The Rate of Proflavin Passage into Single Living Cells with Application to Permeability Studies

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ABSTRACT A technique for the measurement of the uptake rate of proflavin by the single cell in tissue culture has been developed, and the kinetics of the dye transport are discussed in terms of its physicochemical properties. Some applications of the technique to the study of permeability are given.

INTRODUCTION

Most of the work that has been carried out on the passage of substances into the cell has been done on red blood cells, marine eggs, amphibian skin, and nerve. There are few data that apply to mammalian tissue cells other than nerve. Moreover quantitative permeability studies in general have been performed either by placing the cell in a hypotonic medium and noting volume changes (and hemolysis rates in red blood cells) or by using radioactive tracers. In the former the unphysiological environment of the cell is a strong disadvantage in that it may itself affect the cell barrier. The latter suffers from the inability to measure the kinetic process in the isolated cell. The following method has been developed in an attempt to obtain data on this kinetic process in human tissue cells avoiding these disadvantages.

It is known (1) that the diaminoacridine dyes enter living cells and in lower concentrations appear to be non-toxic. Proflavin, among other members of this group, has the characteristics of fluorescence when excited by wave lengths around 4500 A as well as being intensely concentrated by the cell nucleus. It was felt that these properties might be utilized in the evolution of a photometric means of measuring the rate at which the dye enters the cell. Aside from the inherent interest in measuring a rate process in the single cell, it was hoped that characterization of the uptake curve would furnish a criterion for the effects of certain physical and chemical agents on the rate

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of proflavin penetration into the cell. In this paper a method is described for measuring the rate of dye penetration into single human conjunctiva cells in tissue culture. The effect of ether and alcohol on the rate of penetration is determined and an explanation of the kinetics of the process in terms of the percentage ionization of the dye is given.

Materials and Methods

Single cells were exposed to a solution of proflavin (National Aniline) and the increase in fluorescence of the cell as a function of time was photometrically recorded, yielding a curve directly related to the uptake of dye by the cell. The method is simple but it has been necessary to overcome several technical difficulties in order to get what is felt are valid results.

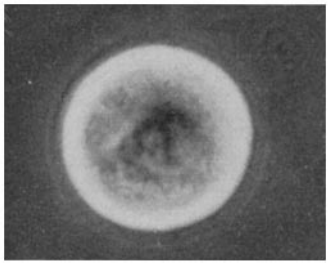

FIGURE 1. Normal human conjunctiva cell (established strain), 5 minutes after attachment to a glass surface. Spherical shape is evident × 2500 phase contrast.

APPARATUS The apparatus consists of essentially a fluorescence microscope with a binocular sidetube and attached phototube, the output of which is fed to a microammeter and thence to an oscilloscope. For details concerning the microscopic assembly see (6), and for details regarding the fluorescence microscope see (1). The light source used in these studies was an AH3 G.E. mercury arc which was found to be the most stable of the various sources tried.

CELLS The cells used in these studies were mostly derived from the human conjunctiva maintained in continuous culture, but single cells from fresh tissue obtained by the method of Puck (2) may also be used. The growth medium was Eagle's basal medium (4) with 10 per cent horse serum. Twelve hours prior to use a bottle with prolific growth was charged with a solution of 0.02 per cent trypsin (Difco) dissolved in Earle's calcium and magnesium-free salt solution (4). After cellular detachment from the bottle walls the suspension was diluted with growth medium so as to contain approximately 5×10^3 cells/cc. The suspension was then placed on a revolving shaker with a cycle of 1 second in an incubator at 37°C. Shaking was continued for 6 to 8 hours to insure recovery from the trypsin treatment. The cells at this time appeared as almost perfect spheres with well defined borders (Fig. 1). The procedure provides a simple means of comparison of cellular size and surface area.

