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Measurement of rapid membrane permeation in cell suspensions by application of a generalized capillary method

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An improved version of the capillary technique for the determination of diffusion coefficients has been developed as a simple method of measuring membrane permeabilities of single cells suspended at relative densities between 0.70 and 0.97. A new, generalized theoretical formulation to describe the diffusion process of a solute in a composite system was derived using a series-parallel-pathway model with explicit consideration of the diffusion pathways inside and between the cells. This renders the technique insensitive to unstirred layer effects. Any single cell population of known size distribution may be investigated. High permeabilities (above $5 \cdot 10^{-3}$ cm/s) can be measured with the greatest precision, but lower permeabilities, down to a limit of about $5 \cdot 10^{-4}$ cm/s, may also be determined by the method. Measurements in erythrocyte suspensions have been made using non-electrolytes such as hexanol, water and ethylene glycol as test solutes. The permeabilities obtained agree with the values obtained by much more sophisticated equipment. Cell shape was shown to be without significant influence on the permeability data obtained. The procedure may become of particular interest for measurement of suspensions of membrane vesicles.

Introduction

Conventional experimental techniques [1] to investigate the membrane permeability of biological membranes are usually limited to the range of high transport rates [2,3]. This is particularly true for measurements of the self-exchange of radioactive tracers [4] across membranes of single cells. Here, the system is in complete equilibrium except for the labelled test solute. The system is supposed to consist of two well-stirred compartments separated from each other by the membrane. Membrane permeation is taken to be the rate-limiting step. For large cells and for high permeabilities, however, diffusive steps in unstirred layers or in the cell interior become increasingly important [2,3]. The procedure has a low time resolution unless highly elaborated rapid sampling devices [3,6–8] are used. More refined techniques using NMR [9–12] or light scattering [13–14] have a higher time resolution but require a high concentration of the labelled component in order to achieve sufficiently high signal amplitudes. Their use in systems under physiological conditions is, therefore, complicated.

As an alternative, membrane permeabilities can be derived from the bulk diffusion coefficient of a test solute in a system consisting of a column of cells packed in a capillary. In this adaptation of a

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classical set-up [15] to biological cells, first introduced by Redwood et al. [16], the single cell exchange process is replaced by a large series of transport steps. Thus, problems arising from the limited time resolution in measuring the exchange across single membranes are avoided. The system in the capillary is strictly unstirred. Measurements result in the determination of an effective diffusion coefficient, D_{eff}, which comprises the influence of the membrane permeability, P_d, as well as that of the two diffusion coefficients D_1 and D_2 of the extra- and intracellular compartments proportionate to their fractional contributions as derived from the fractional extracellular volume V_{rel}. The only assumption resides in the choice of some geometric model to depict the cell suspension in microscopic detail.

The effective diffusion coefficient, D_{eff}, was originally obtained from the tracer concentration profile in the capillary [17]. Osberghaus [18] simplified this experimental procedure by introducing the so-called integral method. Here, instead of measuring the concentration profile in the cell column, the overall change of tracer concentration in the capillary after an appropriate exposure time starting from some known concentration is measured. Both methods provide an effective diffusion coefficient, D_{eff}, for the transport of the test solute through the composite system.

Yet, the applicability of the integral method to lower permeabilities remains limited because of the increasingly longer exposure times required to achieve the necessary concentration decrease of at least 35% [19]. Living cells in a packed state usually cannot endure incubation times of longer than a few hours, due to problems of substrate supply and metabolite disposal.

We, therefore, undertook a further revision [19] of the former approaches [16,18]. Specifically, the theoretical description of the transport through a homogeneous suspension composed of identical units was revised. A more general solution of the diffusion equation is presented which explicitly accounts for the intercellular region. Thus, the former restriction of the method to very closely packed cell suspensions (approx. 97%) becomes unnecessary. As a consequence, lower permeabilities come into the range of the method. Most important, the revised method may now be applied to systems that cannot be very closely packed, such as membrane vesicle suspensions.

Theory

The experimental set-up consists of a narrow glass capillary which is closed on one side (for details see Ref. 20). Thus, the transport problem is reduced to a linear form of the diffusion equation

$$\frac{dc}{dt} = -D \cdot \frac{\partial^2 c}{\partial x^2} \tag{1}$$

where the x direction indicates the net tracer flux direction along the axis of the capillary. D denotes the diffusion coefficient of the test solute, at a concentration c, in the diffusion medium. The boundary conditions are

$$c = c_0 \text{ for } 0 < x < l \text{ and } t = 0$$

$$c = 0 \quad \text{for} \quad x \geq l \text{ and } t \geq 0 \tag{2}$$

The second of these conditions expresses the fact that the capillary is placed into an infinitely large diffusion bath where the tracer concentration does not change with time. To this end, the bath medium has to be stirred.

The solution to Eqn. 1 leads to

$$\bar{c}(\Delta t) = c_0 \cdot \frac{8}{\pi^2} \sum_{\nu=0}^{\infty} \frac{1}{(2\nu+1)^2} \cdot \exp\left[-(2\nu+1)^2 \cdot \frac{D \cdot \Delta t}{l^2}\right] \tag{3}$$

Here, $\bar{c}(\Delta t)$ is the average concentration in the capillary of length l after a diffusion time Δt.

