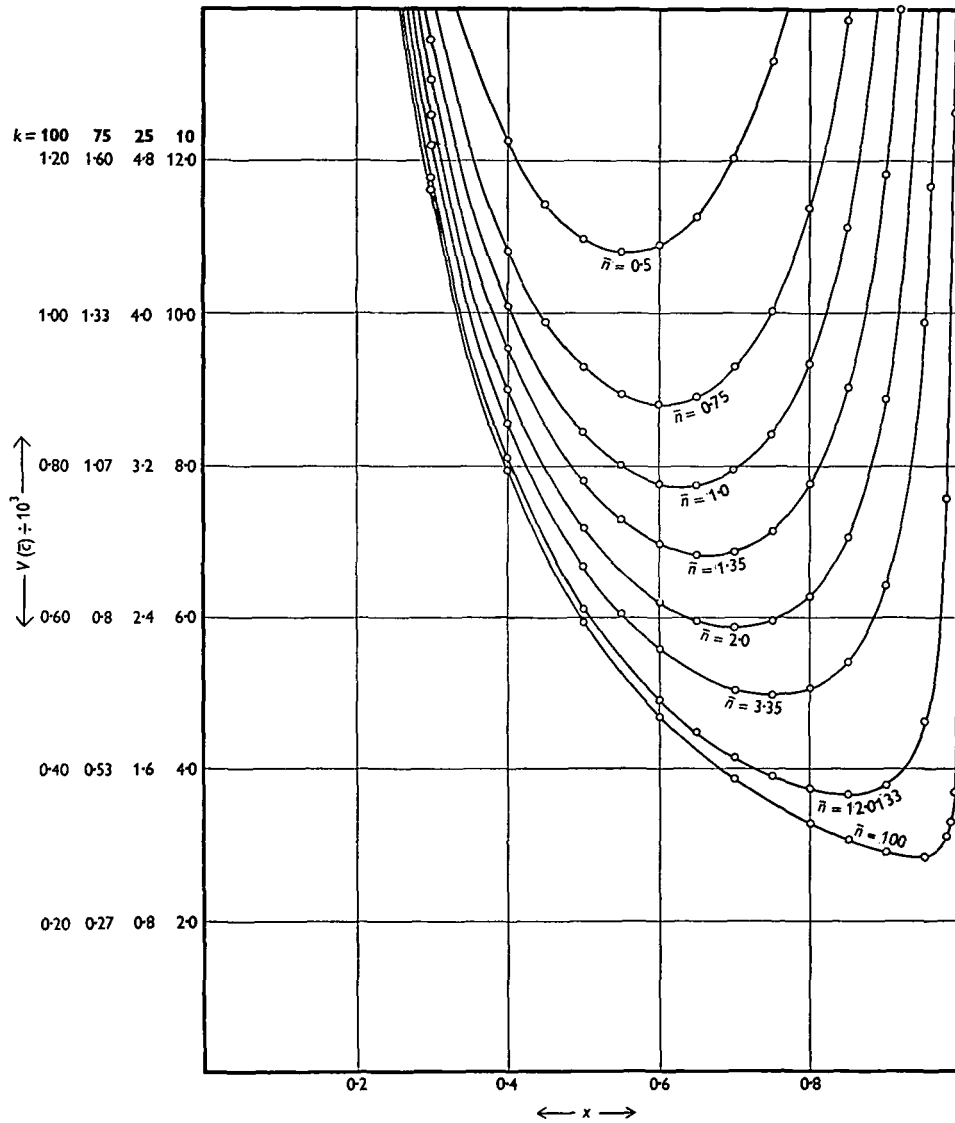


Fig. 2. Relationships between $V(\bar{c})$, the variance of an estimate of \bar{c} (assumed to be $110.5 \mu/\text{sec.}$) and $x = e^{-\lambda\tau}$, for different values of \bar{n} , the average number of spermatozoa counted. Four $V(\bar{c})$ scales are given, appropriate to the cases where $k = 10, 25, 75$ and 100 .

it becomes clear that, with an appropriate change of ordinate scale, the curves in Fig. 2 can be used with any values of k . Four ordinate scales, relevant to

$k = 10, 25, 75$ and 100 , are given in Fig. 2. In this case, for reasons that will be apparent later, \bar{c} was taken as $110.5 \mu/\text{sec}$. By laying a ruler horizontally across the curves in Fig. 2, at some particular value of $V(\bar{c})$, experimentally convenient values of \bar{n} , k and $\tau (= -1/\lambda \log_e x)$, corresponding to that variance, can be selected. For example, if a variance of 1000 is required, $k = 100$, $\bar{n} = 100$ and $x = 0.337$ or 0.999 ; or $k = 100$, $\bar{n} = 2$ and $x = 0.365$ or 0.915 . We can, in fact, draw a system of iso-variance curves or 'isologs', from which the values of \bar{n} , k and τ corresponding to any particular variance can be readily obtained. These curves are given in Fig. 7, which it is convenient to put in the section entitled 'Practical considerations'. The curves relating $V(\bar{c})$ and τ at different levels of \bar{n} are also instructive and are given in Fig. 3.

Loss of information. Previous workers on probability-after-effect do not seem to have realized that if one only observes the number fluctuations in successive *pairs* of observations, a significant amount of information is lost. The point is best made by means of a diagram. Fig. 4*a* refers to the case where a comparison is only made between successive pairs of counts. But a comparison of the numbers in frame 1 and those in frames 3, 4, 5, ... (Fig. 4*b*) provides further information, using the method of Maximum Likelihood. There is yet another source of loss of information. Both in Fig. 4*a* and *b*, comparisons are made between the numbers at different times in the *same* region in the suspension. But apart from the temporal relationships to which reference has been made, spatio-temporal relationships also exist; these can provide further information, a point which is made in Fig. 4*c*. The extraction of this additional information presents formidable mathematical difficulties. I hope to examine these questions later, with Dr H. Ruben.

EXPERIMENTAL PROCEDURE

Bull semen was diluted 1/4 with phosphate buffer (0.6 g. KH_2PO_4 and 6 g. Na_2HPO_4 in 300 ml. water) containing 5 mg. fructose/ml. A drop of this diluted semen was placed on a microscope slide and a no. 1 cover-slip ($\frac{7}{8} \times \frac{7}{8}$ in.) was very gently placed on top of the drop. The size of the drop was so adjusted that after the cover-slip had been put on top of it, the drop spread just to the edges of the largest circle which could be inscribed on the cover-slip, without any pressure being applied. In these circumstances the spermatozoa swim actively in a particular lamina of fluid between the lower surface of the cover-slip and the top surface of the microscope slide.* This point is important, as the analysis involves considerations similar to those used in the kinetic theory of gases, but in two, not three, dimensions. If, therefore, the field examined under the microscope were a cylinder rather than a disk, the theoretical foundation on which the method is based would be wrong, for reasons shown pictorially in Fig. 5. This does not mean that a three-dimensional system cannot be used in other types of experiment, but that the formulae used in this paper would have to be altered.†

* The fact that spermatozoa swim 'best' in particular laminae in the suspension is an interesting phenomenon for which, at present, there does not seem to be any obvious explanation.

