THE LOCOMOTION OF MOUSE FIBROBLASTS IN TISSUE CULTURE

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ABSTRACT Time-lapse cinematography was used to investigate the motion of mouse fibroblasts in tissue culture. Observations over successive short time intervals revealed a tendency for the cells to persist in their direction of motion from one 2.5 hr time interval to the next. Over 5.0-hr time intervals, however, the direction of motion appeared random. This fact suggested that D, the diffusion constant of a random walk model, might serve to characterize cellular motility if suitably long observation times were used. We therefore investigated the effect of "persistence" on the pure random walk model, and we found theoretically and confirmed experimentally that the motility of a persisting cell could indeed be characterized by an augmented diffusion constant, D^*. A method for determining confidence limits on D^* was also developed. Thus a random walk model, modified to comprehend the persistence effect, was found to describe the motion of fibroblasts in tissue culture and to provide a numerical measure of cellular motility.

I. INTRODUCTION

Abercrombie has suggested that the locomotory behavior of malignant cells differs from that of normal cells (1). Embryogenesis and inflammatory processes also dramatize the importance of understanding cellular locomotion. Abercrombie pioneered the use of serial (i.e. "time-lapse") photography for studying locomotory behavior, and he characterized the motility of chick heart fibroblasts in terms of a simple velocity estimate based on the distance traveled in a given unit of time (2). But he recognized that this measurement was "biased" and was only a valid measure of cell motility if the locomotion were linear and uniform (2).

Ambrose noted "that movements of cells when isolated and spreading on a glass are largely at random" (3). We recognized the attractiveness of a two-dimensional random walk model of cell motion. This model could be used to characterize cellular motility in terms of a single constant—D, the diffusion constant. We therefore sought to test the hypothesis that fibroblasts in tissue culture executed a pure two-dimensional random walk.

We measured the angles between successive displacements of mouse fibroblasts

over consecutive 2.5-hr time intervals, and we noted a mild tendency for the cells to persist in their direction of motion. However, we detected no such persistence over successive 5.0-hr intervals. This fact suggested that a random walk model might describe the motion, persistence notwithstanding, if suitably long observation times were employed. We showed theoretically that a "persisting" cell, like a pure random walker, undergoes a mean square displacement proportional to time provided only that suitably long time intervals are used. The proportionality constant is $4D^*$, where D^* is an augmented diffusion constant which subsumes the persistence effect. Thus measurements of the mean square distance traveled as a function of time provided a convenient measure of cellular motility.

The pure two-dimensional random walk model predicts an exponential distribution of displacements (see Appendix I). Surprisingly, this prediction fits the observed data quite well even for time intervals as short as 2.5 hr during which the cells are known to demonstrate persistence. Evidently, the exponentiality property of the random walk is not very sensitive to the persistence effect. We were able to use the proved exponentiality property to estimate D^* by the method of maximum likelihood (see Appendix III).

Thus, mouse fibroblasts were not "pure" random walkers but instead persisted in their direction of motion. By modifying the random walk model to comprehend the persistence effect, we were able to characterize the motility of these persisting cells in terms of D^*, the augmented diffusion constant.

II. THEORY

In this section we outline the pure random walk model. Then we discuss the effect of persistence on this model. Appendix I contains a rigorous derivation of the two-dimensional random walk model, and Appendix II contains an analysis of the persistence effect. Methods for testing the random walk hypothesis are included in the Results section.

A. Pure Two-Dimensional Random Walk Model

We seek an expression for T^2, the square displacement in time t. The model is probabilistic, and we show (see Appendix I) that the probability of a given square displacement is given by

$$f(T^2)\,dT^2 = \frac{1}{4Dt}\exp\left(\frac{-T^2}{4Dt}\right)dT^2, \tag{1}$$

where D is the diffusion constant and f is an exponential probability distribution. From the properties of the exponential distribution we note that the mean square displacement, $\langle T^2\rangle$, is given by

$$\langle T^2\rangle = 4Dt. \tag{2}$$

B. The Effect of Persistence on Square Displacement

It seems intuitively clear that a cell which tends to persist in its direction of motion will undergo a larger displacement in a given time, t, than would the same cell without directional persistence. In Appendix II we confirm theoretically that the mean square displacement of a persisting cell always exceeds that expected of a similar cell without persistence. Indeed, for large t, the persistence effect reduces to the simple relationship

$$\langle T^2 \rangle = 4Dt(1 + \rho) \tag{3}$$

where ρ is a positive constant measuring the persistence tendency and D is the diffusion constant in the absence of persistence. If we define D^*, the augmented diffusion constant, by

$$D^* = D(1 + \rho) = \lim_{t \to \infty} \langle T^2 \rangle / 4t,$$

then, for large t, equation 3 becomes

$$\langle T^2 \rangle = 4D^*t, \tag{4}$$

which is formally identical to equation 2, the equation governing the pure random walk.