This formulation implies a simple experimental procedure to determine the diffusion coefficient D because $\bar{c}(\Delta t)$ and c_0 can be measured as the mean tracer concentrations in the capillary at the beginning and at the end of a diffusion experiment. It can be shown that both very small and very large concentration decreases are not suitable for the determination of the diffusion coefficient. In order to achieve optimal experimental precision, it should be arranged that the ratio $\bar{c}(\Delta t)/c_0$ is larger than 0.25 and smaller than 0.65 [19].

The cell suspension is envisaged (Fig. 1) as an ordered system of identical elementary units each of which is heterogeneous because it contains a cell and its aqueous surroundings. Each unit must

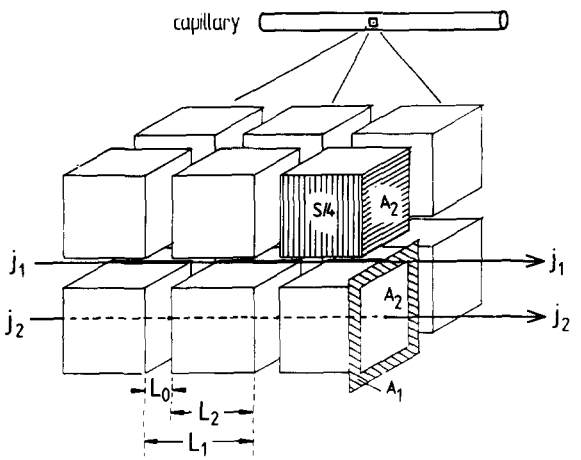

Fig. 1. Simplified model of the cell suspension used for the capillary technique. Cells are envisaged as cube-shaped bodies of length L_2, with intercellular distances L_0 between each other in all directions. Two coupled diffusion streams j_1 (extracellular) and j_2 (intracellular-intercellular) perpendicular to the face surfaces A_1 and A_2 can be distinguished. Local relaxation is provided for by continuous solute exchange across the side surfaces S.

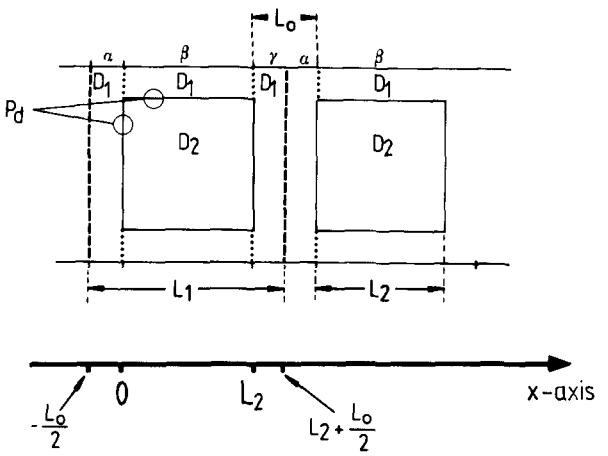

Fig. 2. Top view of the suspension model. Units of length L_1 containing one model cell of length L_2 with distance L_0 to the next one compose the cell suspension. Each unit consists of three portions α, β, γ (as explained in the text) with their respective diffusion resistances represented by D_1, D_2 or a combination of D_1, D_2 and P_d. Direction x indicates the macroscopic diffusion direction of j_1 and j_2 (Fig. 1) parallel to the capillary axis.

be small relative to the whole system because, only under this condition, the concentration gradient changes very slowly with time and the assumption of local quasi-stationarity is justified. Then the solution of the transport problem can be reduced to the consideration of a single unit.

In solving the local transport problem in such a unit, the microscopic heterogeneity that is a characteristic of a suspension is considered by a series-parallel-pathway model. Cube-shaped cells of length L_2 and with distance L_0 from each other are oriented with one face perpendicular to the diffusion stream, j. This total diffusion stream, j, is the sum of two parallel diffusion streams, j_1 and j_2, which are coupled to each other by solute exchange across the face (A_1, A_2) and side (S) surfaces of the cubes.

The cubic model is chosen because it is the best compromise between reality and mathematical simplicity:

(i) The symmetry of this model is approximately the same as that of statistically oriented cells.

(ii) This model reduces a three-dimensional diffusion problem to a one-dimensional one.

A sufficiently dense packing of the cells provides for equal diffusion resistances in the whole macroscopic test volume, say overall in the capillary. To solve the problem in detail we further divided each unit into three portions as indicated in Fig. 2: α, a region in front of the cell; β, the region that contains the cell itself and some part of the parallel extracellular pathway; γ, a region behind the cell.

The diffusion coefficients D_1 and D_2 are those of the extracellular (aqueous) and the intracellular (hemoglobin-containing) diffusion medium, respectively.

The quantity

$$A = A_1 + A_2 \tag{4}$$

is the total face area of the unit perpendicular to the diffusion direction, x, with an extracellular portion, A_1, and a cellular one, A_2. In the case of our geometric model of a cubic cell of length L_2, we take L_2 from

$$L_2 = \sqrt[3]{\overline{V}_{\text{cell}}} \tag{5}$$

with $\overline{V}_{\text{cell}}$ being the mean cell volume.