† In the case where the region under examination is a cylinder of height h as in Fig. 5*b*, the only alteration is that equ. (3) becomes $\lambda = \frac{\bar{c}}{2} \left(\frac{1}{r} + \frac{1}{h} \right)$.

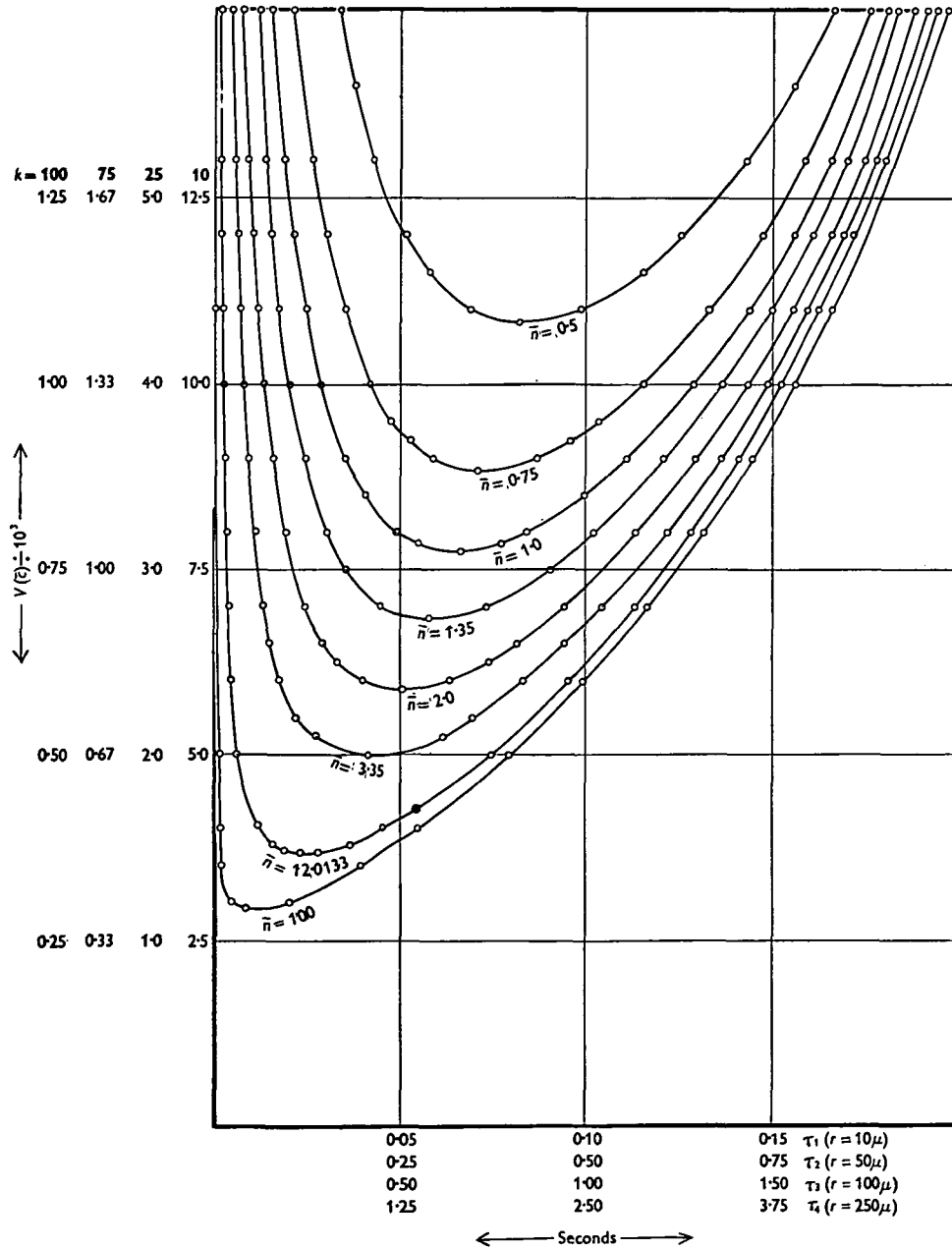


Fig. 3. Relationships between $V(\bar{c})$ and $\tau (= -1/\lambda \log_e x)$ at different levels of \bar{n} . Four $V(\bar{c})$ scales, appropriate to $k = 10, 25, 75$ and 100 , are given. The four τ scales, τ_1, τ_2, τ_3 and τ_4 , correspond to circular (not rectangular) regions on consecutive frames, in which the numbers of spermatozoa are counted.