Unfortunately, such long observation times are not experimentally feasible since the doubling time of these cells is 20–35 hr. We were able to estimate D^*, nonetheless, by modifying the two-parameter persistence model of Fürth (4). Fürth derived a limiting expression for the mean square displacement of a one-dimensional persistent random walker. By extending his results to two dimensions, and by finding a correspondence between D^* and Fürth's parameters, we arrived at the model

$$\langle T^2 \rangle = 4D^* (t - t^*(1 - \exp(-t/t^*))), \tag{5}$$

where t^* and D^* are parameters to be estimated from the data. For large t, this equation reduces to equation 4. However, for only moderately large t, equation 5 simplifies to

$$\langle T^2 \rangle = 4D^*(t - t^*). \tag{6}$$

Because most of the experimentally accessible data is described by this linear locus, equation 6 can be used for estimating D^* and t^* (see section IV E and Appendix III).

III. METHODS

A. Cell Culture and Photography

A line of mouse fibroblasts (Balb 3T3–4/clone 31) was kindly provided by Drs. S. Aaronson and G. Todaro (5). The line was maintained at subconfluent density at 37°C in Dulbecco-

Vogt medium containing 10% fetal calf serum (Hyland Div., Travenol Labs., Inc., Costa Mesa, Calif.), glutamine (2 μmoles/ml), penicillin (50 μg/ml) and streptomycin (50 μg/ml).

T-15 culture flasks (Bellco Glass, Inc., Vineland, N. J., catalog No. 1015, size T-15 flask) were cleaned by soaking in a 70% sulfuric-30% nitric acid bath for 48 hr at room temperature. The flasks were then immersed in a sodium hexametaphosphate detergent solution (Calgolac, produced by Calgon Corp., Pittsburgh, Pa.) which was heated to 90°C and then allowed to cool. The flasks were then rinsed 10 times with distilled water and dry heat sterilized. The T-15 flasks had a bottom surface area of 15 cm^2.

In preparation for time lapse photography, cell monolayers were briefly rinsed with 0.1% trypsin (Grand Island Biological Co., Grand Island, N. Y.) in phosphate-buffered saline; detachment of the cells occurred after 15 min. 1.5×10^5 cells in 0.1 ml of trypsin solution were then transferred to a T-15 flask containing 3.4 ml medium, and the cells were allowed to grow for 24 hr. The supernatant was then decanted and 3.5 ml of fresh medium added 1 hr before time-lapse photography began. The medium filled the T-15 flask to a depth of 2.3 mm.

A thermocouple placed below the medium level of the T-15 flask was attached to an air curtain heat regulator (model 279, Sage Instruments, Inc., White Plains, N. Y.). The microscope was shrouded in a large plastic cover, and the microscope stage and T-15 were in thermal equilibrium at 37°C well before time-lapse photography began.

Serial photographs of a fixed 0.69 mm^2 field were taken at 2-min intervals beginning 1 hr after the final medium change (see above). A Nikon model M phase contrast microscope (Nikon Inc., Instrument Div., Garden City, N. Y.) with 10× objective lens presented an image to the time-lapse camera (model 16S, Arriflex Corporation of America, Woodside, N. Y.) via a Leitz 2.5× projection eyepiece (E. Leitz, Inc., New York) and an Arriflex ⅓× reduction lens. After five days' filming, the medium was changed and filming resumed on another field for five days.

B. Measurements

The films were projected with a Kodak analyzer (model 224A, Eastman Kodak Co., Rochester, N. Y.), at a standard projector-screen distance of 4.46 m. Dimensions on the screen were calibrated by photographing a stage micrometer. 1 cm on the projector screen corresponded to 13.37 μ on the microscope stage and 1 cm^2 to 178.7 μ^2.

A growth curve was obtained by counting the number of cells within a standard 20 x 30 inch screen area as a function of time elapsed.

Each cell within the field of frame *1150* (38.3 hr after beginning of time-lapse photography) was observed for 12.5 hr, and if no mitosis occurred in that period, the position of that cell was charted at specified time lapse intervals. Fig. 1 exemplifies the movement of one cell over three such intervals.

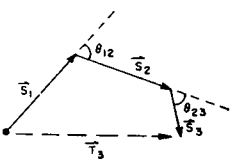

FIGURE 1 Schematic depiction of the motion of a cell over three time intervals. $\mathbf{T}_3$ represents the total displacement, $\mathbf{S}_i$ the segmental displacement corresponding to the ith time interval, and θ_{ij} the intersegmental angle between $\mathbf{S}_i$ and $\mathbf{S}_j$.