The sum of the side faces, S, then is

$$S = 4 \cdot L_2^2 \tag{6}$$

Using the condition of local quasi-stationarity, the diffusion equations for all three regions α, β and γ are solved. Then the boundary and the continuity conditions are taken to couple the results [20], which finally leads to the equation

$$D_{\text{eff}} = \frac{(1+r)}{(1+z)\cdot(1+y)} \cdot \frac{D_2 \cdot A_2}{A} \tag{7}$$

The symbols used herein can be defined thus

$$r = \frac{D_1 \cdot A_1}{D_2 \cdot A_2} \tag{8a}$$

$$z = 2 \cdot [\pi_0 \cdot (1+r) + r \cdot \alpha \cdot \coth(\alpha/2)]^{-1} \tag{8b}$$

$$\pi_0 = \frac{P_d \cdot L_2}{D_2} \tag{8c}$$

$$\alpha = \left[\frac{S}{A_2} \cdot \pi_0 \cdot (1 + r^{-1})\right]^{1/2} \tag{8d}$$

$$y = \frac{\frac{L_0}{L_2}}{1 + \frac{L_0}{L_2}} \cdot \frac{z}{1+z} \cdot \left[\frac{\alpha}{2} \cdot \coth\left(\frac{\alpha}{2}\right) - 1\right] \tag{8e}$$

Eqns. 7 and 8a–e formally resemble the equation that was derived by Perl [16] within the limits of infinitely densely packed cell suspensions, except for the term $(1+y)^{-1}$. In addition, there is an even more important difference inherent in Eqns. 7 and 8a–e: the geometric parameters (see Eqn. 10a and b) as calculated from the measured values of V_1/V are defined differently from those given by Perl [16] or Alpini [21].

D_{eff} in Eqn. 7 is the effective diffusion coefficient that produces, over the total diffusion length $(L_2 + L_0)$ of a suspension unit, the concentration decrease

$$\Delta c = \left| c_\gamma\left(x = L_2 + \frac{L_0}{2}\right) - c_\alpha\left(x = -\frac{L_0}{2}\right)\right| \tag{9}$$

Since each unit is very small as compared to the capillary length l, we are allowed to approximate the macroscopic concentration profile within the capillary by concentration gradients, like that in Eqn. 9. Under this condition, the measured bulk diffusion coefficient D from Eqn. 3 is the same as the diffusion coefficient in the microscopic subsystem, D_{eff}. Thus, the application of Eqns. 7 and 8a–e results in the determination of the desired value of the membrane permeability, P_d.

All the necessary quantities can be measured directly except for the geometric parameters A_1/A_2 and L_0/L_2 that depend on the choice of the geometric model for the microscopic subsystem of the suspension. Once a model is chosen, these parameters can be obtained from the measured value of the relative extracellular volume, $V_{\text{rel}} = V_1/V$. In the case of a cubic model, they are

$$\frac{L_0}{L_2} = (1 - V_{\text{rel}})^{-1/3} - 1 \tag{10a}$$

$$\frac{A_1}{A_2} = (1 - V_{\text{rel}})^{-2/3} - 1 \tag{10b}$$

and $S/A_2 = 4$.

Other shapes, that can be chosen alternatively, are in general not isotropic. In all these cases, the Eqns. 4–6, 10a and 10b have to be replaced by the corresponding relations. Furthermore, the different possible orientations of the cells relative to the diffusion directions have to be considered when the membrane permeability is calculated from Eqns. 7 and 8a–e [19].

Materials and Methods

Materials. All chemicals used were of analytical grade. As radioactive isotopes we used ^3H_2O (5 mCi/ml), [^{14}C]ethylene glycol (4.7 mCi/mmol), [^{14}C]sucrose (10 mCi/mmol) from New England Nuclear and [^{14}C]hexanol (5 mCi/mmol) from Amersham Buchler.

Microliter syringes (Unimetrics, Type UMS Tp 4025) were used as capillaries. The screw cap that usually holds the needle was cut away; the cut face was carefully milled. The capillary length, l, could be adjusted with a precision of ± 0.1 mm by an adjustable scale fixed to the syringe; the measured capillary diameter was 0.802 ± 0.001 mm. The capillaries were filled with a complete syringe (Unimetrics, Type UMS TP 4050). A rack allowed experiments with 16 capillaries at a time with

groups of four immersed into the same diffusion bath containing 2.2 l of diffusion medium at $T = 293.0$ K, respectively.

The diffusion medium (medium D) was composed of isotonic NaCl plus 10% (v/v) isotonic phosphate buffer (NaH_2PO_4/Na_2HPO_4, pH = 7.4). The osmolarity was 330 mosmol/l. The diffusion baths were filled with 2.2 l of medium D and an additional amount of unlabelled test solute in order to measure the self diffusion of the test solute at a defined concentration level. For preincubations of the cells and loading with test solute medium D was also used, but with an additional amount of labelled (radioactive) test solute.