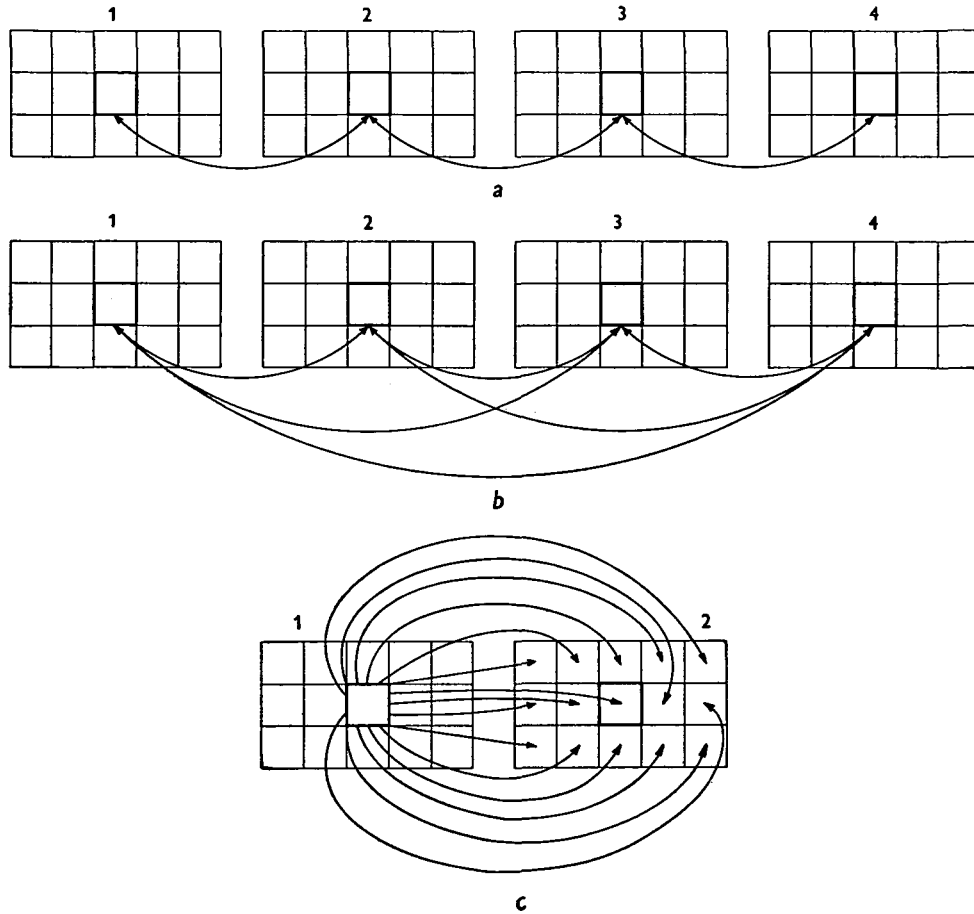


Fig. 4. Three possible ways of comparing the numbers of spermatozoa in selected regions on consecutive frames. A curved line with an arrow on it is a comparison: *a*, the method used in these experiments; *b*, the same as *a*, but region 1 is compared with regions 2, 3, 4, etc., region 2 with regions 3, 4, etc., and so on; *c*, other combinations of comparisons are made between subregions on different frames.

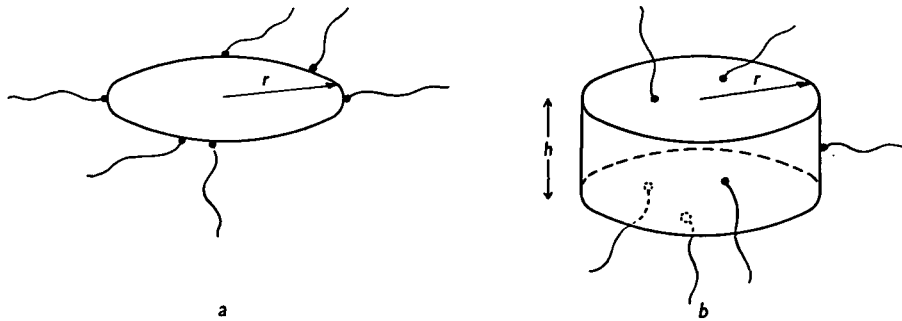


Fig. 5. *a*, spermatozoa 'bombarding' a circle of radius r . The bombardment frequency is $2d\bar{c}r$, where d is the number of spermatozoa per unit area. *b*, spermatozoa 'bombarding' a cylinder of radius r and height h . The bombardment frequency is $\frac{1}{2}D\bar{c}\pi r(r+h)$, where D is the number of spermatozoa per unit volume.

A ciné-film was then taken of the suspension, using a $\times 18$ phase-contrast objective, and a $\times 10$ ocular. The ciné-camera was an Askania 35 mm., with the shutter aperture set at 45° , running at 25.8 frames/sec. The light source was a Point-o-Lite with one Chance heat filter between it and the microscope. In the experiment described in this paper, the suspension was photographed for 20.2 sec., which is equivalent to 521 frames.

All operations were carried out at 37° C. The overall magnification (1590 after projection of the film) was such that the field photographed was a rectangle with sides 337 and 255μ . When plotting the positions of spermatozoa, or counting them, this rectangular field was subdivided into twenty subrectangles each with sides 67.4 and 63.8μ .

RESULTS

Random movements of spermatozoa. As mentioned in the section entitled 'Theory', if the spermatozoa move in random directions, their spatial distribution will be random, in which case the numbers in the field will fluctuate Poisson-wise. This will only apply provided the positions of spermatozoa in the fields under examination are uncorrelated, i.e. provided $\text{corr}(n_1, n_2)$ is small, say 0.05 or less. The time interval between counts which provides a correlation of 0.05 or less can be calculated for a region of any desired size. In these experiments each frame was divided into twenty subframes or rectangles with sides 67.4 and 63.8μ . Assuming an average sperm speed of $125 \mu/\text{sec.}$,

$$\begin{aligned} \text{corr}(n_i, n_{i+1}) &= e^{-\lambda\tau} = \exp - \left\{ \frac{250(67.4 + 63.8)}{3.1416(67.4)(63.8)} \tau \right\} & (6) \\ &= e^{-2.428\tau}. & (6.1) \end{aligned}$$

If $e^{-\lambda\tau}$ is to equal 0.05, τ must equal 1.23 sec. The film speed being 25.8 frames/sec., every 30th frame will have a sufficiently low correlation to allow the numbers of spermatozoa in each subframe to be examined for a Poisson distribution. If the numbers of spermatozoa in the twenty subframes fluctuate Poisson-wise, the numbers in groups of subframes, for example, in whole frames, will also be Poisson-distributed (Fisher, 1948); but if a whole frame is examined, λ , which *inter alia* is a function of the frame size, and therefore of $\text{corr}(n_i, n_{i+1})$, will have different values from those applicable to the subframe case. To test, therefore, for random movement, we select frames sufficiently far apart in time to examine whole and subframes for a Poisson distribution of sperm numbers. A simple calculation similar to that done above for subframes shows that if whole frames as well as subframes are to be examined, $\tau \doteq 4.6$ sec. and every 118th frame may be selected. The actual numbers of spermatozoa observed in such an experiment are given in Table 1. The calculations show that the directions of movements of the spermatozoa are random. As mentioned above, it follows automatically that the sperm numbers will fluctuate Poisson-wise in whole as opposed to subframes, and this can easily be verified by the use of Fisher's dispersion index

$$\chi^2_{\nu-1} = \frac{1}{n} \sum (n_i - \bar{n})^2, \quad (7)$$

or the relationship

$$1 + 2 \sqrt{\left(\frac{2\nu}{(\nu-1)^2}\right)} > \frac{V(n)}{\bar{n}} > 1 - 2 \sqrt{\left(\frac{2\nu}{(\nu-1)^2}\right)}, \quad (8)$$

where ν is the number of observations, in this case 5. The numbers relevant to whole frames are given in Table 1, in the horizontal column which starts with a Σ . Substitution of the appropriate values in these two equations shows that $\chi_4^2 = 2.9044$, making $P = 0.5-0.7$, and $2.5811 > 0.7261 > -0.5811$.

Table 1. *Number of spermatozoa in frames and subframes*

Frame no. ...	2	119	237	355	473
Subframe no. 1	5	6	15	9	9
2	5	10	7	2	7
3	5	6	3	5	9
4	4	5	8	8	6
5	8	9	7	5	7
6	7	3	8	9	4
7	7	8	6	6	7
8	6	7	4	7	4
9	6	4	7	4	8
10	6	5	11	6	6
11	11	6	6	9	11
12	6	9	7	7	5
13	3	3	6	5	5
14	5	7	4	0	6
15	4	5	3	3	7
16	3	9	14	13	6
17	3	8	1	4	3
18	10	6	11	6	9
19	13	8	8	8	10
20	3	3	9	13	9
Σ	120	127	145	129	138

Subframe analysis

No. of sperm per subframe	0-3	4	5	6	7	8	9	10-∞
No. of subframes with that number, o	14	9	12	18	14	10	11	12
Expected no. of subframes with that numer, e	10.5806	10.7979	14.2316	15.6306	14.7225	12.1284	8.8804	13.0280

$$\chi_6^2 = \frac{(o-e)^2}{e} = 3.1096. \quad P = 0.7-0.8$$

These observations show that in these conditions, the movements of spermatozoa are essentially random. If the suspension exhibited much orientation, or if a significant number of spermatozoa in the suspension swam in circles, the Poisson-distribution tests would fail and it would be necessary to attempt more recondite methods of analysis.