If we define the vector $\mathbf{S}_i$, as the directed segment corresponding to the total displacement in the ith time interval and if we define $\mathbf{T}_i$ as the total displacement from the origin to the ith vertex (corresponding to the end of the ith time interval), then the measurements made can be expressed as (*a*) measurements of the angles between successive segments, and (*b*) measurements of the magnitudes of the displacement vectors, $|\mathbf{T}_i|$. A protractor model 183, (The L. S. Starrett Co., Athol, Mass.) was used for angle measurements, and a transparent millimeter ruler for distance measurements.

IV. RESULTS

A. Cell Density

Fig. 2 is a plot of the logarithm of cell density (in cells/mm²) as a function of time. The time interval used for all locomotion studies is also indicated. As shown, the cell density was in the range 90–110 cells/mm² during locomotion studies. The average "contact number" (2) was estimated as 0.73 contacts/cell during locomotion studies (40 contacts were noted among 55 cells). The plot shows further that the cells were growing exponentially and were therefore healthy during locomotion studies.

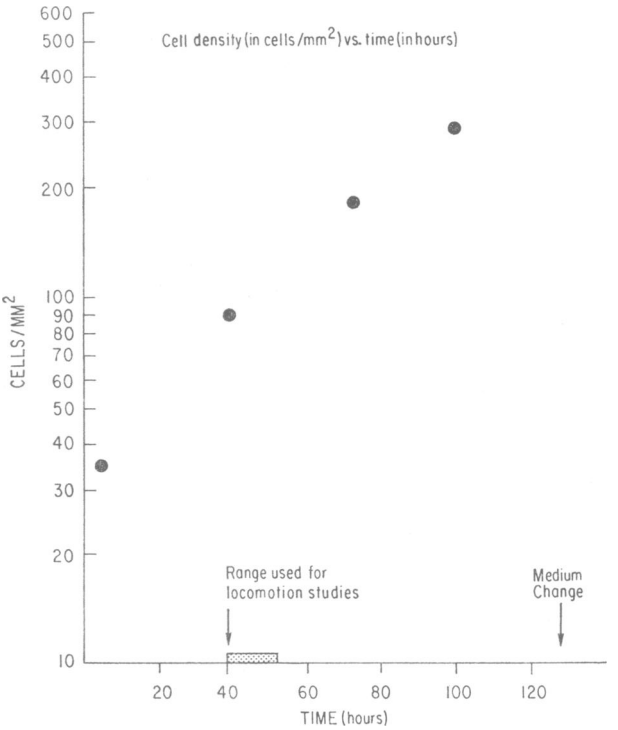

FIGURE 2 Growth curve showing that locomotion studies were conducted during exponential growth at cell densities of 90–110 cells/mm².

B. Intersegmental Angle Measurements

Measurements of the angles between successive segments, S_i, provided the most powerful evidence against the pure random walk hypothesis; these measurements proved that the direction of motion of cells is not perfectly random if the segmental displacements are observed for 2.5-hr time intervals. Fig. 3 is a histogram of 80 such intersegmental angle measurements. Most of the measurements fall within the $-100°$ to $+100°$ range. To test the hypothesis of equiprobable intersegmental angles, we employed the Z statistic used to test whether $K = 0$, where K is a parameter of the "circular normal distribution" (6). Our Z value of 7.84 was highly significant ($Z > 4.57$ is the critical region at the 1% significance level). Thus intersegsegmental angle measurements over successive 2.5-hr time intervals provide strong evidence against a pure random walk model. These measurements demonstrate persistence.

By contrast, intersegmental angle measurements made over 5.0-hr time intervals appear randomly distributed (see Fig. 4). The Z value for these measurements was

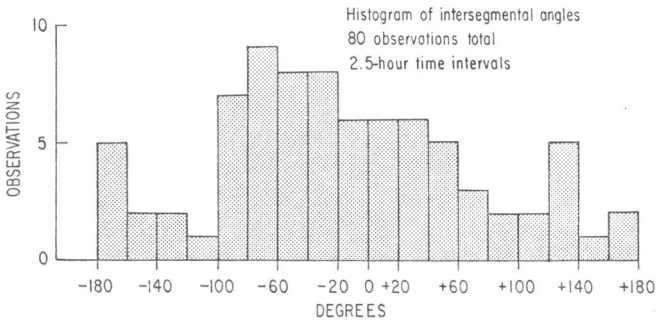

FIGURE 3 Histogram of intersegmental angle measurements made for 2.5-hr observation intervals. The distribution is nonuniform with clustering about 0°.