CHAMBER The chamber in which the studies were carried out was fashioned from a stainless steel plate 2.5 × 7.5 × 0.5 cm. A hole 2 cm. in diameter was drilled

through the center. When a coverslip 2.5 cm. in diameter is placed on the undersurface and a pressed spacer washer with a coverslip of 1.6 cm. diameter and a measured thickness (19 to 20 microns) on its undersurface is placed on top the resulting space between the two coverslips is 75 microns. The surrounding moat with a volume of about 1.5 cc. acts as a reservoir (Fig. 2). This space communicates with the exterior by two 0.15 cm. holes drilled in the 7.5 × 0.5 cm. surface with tight fitting tubes of 2.5 cm. length placed in them.

DYE The dye used, proflavin (National Aniline), is 2.8-diaminoacridine (5). Since it was found that even short exposures of the dye in solution to overhead fluorescent lighting or daylight resulted in significant destruction of its fluorescent properties, the solutions were prepared fresh just before usage and in semidarkness or illumination with safety light, and placed in a brown bottle wherein they kept quite well for several hours. The dry powder was dissolved in Earle's salt solution to a final concentration of 2.9×10^{-6} M. The pH of the system, however, was first

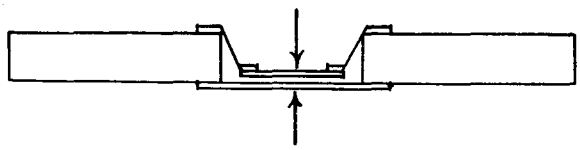

FIGURE 2. Cross-section through test chamber with pressed spacer washer in place resulting in a thin chamber of 75 microns between the two coverslips indicated by the two arrows.

adjusted to the value desired for each particular study. It is crucial that the pH be known and maintained since as will be shown this is a factor of prime importance in the rate of entrance of the dye into the cell. With a bicarbonate buffer system, such as Earle's solution, pH control is sometimes difficult but initial adjustment of the stock solution and clamping off of the tubes leading from the test chamber following dye infusion allow maintenance of a constant pH for appreciable periods.

MEASUREMENTS *Rate of Uptake of Proflavin by the Single Cells* Basically the procedure involves the exposure of the phototube to a single cell and photographically recording the oscilloscopic tracing of the phototube signal as a function of time. The system is first standardized with a No. 3484 Corning filter, the light source being adjusted so that the fluorescence from the filter gives a reproducible reading from day to day. The hole in the steel plate of the chamber is rimmed with a thin film of high vacuum silicone grease (Dow-Corning). In the center of the coverslip attached to the spacing washer is placed about 0.01 cc. of a previously prepared cell suspension. This is placed in an incubator at 37°C. and 100 per cent humidity and allowed to remain for 10 to 13 minutes. By the end of this period the cells have established points of attachment to the coverslip while still remaining essentially spherical. The medium is removed with a fine pipette and the attached cells washed with 0.01 cc. aliquots of Earle's salt solution. The excess solution is removed leaving only a thin film over the cells. The spacing washer is quickly inserted into the steel plate thus closing the chamber and preventing evaporation of the thin film. The cell for study is quickly

localized in the microscope field in a predetermined position. This is done by placing a diaphragm with a central 200 micron hole in the focal plane of the phototube ocular. Coordination of this hole with a finely ruled grid placed in the side tube allows rapid localization of the cell in the center of the field. The diaphragm effectively masks out much of the extraneous fluorescence from the background. A syringe containing the prepared dye at a temperature of 23°C. is then attached to the chamber by a polyethylene tube. A shutter with a $\frac{1}{30}$ second speed is placed between the light source and the condenser. The dye is then infused into the chamber. At this instant the shutter is clicked and a recording of the phototube signal is obtained. This reading is taken as the fluorescent intensity of the cell plus background at time, t_0; and is considered as the point in time of zero dye concentration in the cell. The firm attachment of the cell assures its remaining immobile during the infusion. Exposures are then made at specified intervals. A continuous record is *not* obtainable because