Cell suspensions were prepared from human red blood cells isolated from fresh blood conserves obtained at the local blood bank. Heparin was added as an anticoagulant. The conserves were stored at 277 K and used for diffusion measurements within 2 days. Plasma and buffy coat were separated by centrifugation (10 min, $2800 \times g$) and discarded. The red blood cells were washed three times in medium D by successive centrifugation and dilution until no plasma was left in the sediment.

Vesicle preparation. In order to obtain cell vesicles by heat-fragmentation of human erythrocytes washed red blood cells were suspended in medium D containing 5 mmol NaN_3 to prevent bacterial growth which was favoured by the temperature increase. Albumin was added (330 mg/l) as described by Ponder [22] to stabilize the spherical shape of the vesicles. The suspension was then heated to 51°C for 15 min under continuous shaking. Under these conditions, the cells fragment into 2–3 spherical vesicles [22]. Subsequently, the suspension was sucked up into a syringe through a 0.9 mm diameter needle, squeezed out into a centrifugation tube and centrifuged (Sigma 3E: $1400 \times g$, 3 min, without braking). This procedure was repeated three times. It served to shear off small vesicles adherent to the larger fragments and to separate them from the larger vesicles. Finally the vesicle suspensions were washed five times.

Measurement of the effective diffusion coefficient D_{eff}. The effective diffusion coefficient, D_{eff}, of a solute diffusing through a suspension of cells was measured using the capillary method as described [15,19,23]. Microliter syringes were set to the desired capillary length (e.g., $l = 0.480$–0.960 cm) and mounted on the rack. 1.25 ml washed erythrocytes were suspended in an equal volume of medium D. A small amount of radioactive tracer (approx. 2 μCi) was added and well distributed by thorough stirring. After 30 min of incubation at 20°C to reach equilibrium of all permeant solutes, the so-called diffusion samples and the samples for measuring the relative extracellular volume (see below) were brought to a high packing density $V_{\mathrm{rel}}^0 = (V_1/V)^0$ by centrifugation ($22\,000 \times g$, 30 min). The handling procedure for samples for volume determination is explained below. The supernatant of the diffusion samples was completely removed by suction and the sediment was brought from its tight packing $(V_1/V)^0$ to the desired value of V_1/V by addition of a measured volume, ΔV, of medium D. The suspension was then thoroughly homogenized and sucked into the filling syringe (Unimetrics, UMS TP 4050). The needle was inserted into the capillaries mounted on the rack from above through the open mouth to the bottom of the capillary. Slowly and drawing out the needle simultaneously the capillaries were filled with cell suspension from below to ensure that no air bubbles were left.

An excess drop of suspension on top of the capillary mouth was sheared off with a razor blade and replaced by a drop of medium D to prevent exsiccation of the cell column during the filling procedure. The error in diffusion time resulting from this advanced diffusion start was thought to be negligible, relative to the total diffusion period of approx. 10–20 h.

The initial concentration, c_0, of the labelled solute in the suspension was determined by mixing an exactly measured volume of the tracer-loaded cell suspension with 1.5 ml of destilled water. Proteins in the resulting lysate were precipitated by addition of 50 μl 60% $HClO_4$ and then separated by centrifugation ($11\,500 \times g$, 2 min).

To start the diffusion experiment, the rack with the capillary syringes was lowered into the diffusion baths. Stirring the bath solution was necessary to provide for constant solute concentrations outside the capillaries. The stirring rate had to be very low to avoid an undesired washout of cell suspension from the cell column, which would

result in the determination of artifactually high diffusion coefficients (the so-called Δl effect, see Nanis et al. [24]).

At the end of the diffusion period ($\Delta t \leqslant 24$ h) the capillaries were removed from the bath, their content was emptied into 1.5 ml distilled water and handled in the same way as the c_0 samples to obtain a $\bar{c}(\Delta t)$ sample for each capillary. Radioactivity in both, the c_0 and the $\bar{c}(\Delta t)$ sample, was determined by liquid scintillation counting, using Quickszint 212 (Zinsser Analytics, Frankfurt). The results were normalized to equal suspension volumes.

The effective diffusion coefficient, D_{eff}, was then calculated from the first five terms of Eqn. 3 by an iterative method. Iteration was stopped when the relative deviation between the experimental and the theoretical value of $\bar{c}(\Delta t)/c_0$ was less than 0.01%.

The diffusion coefficients, D_1 and D_2, for the extra- and the intracellular medium, respectively, can, in principle, be obtained by the same experimental technique. In these cases, the capillaries were filled with either the extracellular medium D or a buffered solution of hemoglobin to represent the intracellular medium. A detailed description of these procedures can be found elsewhere [18,25].

Measurement of the relative extracellular volume V_1/V. The total volume, V, of the suspension is the sum of the volume, V_2, of the suspended particles and the volume, V_1, of the suspending medium. The quantity $V_{\text{rel}} = V_1/V$ (relative extracellular volume) was measured by a dilution procedure with an impermeable radioactive volume marker. 2.5 ml of a 1:1 (v/v) suspension of red blood cells in medium D (see above) were mixed with 0.05 μCi [^{14}C]sucrose by careful stirring. The sample was centrifuged (30 min, 22000 $\times g$) to a high packing density, $V_{\text{rel}}^0 = (V_1/V)^0$. The impermeable tracer will be found almost completely in the supernatant, only a small fraction will be left in the intercellular space. A sample, c_A, of 0.5 ml was taken from the supernatant, the rest was carefully and completely removed from the sediment. The sediment was weighed (m_s), rediluted by addition of a known amount V_s (approx. 1 ml) of medium D and stirred. After a further centrifugation a sample, c_S, of 0.5 ml supernatant served to determine the residual radioactivity of sucrose that had been trapped in the primary sediment and that was now almost completely in the new supernatant. The radioactivities of both samples, c_A and c_S, were determined by liquid scintillation counting.