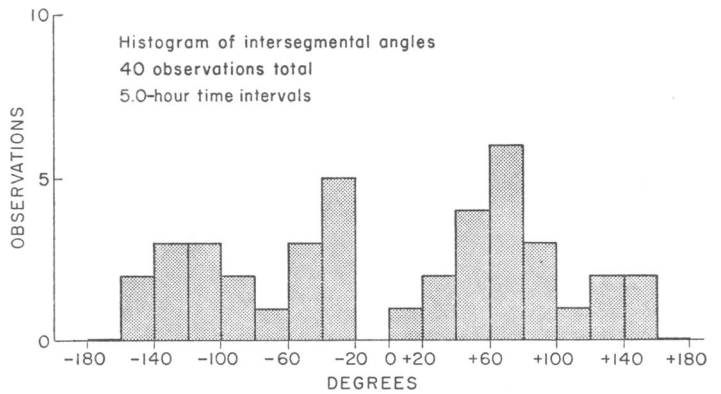

FIGURE 4 Histogram of intersegmental angle measurements made for 5.0-hr observation intervals. The distribution appears uniform.

0.768, well within the acceptance region for the hypothesis of equiprobable intersegmental angles ($Z > 2.977$ is the critical region at the 5% level). Thus the pure random walk hypothesis becomes tenable for observation times greater than 5.0 hr.

C. *Measurements of Square Displacements*

Table I presents the raw measurements (on the projector screen) of the distance traveled by each of 20 cells in five consecutive 2.5-hr time intervals and Fig. 5 is a histogram of the squares of the observed displacements. This histogram appears to follow the exponential distribution of the random walk model (equation 1). To test for exponentiality, a chi-square goodness of fit test with 12 intervals was employed, and the value $\chi^2 = 13.35$ was computed. This is well within the acceptance region ($\chi^2 > 19.67$ defines the 5% critical region). Thus measurements of square displacements over 2.5-hr time intervals provide no evidence against the random walk hypothesis and, indeed, appear to conform to the predicted exponential distribution. Certainly, therefore, measurements of square displacements over larger time intervals should conform to the exponential distribution (since even the more powerful

TABLE I

RAW MEASUREMENTS OF THE DISPLACEMENTS (SEGMENT LENGTHS $|S_i|$) OBSERVED FOR EACH CELL IN EACH OF FIVE SUCCESSIVE 2.5-hr INTERVALS

Original projector screen dimensions (in centimeters) are used.

Cell number	Time interval (hr)				
	0–2.5	2.5–5.0	5.0–7.5	7.5–10.0	10.0–12.5
	cm	*cm*	*cm*	*cm*	*cm*
1	4.5	4.3	5.0	5.4	5.8
2	2.4	7.3	4.0	2.7	4.5
3	2.5	5.1	6.3	7.1	2.8
4	2.1	2.9	8.4	5.1	3.3
5	4.0	1.7	0.9	3.3	4.9
6	6.5	1.0	4.9	0.8	2.2
7	3.6	5.8	3.7	5.6	3.0
8	3.6	4.1	3.6	7.4	5.1
9	7.9	2.8	2.2	5.5	1.9
10	6.6	6.6	3.8	5.3	8.7
11	4.3	5.0	4.3	7.0	6.0
12	1.6	1.8	2.9	1.3	2.5
13	3.3	5.4	4.9	2.5	5.2
14	4.0	3.6	5.2	5.0	2.9
15	4.7	8.6	7.8	7.1	5.2
16	4.0	6.4	3.2	1.0	0.6
17	4.0	4.3	4.6	2.8	3.9
18	5.0	4.4	4.5	3.5	2.7
19	6.8	5.1	3.6	7.2	5.1
20	9.6	8.3	6.5	2.9	2.8

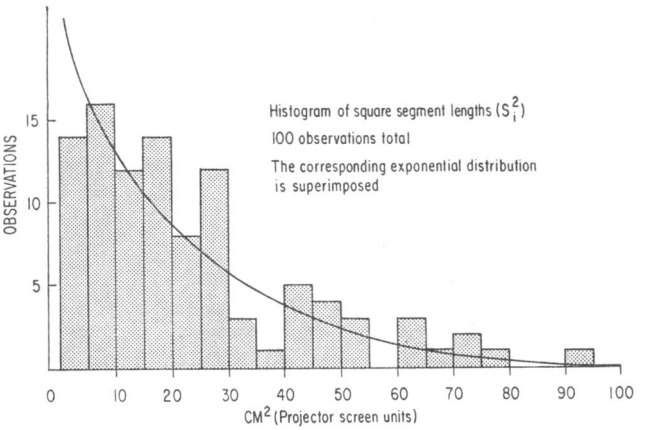

FIGURE 5 Histogram of 100 square displacements measured for 2.5-hr observation intervals. The histogram conforms to an exponential distribution.

tests based on intersegmental angle measurements are consistent with the random walk hypothesis for time intervals greater than 5.0 hr). Thus the test for exponentiality is a less powerful test of the random walk hypothesis than the test based on intersegmental angles, and, even at 2.5-hr time intervals, the distribution of square displacements appears exponential.