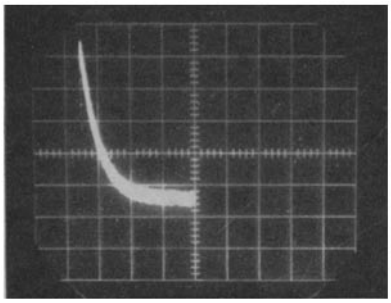

FIGURE 3. Extinction of fluorescence with continuous exposure of the dye solution to the exciting wave lengths. The ordinate is fluorescence and the abscissa is time (18 seconds/division).

of the extinction of fluorescence with prolonged exposure of the dye to the exciting wave lengths. This is clearly shown in Fig. 3 where the fluorescent intensity of the dye is seen to have dropped to one-half its initial value in approximately 8 seconds of continuous exposure. However, brief ($\frac{1}{30}$ second) exposure at an appropriate interval makes this effect negligible.

Since the method of measurement is based on the cellular concentration of the dye, which registers as a gradual increase in fluorescence of the cell above that of the background, it is important that the molarity of dye not be too great and that the chamber not be too thick so the fluorescence arising from the bathing fluid around the cell is minimal. The specifications given above were found to be satisfactory. Care must also be taken to avoid the attachment of more than fifteen or twenty cells to the coverslip. Because of the extreme concentration of the dye by all the cells, any appreciably greater number results in a reduction of the dye concentration in the background, as this is not an infinite reservoir and must be held constant in order that changes in the total readings of cell plus background may be attributed to the cell exclusively. On the other hand if the number of cells is small, their uptake of dye is slow enough to leave the dye concentration in the vicinity of the cell essentially unchanged during the periods of observation used.

For valid comparisons cells of the same size must be taken. This points up the convenience of employing spherical cells which permit rapid and simple determination of surface area. Residual cellular variability remaining when all known conditions have

been controlled is probably due to fluctuations in physiological state and to experimental error.

Because of the predictable nature of the uptake curve and its reproducibility, effects of certain physical and chemical agents on the passage of proflavin into the single cell may be determined. Diethyl ether and ethyl alcohol were studied as examples. Three points on the curve were first obtained as control values. The chamber was then flushed with the test substance dissolved in Earle's solution and four more readings were made. The possibility of dye combination with the test substance with resulting change in mobility was ruled out to some extent by absorption spectroscopy. Diethyl ether and ethyl alcohol caused no significant change in the absorption spectrum of proflavin in concentration of 4 and 5 per cent respectively.

Determination of Average Dye Uptake per Cell per Minute The determination of dye concentration in the individual cell cannot be made directly, because the factor relating fluorescence and concentration inside the cell is not known and may differ from that outside. The relationship may be determined to a first approximation by exposing a large number of cells to a relatively small volume of dye solution and noting the diminution in fluorescence of the solution with time. It is known that the fluorescence of a cell-free solution is proportional to the dye concentration. Thus if the amount of dye extracted is determined and the number of cells that have achieved the extraction is known, the average dye uptake per cell per minute may be found. The procedure is as follows: A suspension of 2×10^7 cells is centrifuged gently and all of the supernate is removed. The cellular clump is carefully sucked up into a finely graduated pipette and one-half (suspension I) is added to 7 cc. of 2.9×10^{-6} M dye solution at pH 6.6 at which pH a negligible amount of dye enters the cells during the 1st minute (see below). The other half (suspension II) is added to a similar solution at pH 7.9. Mild pipetting rapidly results in a suspension of mainly single cells. Suspension I is immediately centrifuged at 2000 R.P.M. for 20 seconds and the supernate is removed. This is used as a control. A small aliquot is removed for cell count from suspension II. After 5 minutes this suspension is also brought to pH 6.6 by bubbling in CO_2 for 15 seconds thus stopping dye uptake; this is followed by centrifugation at 2000 R.P.M. for 20 seconds and removal of the supernate. The fluorescence of these solutions is then determined. These data may be used to obtain the desired results as discussed below.