The relative extracellular volume of the densely packed cell suspension could now be calculated according to

$$V_{\text{rel}}^0 = \left[\frac{m_s}{\rho_{\text{ery}}}\right]^{-1} \cdot \frac{V_s \cdot c_s}{c_A - c_S} \tag{11}$$

if we assume $\rho_{\text{ery}} = 1.096$ g/ml.

Any desired relative extracellular volume V_1/V higher than $(V_1/V)^0$ could then be obtained by adding a volume ΔV of bath solution into the standard packed cell suspension.

The final value follows from

$$V_{\text{rel}} = \frac{V_{\text{tot}} \cdot V_{\text{rel}}^0 + \Delta V}{V_{\text{tot}} + \Delta V} \tag{12}$$

where $V_{\text{tot}} = m_S/\rho_{\text{ery}}$ is the volume of the sediment before dilution.

Results and Discussion

The method was tested in experiments with three permeants that differ in their membrane permeability, P_d, using suspensions of human red blood cells with different relative extracellular volume V_1/V. The main issue was to see if the theory could successfully predict the dependence of D_{eff} on V_1/V, in other words, to find out whether P_d is independent of V_1/V. If this were the case, it would substantiate the chosen model. In our experiments, we used hexanol (1 mmol/l) as a very fast permeating solute, water as a rapid permeant and ethylene glycol (10 mmol/l) as a test solute with relatively low transport rates. The results are presented in the following.

Hexanol

The effective diffusion coefficient, D_{eff}, was measured at different relative extracellular volumes, V_1/V, between 0.05 and 0.3. From each pair of D_{eff} and V_1/V (averages of 4 to 10 single values) we calculated P_d, taking: $D_1 = 0.640 \cdot 10^{-5}$ cm^2/s [26], $D_2 = 0.240 \cdot 10^{-5}$ cm^2/s [25], and $\bar{V}_{\text{cell}}$

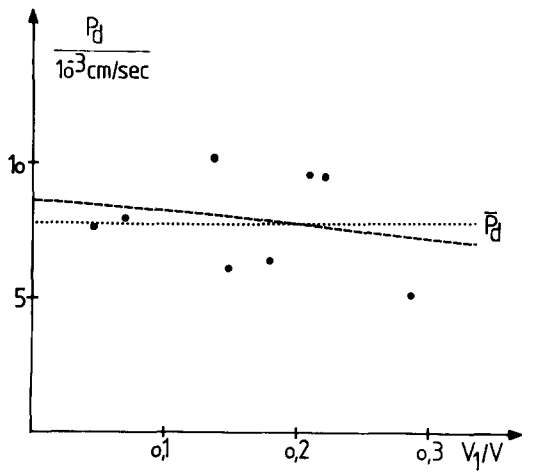

Fig. 3. Diffusion measurements with hexanol. Graph of P_d as a formal function of V_1/V. Linear regression analysis yields the hatched line which slightly deviates from the graph of constant $\bar{P}_d = 7.81 \cdot 10^{-3}$ cm/s (dotted line). On testing the correlation coefficient $r = -0.193$ against the zero hypothesis no significant difference was found at a significance level of $\alpha = 1\%$ which confirms the expected independence of P_d on V_1/V.

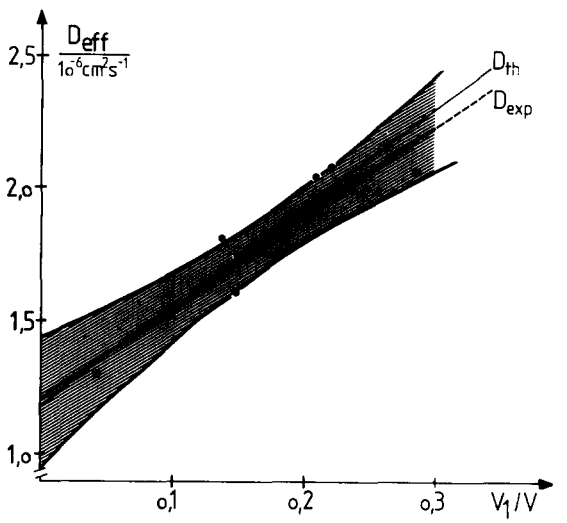

Fig. 4. Diffusion measurements with hexanol. Graph of D_{eff} as a function of V_1/V. Data points are mean values from 4–8 determinations. The dotted line, D_{exp}, is the linear regression line calculated from the measured values, the shaded area represents the 95% confidence interval. The solid line has been calculated from the mean permeability $\bar{P}_d = 7.81 \cdot 10^{-3}$ cm/s by use of Eqns. 7 and 8a–e.