D. Tests for Homogeneity of the Cell Population

Since our data was generated by 20 different cells, we sought to test the hypothesis that all the cells had the same diffusion constant D^* against the alternative hypothesis that the cells had differing diffusion constants. To do so we employed the Moran statistic for testing whether the square displacements observed for each cell were from the same exponential distribution against the alternative hypothesis that they were all from different exponential distributions corresponding to different diffusion constants (see reference 7; references 8–10 are also pertinent). No significant results were obtained, indicating that the variability in square displacements observed from one cell to the next was no greater than expected by chance (given that the samples were from an exponential distribution). This was so for all time intervals observed (from ½ hr to 10 hr). Thus we were justified in regarding square displacements corresponding to each cell observed as independent samples from the same exponential distribution. In other words, we could justify using a single diffusion constant D^* (together with confidence limits) to characterize the motility of the whole population.

E. Estimates of D^*, the Augmented Diffusion Constant

In section III B we defined $\mathbf{T}_i$, the displacement vector, as the total displacement a given cell undergoes in time t, where t is the time from $t = 0$ to the end of the ith

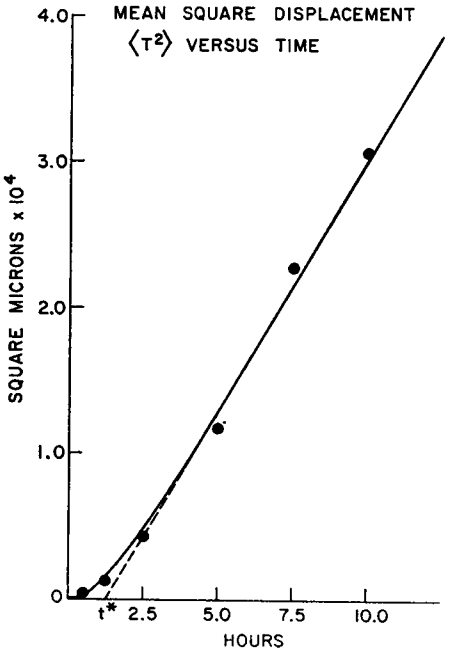

FIGURE 6 Mean square displacement, T^2, is plotted as a function of observation time. The locus of equation 5 with $D^* = 858\ \mu^2/\text{hr}$ and $t^* = 1.23$ hr is also shown (solid line). The dashed line indicates linear extrapolation to the time intercept, t^*.

time interval. $\langle T^2 \rangle_{obs}$, the observed mean square value of 20 such displacements (corresponding to 20 different cells), is plotted against time in Fig. 6. Also plotted is the locus of equation 5 where D^* and t^* were estimated as $D^* = 858$ square μ/hr and $t^* = 1.23$ hr. These estimates are obtained by examining the linear portion of the locus and identifying this with equation 6. The t intercept of this line is t^* and the slope is $4D^*$. Graphical solutions yielded the estimates $D_0^* = 857\ \mu^2$ and $t_0^* = 1.25$ hr. These estimates are good enough for most purposes. A more rigorous maximum likelihood estimation procedure, derived in Appendix III, can be used to estimate the "best" straight line. This procedure yielded the estimates $D^* = 858\ \mu^2/\text{hr}$ and $t^* = 1.23$ hr used to plot the locus of equation 5 in Fig. 6.

In Appendix III we show how the exponentiality property (see section IV C) can be used to determine confidence limits on D^*. Our (conservative) estimate of the 95% confidence interval is thus $521\ \mu^2/\text{hr} < D^* < 1540\ \mu^2/\text{hr}$.