RESULTS

Fig. 4 is a typical record from a human conjunctiva cell. Each signal is a 1/30 second exposure spaced at 1 minute intervals. The first signal is obtained 2 seconds after the dye infusion and is the fluorescence of the background solution. The subsequent increase in fluorescence is due to concentration of the dye by the cell. The total exposure is usually no more than 7/30 second spread over a period of at least 7 minutes. This type of exposure does not appreciably affect the dye as explained previously. The almost perfect linearity of the curve is noteworthy. However, while the curve almost

always tends toward a straight line during the first 5 to 10 minutes, slight irregularities are not rare. Leveling off usually starts 10 minutes after the initial signal as seen in Fig. 7*b*, but is dependent to some extent on pH, occurring more rapidly at higher pH levels.

Since the slope of the curve is constant during the first few minutes, the dye uptake must be constant during this period also if the assumption is made that the factor relating fluorescence to dye concentration inside the cell does not change as the dye accumulates in the nucleus. This problem will be discussed below

EFFECT OF PH Fig. 5 shows the effect of pH on the rate of dye uptake by four different cells which were chosen with the uptake rate equal to the mean for that particular pH. The increase in uptake rate with increased pH

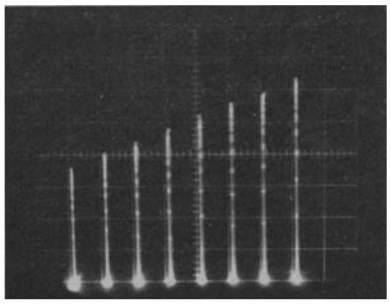

FIGURE 4. Oscilloscopic record from untreated cell at pH 7.9.

is wholly consistent with the physicochemical properties of the dye. It is a relatively strong base with a pK of 9.65.[1] If the Henderson-Hasselbach equation is used as a first approximation, then

$$\mathrm{pH} = \mathrm{p}K + \log \frac{\mathrm{R}}{\mathrm{RH}^+} \qquad (1)$$

in which R is the dye in the un-ionized form and RH^+ is the dye in the charged or ionized form. At pH 7 the ratio is therefore 1/445 giving a concentration for R of about 0.2 per cent of RH^+. If the approximation is made throughout that $RH^+ \approx R + RH^+$, then the concentration of R can be easily computed by multiplication of the calculated ratios by the dye concentration, namely 2.9×10^{-6} M. The error involved in the approximation increases with increasing pH, but for the present data which are carried only to a pH of 8.5 this error is of little consequence. The results are listed in Table I which compares the concentration of R with the slope of the uptake curve.

[1] Spectroscopic evidence (5) indicates that in the ionized form of the dye the proton attaches to the ring nitrogen of the acridine molecule rather than either of the amino groups. The pK values of the amino groups are so low that they need not concern one since the molecule is only singly charged in the pH range used.

It is thus noted that the increase in concentration of R with pH is of the same order of magnitude as the increase in slope of the uptake curves with the same change in pH. This suggests that the effect of pH on the uptake curve is an effect due to the variation of concentration of uncharged dye in the bathing solution with little effect on the cell. The persistent linearity of the curve over a period of several minutes at the various pH values is also noted.