$= 90 \cdot \mu m^3$. The results are plotted in Fig. 3 together with their average

$$\bar{P}_{d,hex} = 7.81 \cdot 10^{-3}\,cm/s$$

Furthermore, a regression line is depicted. Its correlation coefficient, $r = -0.193$, is so low that P_d can rightly be considered to be independent of V_1/V.

In Fig. 4, the relationship between D_{eff} and V_1/V is presented. Here too, the data were fitted by a linear regression analysis. The 95% confidence interval is shown in the graph as a shaded region around the regression line D_{exp}. The line marked D_{th} is calculated from Eqns. 7 ad 8a–e using the average $\bar{P}_d$. It lies completely within the confidence interval. Thus, both kinds of representation of the data show agreement between the model description and the experimental results. The value found for $P_{d,hex}$ agrees well with results reported by Brahm [3]: $P_d = 8.70 \cdot 10^{-3}$ cm/s, which was measured by a continuous fast flow technique at the same extracellular concentration level of 1 mmol/l used in our study. Such high P_d values have to be corrected for the diffusion through the so-called unstirred layers. This implies an additional source of error as the apparent unstirred layer thickness under continuous flow conditions is hard to determined [3,5].

In our completely unstirred system, the influence of the extra-membrane diffusion resistances can be demonstrated if we take, for example, the value about 30% lower $D_1 = 0.452 \cdot 10^{-5}$ cm^2/s for plasma [25] instead of that for saline [26] and again calculate a mean membrane permeability $\bar{P}_d$ from our measurements of D_{eff}. We then end up with an increase of approx. 60% of the permeability to $\bar{P}_d = 13.86 \cdot 10^{-3}$ cm/s. This effect is more pronounced for higher extracellular volumes V_1 (see also Ref. 16) because of the enlarged extracellular diffusion pathlength, L_1. In a somewhat surprising contrast, the method is rather insensitive to errors in the determination of D_2 [16]: a change of a bout 50% in the value of the intracellular diffusion coefficient D_2 hardly affects the value obtained for the membrane permeability P_1. Although diffusion in the intracellular compartment is taken into account in the derivation of our equations, it follows from the mathematical description of the system (see Eqns. 7 and 8a–e) that the influence of D_2 on the

measured effective diffusion coefficient becomes less important with decreasing cell size (see also Ref. 2).

The corresponding diffusion coefficients, D_{eff}, show a linear correlation with the values of (V_1/V) (see Fig. 3) and the measurable quantities $\bar{c}(\Delta t)/c_0$ and V_1/V can be determined with a standard error of less than 5%. Nevertheless, the values of P_d scatter considerably. This is a result of error progression according to Eqns. 7 and 8a–e.

Water

Experiments with water were carried out and evaluated in the same way as with hexanol. ^3H_2O was supposed to be an ideal tracer [27] for the diffusion of H_2O. Therefore no correction for isotope effects was made [6,28]. We obtained a permeability value: $\bar{P}_{d,water} = 4.47 \cdot 10^{-3}$ cm/s with $D_1 = 2.13 \cdot 10^{-5}$ cm^2/s and $D_2 = 1.31 \cdot 10^{-5}$ cm^2/s [19].

This P_d value fits well with data from NMR investigations [9,12,29] but it is higher, by a factor of about two, than the values obtained using a continuous flow method for human red blood cells and their ghosts [7]. Dix and Solomon [30] have recently compiled data for human erythrocyte membrane permeability (P_d) obtained by different groups using different methods. This resulted in a mean permeability of $4.22 \cdot 10^{-3}$ cm/s ($\pm 12\%$ S.D., excluding Brahm's [7] value of $2.4 \cdot 10^{-3}$ cm/s).

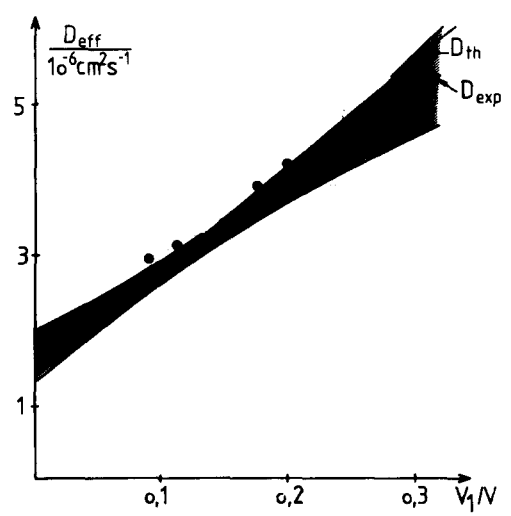

Fig. 5. Diffusion measurements with water. Graph of D_{eff} as a function of V_1/V. Linear regression of the data points (hatched line D_{exp}) results in $\bar{P}_d = 4.47 \cdot 10^{-3}$ cm/s. From this value the straight line D_{th} has been calculated using Eqns. 7 and 8a–e.

The values obtained depend markedly on the choice made for the V/A ratio for the red blood cell as discussed by Brahm [3] and Solomon [31]. Thus, different results may be supposed to result from both the different experimental set-up and different evaluation methods. The function $D_{eff} = f(V_1/V)$ obtained by our method is shown in Fig. 5. Again, the theoretically determined graph, D_{th}, as a function of V_1/V derived from the average permeability $\bar{P}_d$ lies completely within the 95% confidence interval.