V. DISCUSSION

These experiments demonstrate that mouse fibroblasts in tissue culture move neither in a perfectly uniform nor in a perfectly random manner. Rather, they show an intermediate tendency to persist in their direction of motion over successive 2.5-hr time intervals. This persistence effect, demonstrated by intersegmental angle measurements, is not detectable for 5.0-hr time intervals, suggesting that a pure random walk model would describe the motion of the cells over very large time intervals. We therefore investigated the properties of persisting cells theoretically and found

that the mean square displacement of such cells is proportional to time if large enough time intervals are used. Thus we could characterize the motility of such cells in terms of an augmented diffusion constant D^* (see equation 4). Unfortunately, the time intervals accessible to experiment were too short to permit use of the simple equation $\langle T^2 \rangle = 4D^*t$ (equation 4). However, by modifying the one-dimensional model of Fürth (4), we were able to obtain an equation (equation 5) which provided excellent agreement with the observed mean square displacements (see Fig. 6). The two parameters, t^* and D^*, of this model, were estimated graphically and by maximum likelihood procedures (Appendix III).

The exponentiality property predicted by the pure random walk model seems remarkably insensitive to persistence. Square displacements observed for 2.5-hr intervals, during which persistence was demonstrated by intersegmental angle measurements, seem to "fit" the exponential distribution quite well, persistence notwithstanding (see section IV C). Moreover, the cell populations seem homogeneous, justifying the assumption that all observed square displacements are independent samples from the same exponential distribution (see section IV D). These two experimentally supported assumptions (exponentiality and homogeneity) permit one to derive the probability distribution of the mean square displacement, and, in consequence, to obtain maximum likelihood estimates of D^* and t^* (see Appendix III). In addition, the exponentiality property is used for estimating 95% confidence limits on D^* (Appendix III).

The most important tool deriving from this work is an ability to characterize the motility of persisting cells in terms of a single constant, D^*, the augmented diffusion constant.

SUMMARY

Time-lapse cinematography was used to investigate the motion of mouse fibroblasts in culture over a 10.0-hr time interval. For cells in the exponential growth phase at cell densities approximating 100 cells/square mm, it was established that the fibroblasts tend to persist in their direction of motion from one 2.5 hr time interval to the next. A theoretical investigation of persisting cells revealed that their motility could be characterized by a single constant, D^*, the augmented diffusion constant. A two parameter model, based on the work of Fürth (4), was in excellent agreement with observed mean square displacements. Using this model, the augmented diffusion constant, D^*, was estimated by maximum likelihood techniques, and a method for determining confidence limits on D^* was also developed. A random walk model, modified to comprehend the persistance effect, was thus found to describe the motion of fibroblasts in tissue culture.

APPENDIX I

The Pure Two-Dimensional Random Walk Derivation

We seek an expression for the probability function governing T^2, the square of the distance traveled in a given time interval. We begin with the widely known result that $(X - X_0)/2Dt$

has a standard normal distribution where $(X - X_0)$ is the distance traveled from the origin by a particle executing a one-dimensional random walk for a time t, and D is the diffusion constant (11). If there is no persistence, a formally identical and independent description applies to the y coordinate. Hence the quantity $(X - X_0)^2/2Dt + (Y - Y_0)^2/2Dt$ is the sum of the squares of two independent standard normal distributions and is therefore distributed as a chi-square distribution with two degrees of freedom (12). Equation AII 1 follows.

APPENDIX II

The Effect of Persistence

Consider a cell which traces out a path of connected segments, $\mathbf{S}_1, \mathbf{S}_2, \cdots \mathbf{S}_n$ as shown in Fig. 1. We assume the motion is confined to the plane. The total displacement vector $\mathbf{T}$ is given by $\mathbf{T} = \mathbf{S}_1 + \mathbf{S}_2 + \cdots \mathbf{S}_n$. The square displacement is thus

$$\mathbf{T}\cdot\mathbf{T} = T^2 = \sum_{i=1}^{n} \mathbf{S}_i \sum_{j=1}^{n} \mathbf{S}_j ,$$

or

$$T^2 = \sum_{i=1}^{n} \sum_{j=1}^{n} |\mathbf{S}_i|\,|\mathbf{S}_j| \cos\theta_{ij} , \qquad \text{AII 1}$$

where θ_{ij} is the angle between the ith and jth segments. If we assume that θ_{ij} and $|\mathbf{S}_k|$ are uncorrelated for all i, j, k, the mean square displacement is given by

$$\langle T^2\rangle = \langle S^2\rangle \sum_{i=1}^{n} \sum_{j=1}^{n} \langle\cos\theta_{ij}\rangle. \qquad \text{AII 2}$$

Since $\theta_{ii} = 0$, $\langle\cos\theta_{ii}\rangle = 1$ and $\langle\cos\theta_{ij}\rangle = \langle\cos\theta_{ji}\rangle$,

$$\langle T^2\rangle = \langle S^2\rangle n + \langle S^2\rangle 2 \sum_{i<j} \sum_{j=1}^{n} \langle\cos\theta_{ij}\rangle. \qquad \text{AII 3}$$