Distribution of speeds. A new method of estimating the mean speed of a suspension must be compared with some different way of measuring the same

quantity. Accordingly, the 25.8 frames/sec. ciné-film was projected frame by frame on to squared paper and the outline of the head of each spermatozoon was drawn in. The consecutive positions of each head were then joined by straight lines and their lengths measured, from which the speeds of each spermatozoon were calculated. At this film speed and with the magnification used, 1590, the ambiguities about the consecutive positions of particular spermatozoa mentioned earlier did not arise. Mr M. J. Hubbard and Miss G. E. Bending, a research

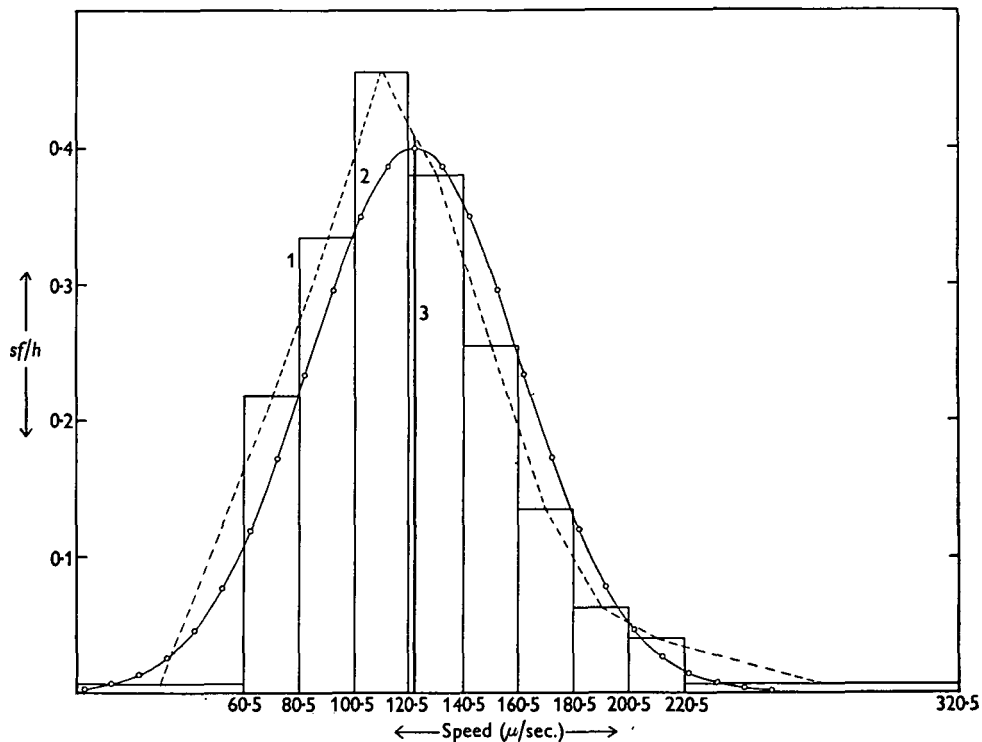


Fig. 6. 1, sperm speed histogram and frequency polygon; 2, normal distribution with the same mean and standard deviation; 3, mean; s , standard deviation; f , relative frequency; h , cell width. Total number of sperm tracks measured, 433.

assistant provided by the Medical Research Council, carried out this tedious and time-consuming work under my direction. The distribution of sperm speeds is shown in Fig. 6. The mean and standard deviation of the distribution are 122.7 and 38.6 μ /sec. The normal distribution with the same mean and standard deviation is included in Fig. 6, to show how much the sperm speed distribution diverges from normality. It will be observed that, as might be expected on theoretical grounds, it is both skew and leptokurtic. There are, of course, a certain number of dead or motionless spermatozoa in the field. If these are taken into consideration, as they are in the probability-after-effect method of estimating the mean speed, the mean becomes 117.2 μ /sec.

Mean speed by probability-after-effect method. To illustrate how calculations are performed, we shall examine the number of spermatozoa in a rectangle with sides 67.4μ and 127.6μ in the middle of every seventh frame, using frames 2, 9, 16, 23, 30, 37, 44, 51, 58 and 65 for this purpose. This means that k in equ. (5) is 10, which is too low for reasonable precision but convenient for explaining the method. The procedure is shown in Table 2. The estimate of the mean speed

Table 2. Estimate of mean speed and standard error using probability-after-effect principle

Frame no.	k	No. of sperm, n , in rectangle	$ \delta $	δ^2	
2	1	9	1	1	$\bar{\delta}^2 = 2\bar{n}(1 - e^{-\lambda\tau}) \quad (2.1)$ $\lambda = \frac{2\bar{c}(x' + y')}{\pi x' y'} \quad (3)$ <p>Film speed, 25.8 frames/sec. $\tau = 0.27$ sec. (every seventh frame) $x' = 67.4, y' = 127.6$</p> Substituting, <div style="border: 1px solid black; padding: 2px; display: inline-block;"> $\bar{c} = 103.7$ </div>
9	2	10	5	25	
16	3	15	4	16	
23	4	11	1	1	
30	5	12	0	0	
37	6	12	3	9	
44	7	9	1	1	
51	8	10	4	16	
58	9	14	1	1	
65	10	15			

$\bar{n} = \frac{1}{k} \sum n = 11.7$	$\bar{\delta}^2 = \frac{1}{k-1} \sum \delta^2 = 7.7778$	
$V(\bar{c}) = \frac{\bar{c}^2}{k\bar{n}(x \log_e x)^2} \left\{ \frac{\bar{n}(1-x)^2(3+x)}{(1+x)} + x(1-x) \right\}, \quad (5)$ <p style="text-align: center;">where $x = e^{-\lambda\tau}$.</p> Substituting, $V(\bar{c}) = 3872$; standard error 62.2.		

Table 3. Variation in estimate of mean speed, \bar{c} , and the standard error, s , of the estimate, with k , the number of sperm counts. \bar{c} and s in μ /sec.

k	\bar{c}	s
10	103.7	62.2
25	76.0	27.2
50	99.0	26.3
75	110.5	24.5

is 103.7 with an associated standard error of 62.2. To get a more precise estimate of \bar{c} , the same calculations were carried out, but with $k = 75$. In this case $\bar{n} = 12.0133$ and $\bar{\delta}^2 = 8.3649$, which, on substitution in equ. (2.1), provide an estimate of \bar{c} equal to 110.5μ /sec., which is in good agreement with the value obtained by the 'plotting method'. The standard error was 24.5. It is of interest to examine how in practice \bar{c} and its precision vary with k ; this is done in Table 3. In the first case examined, multiplication of the standard error by 1.6449, obtained from the normal tables, determines an interval within which \bar{c} lies with a fiducial probability of 90%; but one end of this interval is negative. Negative values of \bar{c} have no meaning

and arise because, for small k , the distribution of the estimate of \bar{c} departs from normality.