Ethylene glycol

The experimental procedure used to measure the effective diffusion coefficient of ethylene glycol in a cell column was the same as that described above except for the addition of NaN_3 (5 mmol/l) to medium D. This was supposed to prevent bacterial degradation of the probe in the system during the long exposure time of up to 24 h. Such long incubation times were necessary in order to obtain sufficient concentration decreases in the capillaries and, thus, low experimental error. We obtained: $\bar{P}_{d,glycol} = 0.897 \cdot 10^{-3}$ cm/s, using $D_1 = 1.035 \cdot 10^{-5}$ cm^2/s and $D_2 = 0.654 \cdot 10^{-5}$ cm^2/s [18].

This value for P_d is about three times higher than that given by Osberghaus et al. [18] who used the same experimental technique but the approach of Perl [16] for evaluation. Although our approach is expected to yield somewhat higher permeabilities the theoretically inherent differences between both approaches can hardly explain the large discrepancy. Other values reported, derived from first order kinetics measurements under different conditions are also 2- to 10-times lower than ours [8,13,32,33]. No satisfactory explanation is presently at hand to account for this discrepancy. If the findings of Mayrand et al. [8] and Levitt et al. [13] that the ethylene glycol transport can be inhibited will be further substantiated, the transport rates depend on the concentration of the substrate. Thus, they will cover a broad range of apparent permeability values. Our results, obtained at a concentration of 10 mmol/l, again

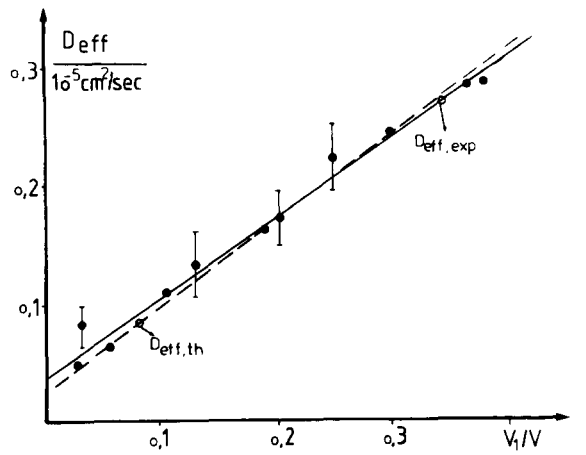

Fig. 6. Diffusion measurements with ethylene glycol. Graph of D_{eff} as a function of V_1/V. The regression line of the experimental points (straight line) and the theoretical graph (dotted line) have been outlined. Some of the data points are given with error bars which indicate the maximal error resulting from error progression in calculating the value of D_{eff}. The errors made in the determination of the $\bar{c}(\Delta t)$, c_0, l and Δt values are considered. The independent relative error in the value of V_1/V was about 3%.

demonstrate (see Fig. 6) a good agreement between the calculated theoretical and the experimentally obtained data.

All experiments presented here have been evaluated using a cubic model ($S/A_2 = 4$) to represent the red blood cell. We preferred this shape as a good approximation because of the random orientation of the disc-shaped cells in the suspension. To check our model, we also did some further control calculations using a quader model. To this end, the different possible orientations of the model cell (in the case of our quader: $S/A_2 = 3$ and $S/A_2 = 8$) with regard to the diffusion (x) axis have to be considered when Eqns. 7 and 8a–e are solved. The results are compared to those obtained for the cubic model in Table I, columns 3 and 4. The difference in P_d values resulting from the choice of the different geometric model is in the order of 10%. We think this justifies working with the cubic model which is easier to handle.

Vesicle experiments

Some additional investigations were carried out on a system in which the cubic model is a much

TABLE I

THE INFLUENCE OF TWO GEOMETRIC MODELS TO DESCRIBE A CELL SUSPENSION ON THE PERMEABILITY VALUES OBTAINED

	D_1 (10^{-5} cm²/s)	D_2 (10^{-5} cm²/s)	Cube P_d (10^{-3} cm/s)	Quader P_d (10^{-3} cm/s)
Hexanol	0.640	0.240	7.81	8.73
Water	2.130	1.310	4.47	5.15
Ethylene glycol	1.035	0.654	0.897	1.04

better model for reality than in the case of erythrocytes: water diffusion was measured in a suspension of heat-fragmented red blood cells. Heat application (15 min, 51° C) leads to a frag-