We define $\alpha_k = \langle\cos\theta_{ij}\rangle$ where $k = |j - i|$. $(k - 1)$ is thus the number of intervening segments between the ith and jth segments, and α_k measurements the directional persistence connecting those segments. In terms of α_k,

$$\langle T^2\rangle = n\langle S^2\rangle + \langle S^2\rangle(2) \sum_{k=1}^{n-1} \alpha_k(n - k). \qquad \text{AII 4}$$

We consider the case of the pure random walk. Thus $\alpha_k = 0$ for $k = 1, 2, \cdots (n - 1)$. Then $\langle T^2\rangle = n\ \langle S^2\rangle$. We now convert to a continuous time scale by introducing the variable τ, the average time required by a cell to trace out a microscopic segment, $\mathbf{S}_i$. Thus t, the macroscopic continuous time variable, is given by $t = n\tau$, where n is the number of segments traced out in time t. We express D, the diffusion constant, as the limit

$$D = \lim_{\substack{\langle S^2\rangle \to 0 \\ n\to\infty \\ \tau\to 0}} \frac{\langle S^2\rangle}{4\tau}. \qquad \text{AII 5}$$

Thus, in the limit,

$$\langle T^2 \rangle = \langle S^2 \rangle n = 4Dt,$$

which agrees with equation 12.

We now consider the case of total persistence (i.e. uniform motion). Thus $\alpha_k = 1$ for $k = 1, 2, \cdots (n-1)$. It follows that

$$\langle T^2 \rangle = n\langle S^2 \rangle + 2\langle S^2 \rangle \sum_{k=1}^{n-1} (n-k),$$

$$\langle T^2 \rangle = n\langle S^2 \rangle + 2\langle S^2 \rangle \left\{ n(n-1) - \sum_{k=1}^{n-1} k \right\},$$

$$= n\langle S^2 \rangle + 2\langle S^2 \rangle \left\{ n(n-1) - \frac{n(n-1)}{2} \right\},$$

$$= n^2 \langle S^2 \rangle.$$

In the case of uniform motion we are justified in taking the limit

$$\lim_{\substack{|\mathbf{S}| \to 0 \\ \tau \to 0}} \frac{|\mathbf{S}|}{\tau} = V, \qquad \text{AII 6}$$

where V is a velocity constant. Thus

$$\langle T^2 \rangle = \left(\frac{t}{\tau} \right)^2 V^2 \tau^2 = V^2 t^2.$$

Thus in the case of complete persistence we get the mean square displacement expected for uniform motion.

Now we consider the intermediate case in which $\alpha_k > 0$ for $k = 1, 2, \cdots m$ and $\alpha_k = 0$ for $k > m$. It is assumed that m is finite and $m < (n-1)$. This case represents a statistical "tendency to persist." Rewriting equation AII 4,

$$\langle T^2 \rangle = n\langle S^2 \rangle + 2\langle S^2 \rangle n \sum_{k=1}^{m} \alpha_k - 2\langle S^2 \rangle \sum_{k=1}^{m} k\alpha_k . \qquad \text{AII 7}$$

Since

$$n \sum_{k=1}^{m} \alpha_k > \sum_{k=1}^{m} k\alpha_k,$$

$\langle T^2 \rangle > n \langle S^2 \rangle$. Taking the usual limits ($\langle S^2 \rangle \to 0$, $n \to \infty$, $\tau \to 0$, $n\tau = t$) we find $\langle T^2 \rangle < 4Dt$. In other words, the mean square displacement exceeds that expected in the absence of persistence. Rewriting equation AII 7 under the same limiting conditions, we find

$$\langle T^2 \rangle = 4Dt \left(1 + 2 \sum_{k=1}^{m} \alpha_k \right) - (2/n) \sum_{k=1}^{m} k\alpha_k .$$

We note that both summations are over finite sequences and thus converge. Since $n = t/\tau$, n gets large as t increases for any fixed (small) τ. Thus, in the limit as

$$t \to \infty, \langle T^2 \rangle = 4Dt\left(1 + 2\sum_{k=1}^{m} \alpha_k\right). \qquad \text{AII 8}$$

This corresponds to equation 3 if we identify ρ with $2\sum_{k=1}^{m} \alpha_k$. If there is no persistence, all $\alpha_k = 0$ and $\rho = 0$. Otherwise, the effect of persistence is to increase the (limiting) proportionality relating $\langle T^2 \rangle$ to t. This fact justifies the concept of D^*, the augjmented diffusion constant, and

$$D^* = \lim_{t \to \infty} \langle T^2 \rangle / 4t = D(1 + \rho).$$

APPENDIX III

Maximum Likelihood Estimation of D^* and t^* and Estimation of Confidence Limits on D^*