Table II shows the degree of reproducibility of the method at two different pH values. The standard deviation of the mean uptake is substantially less

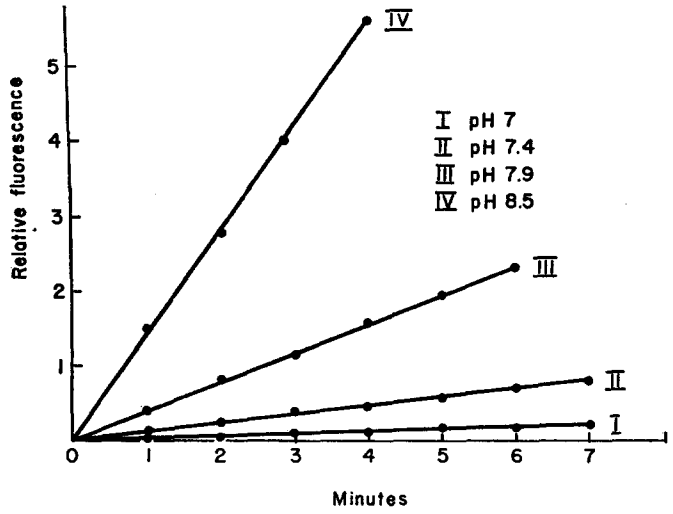

FIGURE 5. Effect of pH on the slope of the uptake curve.

than the change in uptake rate that occurs with a pH change of 0.5 pH unit. One cm. on the oscilloscope screen was arbitrarily chosen as equal to 1 fluorescence unit.

EFFECTS OF ETHER There has been some inconsistency in the literature as to the effect of ether on permeability (7). Fig. 6 shows that in a concentration of 2 per cent ether has a facilitating effect on the rate of dye penetration. These records were taken from the same cell prior to and after the administration of ether. The maintenance of the linear character of the uptake curve during the first few minutes allows the introduction of a perturbation such as ether into the system at some point during this time and notation of the change in slope. The 2 per cent concentration of ether does not kill the cell since addition of this concentration made up in growth medium has no noticeable effect on growing cultures following removal after 2 hours. A 4 per cent solution of ether has the effects shown in Figs. 7*a* and 7*b*. In Fig. 7*a* a marked increase in the uptake rate is seen at pH 7. The

irregularity of the curve is pronounced. The slope of the curve is greater between minutes 5 and 10 than between minutes 0 and 5 possibly due to a cumulative effect of the ether on the area available for passage. At pH 7.9

TABLE I

RELATIONSHIP BETWEEN PH, CONCENTRATION OF UN-IONIZED DYE, AND SLOPE OF UPTAKE CURVE

pH	Approx. per cent of total concentration in un-ionized form (R)	Slope (relative fluorescence units/min.)
7	0.2	0.05
7.4	0.5	0.14
7.9	1.7	0.38
8.5	7.2	1.62

TABLE II

REPRODUCIBILITY OF UPTAKE CURVE

pH	No. of cells	Mean uptake/5 min.	Standard deviation
			per cent
7.4	10	0.5	16
7.9	10	1.8	17

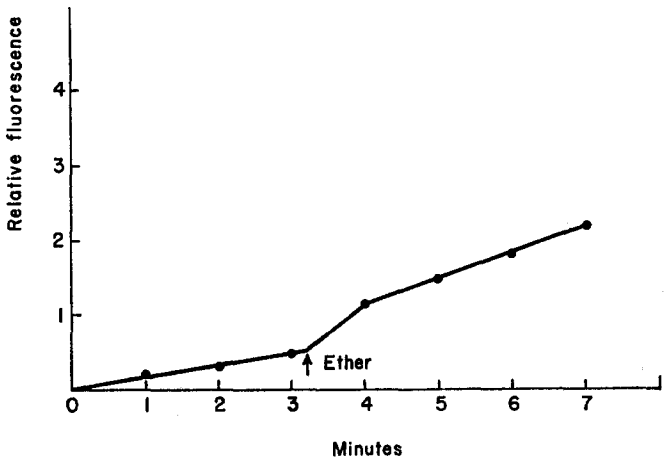

FIGURE 6. Effect of 2 per cent ether on the uptake curve at pH 7.4.