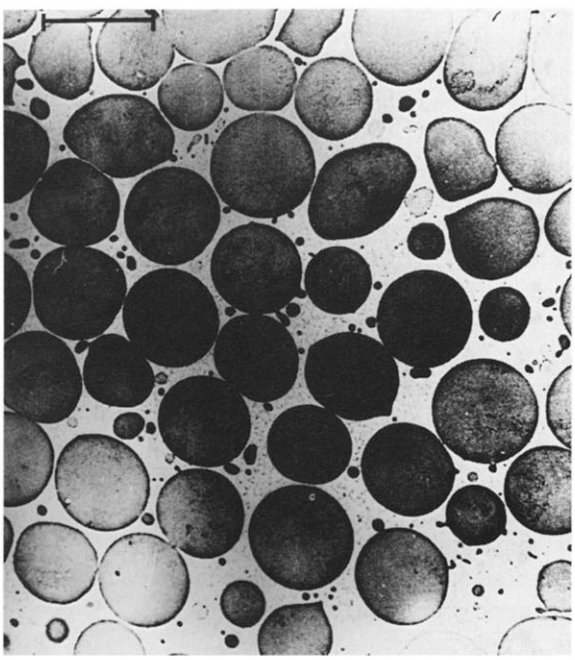

Fig. 7. Electron micrograph of a spherical vesicle population. Electron microphotograph from a section made in a fixed population of heat-fragmented red blood cells. The bar marks a length of 5.0 μm. Probes were made from drops of packed vesicles injected into a buffered fixation solution containing formaldehyde and glutaraldehyde. They were then embedded in epoxide and dried with alcohol and propylene oxide. Post-fixation was performed with OsO_4. Finally, the sample was contrasted by uranyl acetate and sectioned for examination with an electronic microscope.

mentation of the erythrocyte into essentially spherical vesicles [22]. A typical population of such vesicles is represented in the electron micrograph of Fig. 7. The vesicles could be packed to an extracellular volume of 17.5%, about 8% denser than the densest possible packing of homogeneous spheres. This could be due either to an inhomogeneous population or deformability of the vesicles. Since the two semi-axes of the vesicle cross sections were nearly equal the suspension must consist of spherical vesicles with different radii. The varying diameters of the cross sections are partly due to the random stacking of the vesicles and partly caused by different vesicle sizes. It was, therefore, necessary to determine a mean spherical radius as the geometric characteristic of the vesicle population. For this purpose electron micrographs, like that in Fig. 7, were evaluated to obtain a cell section histogram of vesicle populations that were used in a diffusion experiment. From such histograms a mean radius, R_m, of the vesicles can be calculated [34]. A value of $R_m = 1.86\ \mu m$ was found corresponding to a volume of $V_m = 27.0\ \mu m^3$. The geometric model, thus, gave a side length $L_2 = 3\ \mu m$ for the cubic model cell of the suspension.

The water permeability of spherical vesicles from heat-fragmented human red blood cells as derived from diffusion experiments was found to be $P_{d,sph} = 5.24 \cdot 10^{-3}$ cm/s. Within the experimental limits of about 20%, this value is in reasonable agreement with the value, $\bar{P}_{d,ery} = 4.47 \cdot 10^{-3}$ cm/s, that had been measured for native erythrocytes. We, therefore, conclude that the choice of a cube as a cell model in a random suspension is justified. Moreover, these results demonstrate that the new version of the bulk diffusion approach presented here allows measurements on membrane vesicles. This was not possible by the original approach of Perl [16] that required maximal packing of the cells.

Conclusion

An improved description for the diffusion in a composite system has been introduced that expands the applicability range of the capillary method for measuring membrane permeabilities in cell suspensions. The improvement consists in the explicit consideration of the formerly neglected intercellular diffusion path, L_0, in x direction [16] (Fig. 1) when the diffusion equation is solved. The restriction [16,18,25] to very closely packed suspensions ('columns of cells') can thus be avoided. The only theoretically founded requirements left are (i) the suspension must be macroscopically homogeneous and (ii) it must, on a microscopic scale, consist of very small suspended particles.

Condition (i) is always fulfilled provided that the cell suspension packing is denser than the natural sedimentation density of the cell population under study. The suspension may then be assumed to consist of identical units. The second requirement is related to the length, L_2, of the cell species investigated. This value has to be small as compared to the capillary length, l, in order to achieve sufficiently short local relaxation times. Only under this condition, is local quasi-stationarity within all the microscopic units granted.

Under these conditions, the transport problem can be reduced to the solution of the diffusion problem for a single unit and our more generalized approach will be valid. This includes the consideration of all diffusion steps at any packing density level. It does not, therefore, suffer from unstirred layer effects.

Above the sedimentation density the extracellular volume, V_1, may vary within a broad range the limits of which depend on the suspension under study. In the case of human red blood cells, we have shown here that the relative extracellular volume, V_1/V, may be increased up to 35%.

As an important consequence, cell types that cannot be packed very closely, as well as membrane vesicles, can now be investigated by the linear bulk diffusion method. Furthermore, the value of D_{eff} that has to be measured can be influenced by a variation of the value of V_1/V. Increasing the extracellular volume V_1 results in higher values of D_{eff}. This additional degree of freedom may be used to shift the measuring time, Δt, into an adequate range (usually 10–20 h) with regard to the limited lifetime of cells in vitro without a loss of experimental precision due to an insufficient concentration change in the capillary. By this means, lower permeabilities come into the range of the technique.

As for high membrane permeation rates, the

technique is not limited at all. Appropriate concentration decreases can be obtained either by increasing the capillary length or by reducing the measuring time. Thus, the fundamental problems that have been discussed previously have been overcome by a new formulation of the mathematical approach to describe a diffusion process in a composite system like a cell suspension.

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