Homogeneity. If the probability-after-effect method of measuring the mean speed of a suspension is used in a series of experiments, it is of course important to establish that the sampling procedure is satisfactory. 'Sampling procedure' refers to everything that happens between collecting the original ejaculate and estimating the mean speed of the test suspension. Suppose, for example, that we estimate the mean speeds of spermatozoa in five subsamples, derived by 'identical'

Table 4. Estimate of mean speed, \bar{c} , and variance, $V(\bar{c})$, in five subsamples taken consecutively from one sample of semen diluted 1/4 with fructose phosphate buffer

\bar{c}	$V(\bar{c})$
133.46	473.69
145.35	701.68
136.49	589.98
112.51	371.26
122.43	392.77

methods from a parent sample (Table 4). If the sampling procedure is satisfactory, these five mean speeds should be mutually consistent and this can be subjected to quantitative verification. The best joint or pooled estimate of \bar{c} from the data in Table 4 is

$$\bar{c}' = \frac{\sum_{i=1}^5 I_i \bar{c}_i}{\sum_{i=1}^5 I_i}, \quad (9)$$

where $I_i = 1/V(\bar{c}_i)$. $\sum_{i=1}^5 I_i (\bar{c}_i - \bar{c}')^2$ is distributed as χ^2 on four degrees of freedom, and substitution of the values in Table 4 shows that $\chi_4^2 = 1.34$ from which P lies between 0.80 and 0.90. This means that there are inadequate grounds for rejecting the hypothesis that the five estimates of mean speed were obtained from one parent sample; in other words, the subsampling procedure was satisfactory.

PRACTICAL CONSIDERATIONS

As this method of estimating the mean speed, i.e. the *true* activity, of a sperm suspension may be of some practical interest, the details of making measurements and estimating mean speeds from them are summarized in this section.

(1) Take a ciné-film of the sperm suspension, preferably but not necessarily diluted 1/4. The film speed should be between 5 and 10 frames/sec. Phase-contrast lenses should be used. A cover-slip should be gently placed on top of the drop of sperm suspension, as described in the section 'Experimental procedure'. Alternatively, fragments of any innocuous material of suitable thickness can be placed between the cover-slip and the microscope slide to keep the depth of the suspension constant.

(2) Select a region of known area and perimeter in the microscope field (on the ciné-film).

(3) Find the value of t which makes λt equal to or greater than 2.996. In the case of bull semen, if the region (see (2)) is a rectangle with sides x' and y' ,

$\lambda = 64(x' + y')/x'y'$. For a square with side x' , $\lambda = 128/x'$; and for a circle of radius r , $\lambda = 64/r$.

(4) Divide up the field into about twenty subregions of equal area and perimeter and test whether the numbers of spermatozoa in these subregions are distributed Poisson-wise (as in Table 1, p. 187), using frames which are t sec. apart, t having been determined in (3). Assuming that the Poisson distribution test is positive, proceed as follows.

(5) Select a region whose area is such that on the average it contains about ten spermatozoa. The size of the region depends on the microscope magnification, the sperm density and the method of projecting the film. If the semen contains about 10^9 sperm/ml. and is diluted $1/4$, a rectangle with sides 70 and 125μ or a circle with radius 50μ will contain about ten spermatozoa.

(6) Count the number of spermatozoa in this region on k consecutive frames. If $k = 75$ and the average number of spermatozoa in the region is about ten, this will take some 2 hr.

(7) Calculate \bar{n} , the average number of spermatozoa in the region, and $\overline{\delta^2}$, the average of the square of the differences between the number of spermatozoa in regions on consecutive frames.

(8) If the region is a rectangle with sides x' and y' , a square with side x' , or a circle of radius r , the mean speed of the sperm suspension, \bar{c} , is given by

$$\left. \begin{aligned} \bar{c} &= -\frac{\pi x' y'}{2\tau(x' + y')} \log_e \left(1 - \frac{\overline{\delta^2}}{2\bar{n}} \right), \\ \bar{c} &= -\frac{\pi x'}{4\tau} \log_e \left(1 - \frac{\overline{\delta^2}}{2\bar{n}} \right), \\ \bar{c} &= -\frac{\pi r}{2\tau} \log_e \left(1 - \frac{\overline{\delta^2}}{2\bar{n}} \right), \end{aligned} \right\} \quad (10)$$

where τ is the time interval between successive photographs.

(9) The precision or accuracy of the estimate of \bar{c} depends on the average number \bar{n} of spermatozoa in the region of the field selected for examination, the time interval τ between successive photographs, the number of photographs examined k , and the size of the region. These facts are illustrated in Fig. 7, which requires some explanation. Consider the curve labelled IV. Any point on this curve has associated with it a particular value of \bar{n} and of τ_1 . For example, the point P has co-ordinates $\bar{n} = 2$ (the antilogarithm of 0.3), and $\tau_1 = 0.114$ sec. All pairs of values of \bar{n} and τ_1 on this curve will provide estimates of \bar{c} with the same variance 8000 (if ten photographs are examined), or standard error 89.4. A user of the curves in Fig. 7 decides on the variance which he considers adequate. The relevant curve provides a set of alternative values of \bar{n} and τ_1 which will give estimates of \bar{c} with the desired variance. The variance decreases as the number of frames counted is increased. Each curve in Fig. 7 is therefore labelled according to the variance associated with it *and* according to k , the number of frames counted. Four different values of k , 10, 25, 75 and 100, are considered.