(Fig. 7*b*) the dye passes into the cell more rapidly than into the normal cell; however, after 10 minutes there is marked decrease in fluorescence. This interesting difference between the two uptake curves at pH 7 and 7.9 may be explained as follows: The data suggest that it is the uncharged form of the dye that enters the cell. It is postulated that upon entry into the cytoplasm the dye resumes its ionized form as governed by the Henderson-Hasselbach

equation. At the pH of the cellular interior this is probably almost 100 per cent ionized (RH^+). It is probable that a replacement reaction between the singly charged dye molecule and a proton on the nucleic acid then ensues

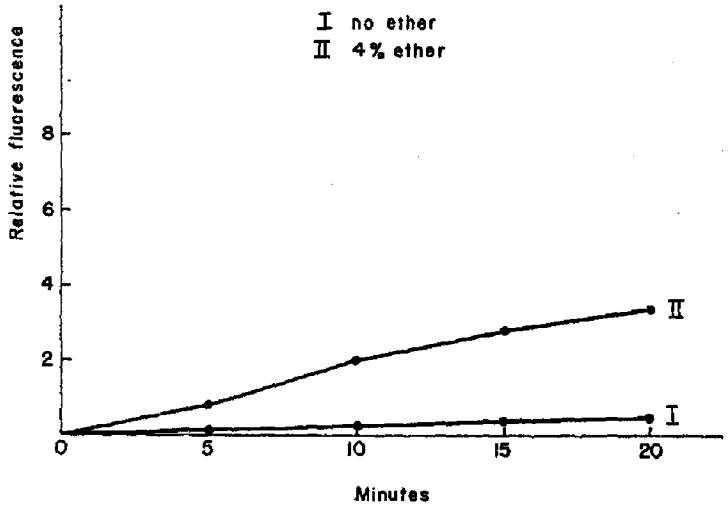

FIGURE 7*a*. Effect of 4 per cent ether at pH 7.

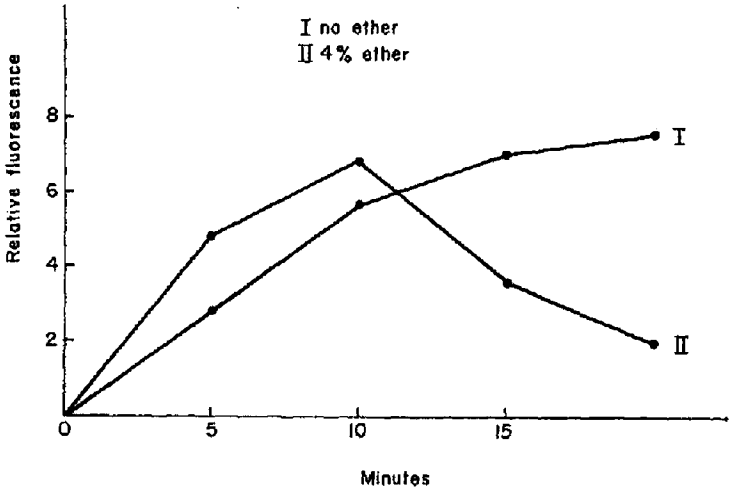

FIGURE 7*b*. Effect of 4 per cent ether at pH 7.9.

with equilibrium far in the direction of the associated complex. The ether in a concentration of 4 per cent affects the cell membrane so drastically that at pH 7.9 the cell cannot maintain its internal pH against the negative H ion concentration gradient. As the internal pH rises, the equilibrium between the R and RH^+ forms of the dye is shifted in the direction of the former with some resultant dissociation of the complexes, DNA^-–RH^+; RNA^-–RH^+

that have already formed and measurable loss of fluorescence. On the other hand at pH 7 the addition of ether allows more rapid entry of the dye but any tendency of the cellular milieu towards pH 7 does not cause a marked shift in the equilibrium between the R and RH forms of the dye since this pH is probably very close to or even lower than that of the normal cellular interior. Thus there is no dissociation of the formed dye–nucleic acid complexes with the passage of time.