A. Maximum Likelihood Methods. We recall from Section IV C that the individual T_i^2 are exponentially distributed with parameter λ, say. Thus the mean value of a series of n independent observations, T_i^2, will have the probability distribution

$$f(\langle T^2 \rangle)\, d\langle T^2 \rangle = \left(\frac{2n}{\lambda}\right)\left(\frac{2n\langle T^2 \rangle}{\lambda}\right)^{n-1} \frac{\exp\left(\frac{-n\langle T^2 \rangle}{\lambda}\right)}{\Gamma(n)2^n}. \qquad \text{AIII 1}$$

If several mean values $\langle T^2 \rangle_r$ are observed, each corresponding to a time t_r with n_r observations, and if we set $\langle T^2 \rangle_r = 4D^*(t_r - t^*)$, then assuming the $\langle T^2 \rangle_r$ are independent, the likelihood function, L, can be written

$$L = \prod_{r=1}^{m} f(\langle T^2 \rangle_r). \qquad \text{AIII 2}$$

Defining $Q = \ln(L)$, we note

$$Q = \sum_{r=1}^{m}\left\{\frac{-n_r\langle T^2 \rangle_r}{4D^*(t_r - t^*)} - n_r \ln\left(D^*(t - t^*)\right)\right\} \qquad \text{AIII 3}$$

plus terms which are independent of D^* and t^*. Solving $\partial Q/\partial D^* = 0$ and $\partial Q/\partial t^* = 0$ we get the maximum likelihood estimators $\hat{D}^*$ and $\hat{t}^*$. Because these equations are nonlinear, numerical methods are necessary for solution. In particular, one can repeatedly solve the following set of equations:

$$-\frac{\partial Q}{\partial D^*} = \frac{\partial^2 Q}{\partial^2 D^*}(\hat{D}^*_{j+1} - \hat{D}^*_j) + \frac{\partial^2 Q}{\partial t^* \partial D^*}(\hat{t}^*_{j+1} - \hat{t}^*_j),$$

$$-\frac{\partial Q}{\partial t^*} = \frac{\partial^2 Q}{\partial D^* \partial t^*}(\hat{D}^*_{j+1} - \hat{D}^*_j) + \frac{\partial^2 Q}{\partial^2 t^*}(\hat{t}^*_{j+1} - \hat{t}^*_j).$$

All partial derivatives are evaluated at $\hat{t}_j^*$ and $\hat{D}_j^*$, and the equations above are repeatedly solved to obtain the improved estimates $\hat{t}_{j+1}^*$ and $\hat{D}_{j+1}^*$ until satisfactory accuracy is obtained. Graphical estimates $\hat{D}_0^*$ and $\hat{t}_0^*$ (see section IV E) provide convenient initial values.

B. Estimation of Confidence Limits on D.* Again we rely on the fact that T_i^2 is exponentially distributed with parameter λ_r, and, using moment generating methods, we derive

$$\frac{\langle T^2\rangle_r}{\lambda_r} = \frac{\chi^2_{2n_r}}{2n_r}, \qquad \text{AIII 4}$$

where n_r is the number of independent observations used in calculating the mean value $\langle T^2\rangle_r$, and $\chi^2_{2n_r}$ is the chi-square distribution with $2n_r$ degrees of freedom. Defining $p_1 = 2n_r/\chi^2_{2n_r,0.025}$ and $p_2 = 2n_r/\chi^2_{2n_r,0.975}$, we find 95% confidence limits on λ_r from

$$\langle T^2\rangle_r p_2 < \lambda_r < \langle T^2\rangle_r p_1 . \qquad \text{AIII 5}$$

Identifying $\lambda_r = 4D^*(t_r - t)$, we find

$$\frac{\langle T^2\rangle_i}{4(t_r - t^*)} p_2 < D^* < \frac{\langle T^2\rangle_r}{4(t_r - t^*)} p_1 . \qquad \text{AIII 6}$$

If t^* were known exactly, then equation AIII 6 would define 95% confidence limits of D^*. Actually, our estimate of t^* is subject to a small uncertainty. However, we know from graphical evidence that $(t_r - t^*)$ is subject to less than 10% uncertainty. Thus a conservative estimate of 95% confidence limits on D^* is given by

$$(0.9)\frac{\langle T^2\rangle_r}{4(t_r - t^*)} p_2 < D^* < (1.1)\frac{\langle T^2\rangle_r}{4(t_r - t^*)} p_1 . \qquad \text{AIII 7}$$

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