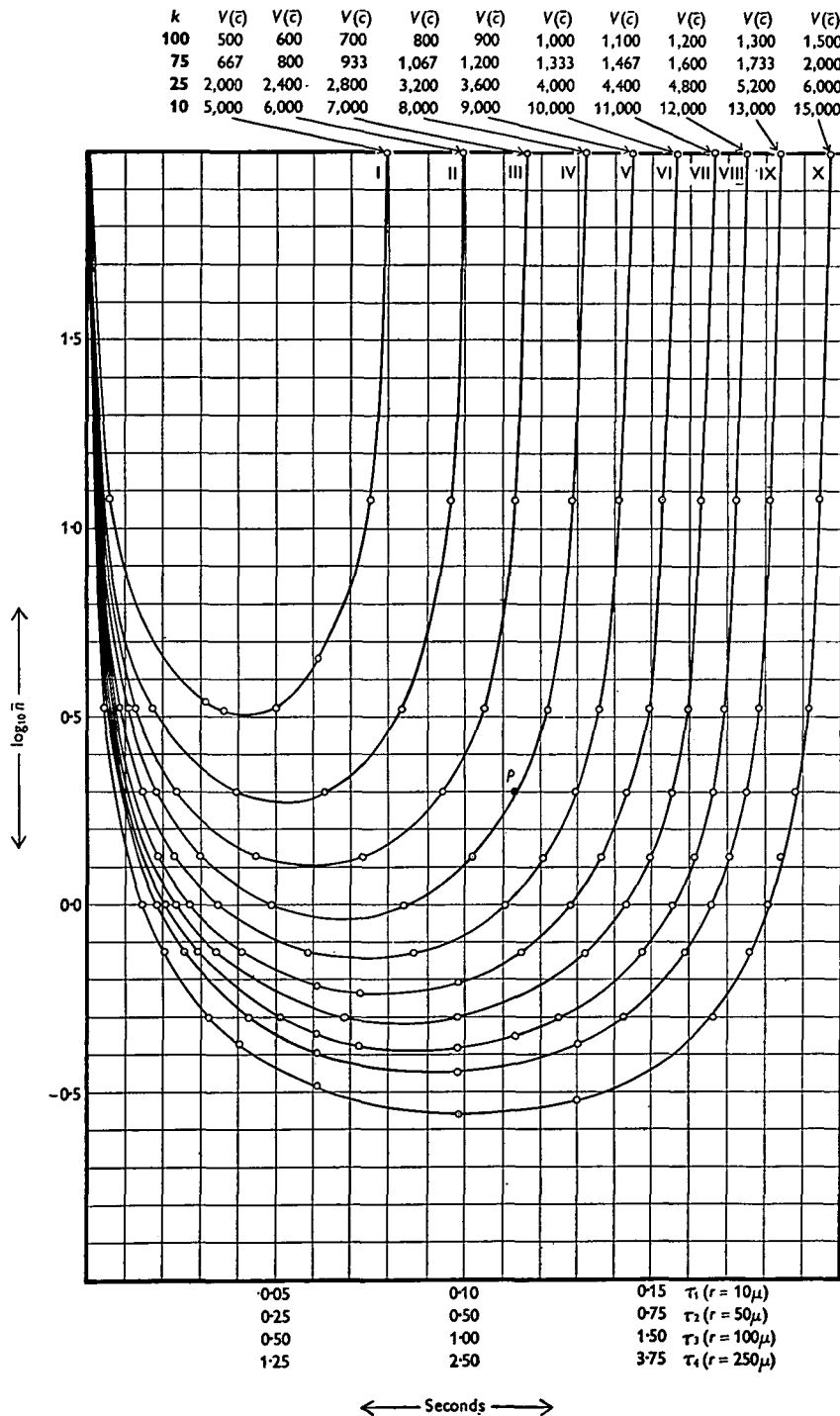


Fig. 7. The relationships between \bar{n} , the average number of spermatozoa counted in a circular region of known radius r , and the time interval τ between consecutive photographs. Any point on any one of the curves I-X provides values of \bar{n} and τ , which, when substituted in the equation for the estimate of mean speed, will produce an estimate of \bar{c} with known variance $V(\bar{c})$. The variances appropriate to curves I-X are given above the curves, in four rows, according to k , the number of counts of spermatozoa made. The four τ scales correspond to circular regions of different radii.

We must now consider how the variance changes with \bar{n} and τ . For the purposes of tabulating variances as in Fig. 7, it is convenient to consider circular regions. The four abscissa scales, τ_1 , τ_2 , τ_3 and τ_4 , correspond respectively to circular regions with radii 10, 50, 100 and 250 μ .

The curves in Fig. 7 are based on a mean speed of 110.5 μ /sec. In practice, however, Fig. 7 should be used as a guide, which will save much time, to the conditions of the experiment. The actual variance relevant to an experiment should be estimated by means of the equation

$$V(\bar{c}) = \frac{\bar{c}^2}{k\bar{n}(x \log_e x)^2} \left\{ \frac{\bar{n}(1-x)^2(3+x)}{(1+x)} + x(1-x) \right\},$$

where $x = e^{-\lambda\tau}$. There are, of course, many published tables giving the values of e^{-z} for different values of z , from which $e^{-\lambda\tau}$ can be quickly calculated.

(10) A simple method of testing the adequacy or inadequacy of the sampling procedure is given in the section entitled 'Homogeneity' on p. 190.

DISADVANTAGES OF THE METHOD

The main disadvantage is the expense involved in taking ciné-micrographs as opposed to photo-micrographs. It is doubtful whether the size of the region examined can be increased sufficiently, by reduction of magnification, to make τ suitable for ordinary photo-micrography. Figs. 7 and 3 are deceptive in this respect, as they superficially suggest that 5 sec. intervals between photographs might be achieved. This is not the case unless the sperm suspension is over-diluted. Whether the refinements outlined in the paragraph headed 'Loss of Information' on p. 183 will make it possible to have 5-10 sec. intervals between photographs, thus obviating the need for cinematography, is not yet known.*

APPENDIX

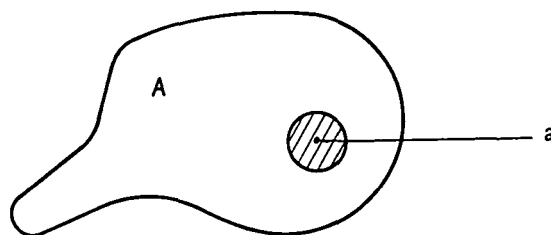


Fig. 8.

1. Consequences of the assumption of random movement

Let there be n spermatozoa in A (Fig. 8). If the movements of the spermatozoa are random, the probability that a particular spermatozoon will, at some particular instant, be in a , is equal to a/A . This statement implies random location of spermatozoa which is a consequence of their random movement.

* In any case, the longer the time interval between photographs, the greater is the chance of the 'mean free time' of the spermatozoa being shorter than the time interval, with consequent underestimation of the mean speed.