EFFECT OF ALCOHOL Fig. 8 shows the effect of perfusion of the chamber with 5 per cent ethyl alcohol in Earle's solution; a marked decrease in the slope of the curve is noted. The experimental data do not allow any extensive

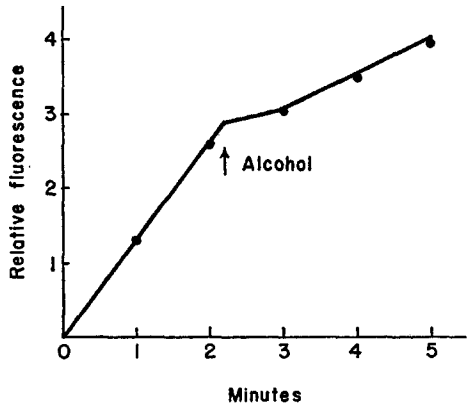

FIGURE 8. Effect of 5 per cent alcohol at pH 8.5.

interpretation of the nature of the alcohol effect but it may be postulated that this concentration of alcohol has a "hardening" effect on the membrane perhaps through its interaction with protein components.[2]

AVERAGE DYE UPTAKE PER MINUTE PER CELL AND THE CALCULATION OF A PENETRATION COEFFICIENT The positive correlation between the concentration of un-ionized form of the dye and the increase in slope of the uptake curve with change in pH is consistent with the assumption that the concentration difference of R across the membrane is the prime driving force involved in the flux of dye into the cell. It is reasonable, therefore, to apply the following equation to the system:

[2] In these experiments utilizing the same cell to determine the effect of a substance it is not possible to state accurately that the final concentration of the substance is precisely the same as the concentration in the perfusate. In the chambers used for the present work it is difficult to effect a total fluid exchange between the two closely approximated coverslips; however, perfusion of the chamber first with a fluorescent solution and then with a non-fluorescent solution results in a disappearance of practically all of the fluorescence, thus indicating a fairly good exchange.

$$\frac{dn}{dt} = K\Delta c = K(c_0^* - c_i^*) \tag{2}$$

in which K is a constant determined by the impedance of the membrane to passage of dye, c_0^* is the concentration of un-ionized dye in the bathing solution and, c_i^* is the concentration of the un-ionized form inside the cell. dn/dt, the slope of the uptake curve, is constant for several minutes beyond time, t_0, which is the moment of dye infusion. The concentration, c_i^*, is zero in the cell at t_0; it must therefore remain zero or the slope would decrease as free dye built up in the cell with resulting decrease in the concentration difference across the membrane. Thus it is postulated that the dye combines essentially irreversibly with cellular components as long as the curve remains linear.

It is now possible to write an equation relating the disappearance of dye from the external solution to the uptake by the cells, when a large enough number of cells are present to result in a measurable diminution in the concentration of the external solution. The *total* change of dye content ($R+RH^+$) at any instant is equal to Vdc_0/dt, in which V is the volume of the solution and dc_0/dt is the rate of change in concentration of the solution. This change is equal to the amount of dye that enters the cells, namely K' ($0.017\ c_0$) which is equation (2) except that K' is K multiplied by the total number of cells; and c_0, the outside concentration is here a function of time. The internal cellular concentration is taken as zero as already explained. The factor, 0.017, converts c_0 to c_0^* which is the concentration of the un-ionized dye form (R) at pH 7.9 (see Table I). Equating gives:

$$-Vdc_0/dt = K'(0.017c_0) \tag{3}$$

The minus sign is necessary to keep the left side positive since the slope, dc_0/dt, is negative. Experimental data obtained from using the procedure described on page 857 are given in Table III.

It is seen that the concentration of the solution fell to about one-half in 5 minutes at pH 7.9. The control solution gave the same reading as the freshly prepared dye solution with no cells. Equation (3) may be integrated. Using the above data and separating variables give:

$$-0.007/0.017 \int_{2.9\times10^{-6}}^{1.4\times10^{-6}} dc_0/c_0 = K' \int_0^5 dt \tag{4}$$

This gives a value for K' of 6.0×10^{-2} liters/minute. Dividing by the total number of cells gives K as 6.1×10^{-9} liters/minute for the average single

cell. Substituting this into equation (2) and taking c_i^* as zero gives:

$$dn/dt = 6.1 \times 10^{-9} (0.017 \times 2.9 \times 10^{-6}) \quad (5)$$
$$= 3.0 \times 10^{-16} \text{ moles/minute}$$

This is the amount of dye entering the single cell per minute as long as the curve remains linear.

THE EFFECT OF TEMPERATURE Preliminary experiments have shown that a rise in temperature of 10°C. approximately doubles the rate of dye uptake. Further work as to the meaning of this rate change is in progress.

TABLE III

pH	Cell count 10^{-6}	Time of cell-solution contact	Concentration of dye remaining in solution
		min.	*moles/liter* $\times 10^{-6}$
6.6	0		2.9
6.6	9.7	0.6	2.9
7.9	9.7	5.0	1.4

DISCUSSION

This work has been carried out postulating that there is a direct proportionality between the amount of dye entering the cell and the increase in fluorescence as here recorded (at least in the linear portion of the curve). Because of the concentration of the dye by the cell nucleus with possible change in its fluorescent properties, it is important to establish that the linear records obtained are an accurate representation of the actual process; *i.e.*, that the passage of dye into the cell is a linear function of time. The amount of dye entering the cell is a function of the concentration difference of the unionized form that exists across the membrane. This difference is a maximum at time, t_0. Therefore two possibilities exist: It must either be maintained at this maximum value (no free dye accumulates in the cell) or it must decrease, the free dye building up in the cytoplasm. If the difference is maintained constant then the passage of dye into the cell will be constant and a plot of bound intracellular dye concentration as a function of time would be a straight line. Conversely a continually decreasing concentration difference would result in a plot with a continually decreasing slope. To experimentally obtain a linear relationship between fluorescence and time from a process that is represented by a curve of decreasing slope it is necessary to assume that at the dye becomes more concentrated by the nucleus there results an *increase* in the proportionality factor relating concentration and fluorescence. This is opposite to what happens in solution. It is more likely that the re-

maining possibility is the true one, namely, the dye uptake is a linear function of time and the experimentally obtained straight line is an accurate representation of this process, points on the curve related to the actual cellular dye concentration by a constant.

The meaning of the increase in rate of dye penetration by a factor of 2 from a temperature rise of 10°C. is not determined in the present work. If the passage of the dye into the cell were effected by simple diffusion then the modified Fick equation would apply, namely:

$$dn/dt = (DA/\Delta x)\Delta c \qquad (6)$$

in which D is the diffusion coefficient; A, the effective area; Δx, the membrane thickness, and Δc the concentration gradient across the membrane. Although the diffusion coefficient would not be expected to change by so large a factor for this temperature rise it is conceivable that A or x might be sufficiently modified to bring about a Q_{10} of 2. Another possibility that exists is that the dye passage is enzyme-mediated.

Throughout this report the nuclear membrane and nucleolus have not been mentioned. Although these need further study, it is felt that they do not affect the reported results in general. Some workers in the field of nuclear physiology feel that the nuclear membrane does not maintain the same cation selectivity as does the plasma membrane (8). The preceding studies have been carried out using this hypothesis; in other words the major barrier to dye passage has been considered to be located at the cytoplasmic-medium interface.

The toxicity of the diaminoacridines has been investigated by DeBruyn (1). Partially hepatectomized rats showed no demonstrable difference in the time and character of liver regeneration whether or not they were fed the dyes although the dyes were demonstrable in the cells by fluorescence microscopy. No evidence of toxicity has been detected in tissue cultures exposed to the dye, proflavin, in the concentrations used in the above experiments.

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