The probability $\Pr\{x\}$ that the number of spermatozoa observed in a at some instant of time will be x is given by

$$\Pr\{x\} = \binom{n}{x} \left(\frac{a}{A}\right)^x \left(1 - \frac{a}{A}\right)^{n-x} \quad (1)$$

$$= \frac{n!}{x!(n-x)!} \left(\frac{a}{A}\right)^x \left(1 - \frac{a}{A}\right)^{n-x} \quad (1.1)$$

$$= \frac{1}{x!} n(n-1)(n-2)\dots(n-x-1) \left(\frac{a}{A}\right)^x \left(1 - \frac{a}{A}\right)^{n-x} \quad (1.2)$$

$$= \frac{1}{x!} \frac{n(n-1)(n-2)\dots(n-x-1)}{n^x} \left(n \frac{a}{A}\right)^x \left(1 - \frac{a}{A}\right)^{n-x} \quad (1.3)$$

$$= \frac{1}{x!} 1 \left(1 - \frac{1}{n}\right) \left(1 - \frac{2}{n}\right) \dots \left(1 - \frac{x-1}{n}\right) \left(n \frac{a}{A}\right)^x \left(1 - \frac{a}{A}\right)^{n-x} \quad (1.4)$$

Let $A \rightarrow \infty$ and $n \rightarrow \infty$ in such a way that the average number of spermatozoa per unit area remains finite, i.e. $\lim_{\substack{n \rightarrow \infty \\ A \rightarrow \infty}} \frac{n}{A} = k$ ($k \neq 0$). Then

$$\Pr\{x\} \rightarrow \frac{1}{x!} 1 (ka)^x \left(1 - \frac{ka}{n}\right)^n \left(1 - \frac{ka}{n}\right)^{-x} \quad (2)$$

$$\rightarrow \frac{1}{x!} (ka)^x e^{-ka} \times 1 \quad (2.1)$$

$$\rightarrow \frac{1}{x!} \lambda^x e^{-\lambda}, \quad \lambda = ka. \quad (2.2)$$

x is therefore distributed Poisson-wise with parameter ka , which equals the average number of spermatozoa in a .

2. Proof that $E(\delta^2) = 2E(n)(1 - e^{-\lambda\tau})$

(1) Let the number of spermatozoa observed in an area a be n_1 at time t_1 , and n_2 at time t_2 , where $t_2 - t_1 = \tau$.

(2) Let $n_1 - n_2 = \delta$.

(3) Let the probability that a particular spermatozoon, in a at time t , will leave in the interval $(t, t + \delta t)$ be $\lambda \delta t + o(\delta t)$.

(4) Let the probability that one spermatozoon will arrive in a during $(t, t + \delta t)$ be $\mu \delta t + o(\delta t)$.

Note. (3) and (4) imply that the spermatozoa do not drift or age.

(5) Let $E(n)$ be the average number of spermatozoa in a .

The average number of spermatozoa leaving a in δt is $\lambda E(n) \delta t$, while the average number entering a in the same time is $\mu \delta t$. When the system is in equilibrium,

$$\lambda E(n) = \mu. \quad (1)$$

Since $E(n_1) = E(n_2)$, $V(n_1) = E(n_1^2) - E^2(n_1)$, $V(n_1) = V(n_2)$
 and $\text{corr}(n_1, n_2) = \{E(n_1 n_2) - E(n_1) E(n_2)\} / \{V(n_1) V(n_2)\}^{\frac{1}{2}}$,
 $E(\delta^2) = 2V(n_1) \{1 - \text{corr}(n_1, n_2)\}$. (2)

If the number of spermatozoa in a is distributed Poisson-wise, $V(n_1) = E(n_1) = E(n)$ and $\text{corr}(n_1, n_2) = e^{-k\tau}$, where k is a constant.* To determine k , consider an infinitesimal value of τ , δt , so that $e^{-k\tau} = 1 - k\delta t + o(\delta t)$. Let n_1 and n_2 be the number of spermatozoa present at t and $t + \delta t$ and $p(n_1, n_2)$ be the probability of these numbers. δt is selected in such a way (i.e. so small) that the only values of $p(n_1, n_2)$ which are not zero are

$$\left. \begin{aligned} p(n_1, n_1 + 1) &= p(n_1) \mu \delta t, \\ p(n_1, n_1 - 1) &= p(n_1) \lambda n_1 \delta t, \\ p(n_1, n_1) &= p(n_1) \{1 - (\mu + \lambda n_1) \delta t\}. \end{aligned} \right\} \quad (3)$$

Note. For fixed n_1 , the average number of spermatozoa leaving a in δt is $\lambda n_1 \delta t$, while the average number entering a is $\mu \delta t$. Hence the probability of none entering and none leaving a in δt is $1 - (\mu + \lambda n_1) \delta t$.

Using eqs. (1) and (3),

$$E(n_1 n_2) = -\lambda E(n) \delta t + E(n) + E^2(n). \quad (4)$$

As in a Poisson distribution

$$\text{corr}(n_1, n_2) = \{E(n_1 n_2) - E^2(n)\} / E(n). \quad (5)$$

From equ. (4) $\text{corr}(n_1, n_2) = 1 - \lambda \delta t$. (6)

But $\text{corr}(n_1, n_2) = e^{-k\tau} = 1 - k \delta t$.

Hence $k = \lambda$ and $E(\delta^2) = 2E(n) (1 - e^{-\lambda\tau})$. (7)

Consider a spermatozoon initially within a . Divide the time interval $t, t + \tau$ into N subintervals of length τ/N . The probability $(1 - P)$ that the spermatozoon will remain within a , throughout the interval under consideration, is

$$\lim_{n \rightarrow \infty} \left(1 - \frac{\lambda\tau}{N}\right)^N = e^{-\lambda\tau}. \quad (8)$$

Hence $E(\delta^2) = 2E(n) P$, (9)

where P is the probability that a spermatozoon will have left a during the interval, but may be back again in a at the end of the interval. Chandrasehkar defines P in a different and slightly less accurate way. Mr D. V. Lindley, of the Statistical Laboratory, Cambridge, drew my attention to this point.

3. Meaning of λ

Let Y = number of spermatozoa arriving in a in δt , Y taking values 0, 1, 2, ...
 If δt is sufficiently small,

$$\Pr\{Y = 1\} = \mu \delta t, \quad (1)$$

$$\Pr\{Y = 0\} = 1 - \mu \delta t, \quad (2)$$

* See Appendix, 4, for the proof of this relationship.

$\Pr\{Y=m\}$, $m \geq 2$ being neglected,

$$E(Y) = (0 \times \overline{1 - \mu \delta t}) + (1 \times \mu \delta t), \quad (3)$$

$$= \mu \delta t. \quad (3.1)$$

$E(Y)$ is the average number of spermatozoa arriving in a in δt . But from kinetic theory, the average number of spermatozoa which collide with the periphery of a , i.e. arrive in a , in δt is

$$Z \delta t l, \quad (4)$$

where $Z = d\bar{c}/\pi$ and l is the length of the periphery of a . d is the number of spermatozoa per unit area.

$$\text{Hence} \quad \mu \delta t = Z \delta t l, \quad (5)$$

$$\text{or} \quad \mu = Zl. * \quad (5.1)$$

Suppose a is a rectangle with sides x' and y' . Then

$$l = 2(x' + y') \quad \text{and} \quad \mu = 2d\bar{c}(x' + y')/\pi.$$

Now $\lambda = \mu/\nu$, where $\nu = dx'y'$. Then

$$\lambda = \frac{2\bar{c}(x' + y')}{\pi x' y'}, \quad (6)$$

$$\text{or, in the case of a square,} \quad \lambda = \frac{4\bar{c}}{\pi x'}. \quad (6.1)$$

$$\text{If } a \text{ is a circle of radius } r, \quad \lambda = \frac{2\bar{c}}{\pi r}. \quad (7)$$

4. Collision frequency in two dimensions

Consider a cm.^2 of a suspension of moving spermatozoa. Each spermatozoon has a velocity vector associated with it and each spermatozoon will have a representative point in velocity space with rectangular coordinates c_x, c_y . An element of area, surrounding a typical point \mathbf{c} in velocity space, is denoted by $\delta\mathbf{c}$. The points in velocity space will have a particular density, $f(\mathbf{c})$, which in general will vary from point to point and time to time. The number of points in $\delta\mathbf{c}$ will be $f(\mathbf{c}) \delta\mathbf{c}$. If instead of a unit area we start with an element of area $\delta\mathbf{r}$, the number of points in $\delta\mathbf{c}$ will be $f(\mathbf{c}) \delta\mathbf{c} \delta\mathbf{r}$. In $\delta\mathbf{c}$ there will be $f(\mathbf{c}) \delta\mathbf{c} \delta\mathbf{r}$ spermatozoa with velocities in the velocity class $\delta\mathbf{c}$. The relationship between the velocity space density and the spermatozoa's speed frequency function $g(c)$ is, for unit area and an annulus $\delta\mathbf{c}$ of width δc ,

$$f(\mathbf{c}) \delta\mathbf{c} = dg(c) \delta c, \quad (1)$$

where d = number of spermatozoa per unit area. Equ. (1) can be written

$$f(\mathbf{c}) = \frac{dg(c)}{2\pi c}. \quad (1.1)$$

* There are certain mathematical assumptions 'hidden' in this equation; they will be discussed elsewhere, as will be the implications of the approximate nature of the model.

Consider an infinitesimal element of length ds , with spermatozoa moving through ds , with velocities in the velocity class $\delta\mathbf{c}$. The number of spermatozoa of class $\delta\mathbf{c}$ in a strip of area $ds \cos \psi c dt$ will be

$$f(\mathbf{c}) dsc \cos \psi dt d\mathbf{c} \tag{2}$$

$$=f(\mathbf{c}) dsc_n dt d\mathbf{c}, \tag{2.1}$$

where c_n = component of c in the direction perpendicular to ds and $\delta\mathbf{r} = ds \cos \psi c dt$. The number of spermatozoa N of all classes passing from one side of ds to the other, in the same direction (i.e. $c_n > 0$), is

$$N = ds dt \int_{c_n > 0} f(\mathbf{c}) c_n d\mathbf{c}. \tag{3}$$

Making c_n identical with c_y ,

$$N = ds dt \int_{c_y > 0} f(\mathbf{c}) c_y d\mathbf{c} \tag{3.1}$$

$$= ds dt \int_{c_y > 0} \frac{dg(c)}{2\pi c} c_y d\mathbf{c}. \tag{3.2}$$

In polar co-ordinates

$$N = \frac{dds dt}{2\pi} \int_0^\infty \int_0^\pi g(c) c \sin \theta d\theta dc. \tag{3.3}$$

The number of spermatozoa passing through unit length in unit time (from one side to the other) is therefore

$$Z = \frac{d}{\pi} \int_0^\infty cg(c) dc \tag{4}$$

$$= \frac{d\bar{c}}{\pi}. \tag{4.1}$$

5. $\text{corr}(n_1, n_2) = e^{-kt}$

Let n_1, n_2, n_3 spermatozoa be observed at $t, t+t_1, t+t_1+t_2$. Let $p(n_1 | n_2)$ denote the probability of n_1 at t , given n_2 at $t+t_1$ (and other expressions similarly, e.g. $p(n_3 | n_2, n_1)$). Then

$$p(n_3 | n_2) = p(n_3 | n_2, n_1) \tag{1}$$

and $E(n_1 n_3 | n_2)$, the average of $n_1 n_3$ for fixed n_2 ,

$$= \sum_{n_1=0}^\infty \sum_{n_3=0}^\infty n_1 n_3 p(n_1, n_3 | n_2) \tag{2}$$

$$= E(n_1 | n_2) E(n_3 | n_2). \tag{3}$$

Hence the partial correlation coefficient of n_1 and n_3 for fixed n_2 is zero and by standard formulae for partial correlation coefficients,

$$\text{corr}(n_1, n_3) = \text{corr}(n_1, n_2) \text{corr}(n_2, n_3), \tag{4}$$

provided $E(n_2 | n_1) = an_1 + b$.

The implication of equ. (1) is that once n_2 is known, n_1 has no predictive value in relation to n_3 . This is known as the Markov property (see, for example, Bartlett, 1950).

Let $g(t)$ be the correlation coefficient of a system of spermatozoa at two times, t apart. Equ. (4) can be written

$$g(t_1 + t_2) = g(t_1) g(t_2), \quad \text{for all } t_1 \text{ and } t_2 \geq 0. \quad (5)$$

The only solution of this equation with $g(0) = 1$ and $g(t) \leq 1$ is

$$g(t) = e^{-kt}. \quad (6)$$

SUMMARY

1. A new and quantitative method of estimating the average speed of a suspension of spermatozoa has been developed. The method, in which the phenomenon known as probability-after-effect and kinetic theory principles are made use of, can be applied to any system of organisms which move in random directions, in one, two or three dimensions.

2. The experimental procedure is to place a drop of sperm suspension on a microscope slide with a cover-slip on top of the drop, and photograph the spermatozoa at known time intervals.

3. The number of spermatozoa in a region of known size is counted on each photograph. The estimated mean or average speed of the sperm suspension, \bar{c} , is given by the equation

$$\bar{c} = -(\pi r / 2\tau) \log_e \left\{ 1 - \frac{\overline{\delta^2}}{2\bar{n}} \right\},$$

where r is the radius of a circular region in which the number of spermatozoa is counted, τ is the time interval between photographs, $\overline{\delta^2}$ is the average of the square of the differences between the numbers of spermatozoa counted on consecutive photographs, and \bar{n} is the average number of spermatozoa in the circular region.

4. Appropriate formulae for non-circular regions and for the precision of estimates of \bar{c} are given.

5. A method of testing whether the directions of movement of spermatozoa are random has been applied to bull semen diluted 1/4 with phosphate buffer containing fructose. The movements were found to be random.

6. The distribution of sperm speeds was determined and found to be somewhat skew and leptokurtic, with mean 123 μ /sec. and standard deviation 39. If dead or motionless spermatozoa were included, the mean speed became 117 μ /sec.

7. Using the probability-after-effect equation given in (3), the mean speed of the suspension, including dead or motionless spermatozoa, was found to be 111 μ /sec., with a standard error of 24.5. The standard error and therefore the precision of the estimate is under the control of the experimenter.

8. Practical instructions for carrying out measurements are summarized in a separate section.

I am greatly indebted to Prof. M. S. Bartlett for reading the typescript of this paper and making a number of important comments; also to the Medical Research Council for the provision of a laboratory assistant.

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