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A MICROFLUORIMETRIC STUDY OF TRANSLATIONAL DIFFUSION IN ERYTHROCYTE MEMBRANES*

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SUMMARY

A method is described which permits quantitative study of translational diffusion in the membranes of single cells. Human erythrocytes were labelled with fluorescein isothiocyanate and then hemolyzed, which yielded ghosts of normal shape and strong fluorescence. By application of sodium dodecylsulfate–polyacrylamide gel electrophoresis it was found that a very large part of fluorescein isothiocyanate was bound to the proteins of the erythrocyte membrane. In a fluorescence microscope, single ghosts were exposed to a sharply bounded intensive beam of light in such a manner that in each case only one half of the ghost was bleached. By microscopic measurements it was studied whether fluorescent material would diffuse from the unbleached part of the membrane into the bleached part and vice versa. However, within the measuring time of 20 min at room temperature a significant degree of such a diffusion could not be detected. In order to evaluate the experimental data quantitatively, the diffusion equation for a spherical surface was solved, and the obtained solution furthermore was integrated over the hemispheres. By these means a value of $3 \cdot 10^{-12}$ cm^2/s was derived from the experimental data as an upper limit for the diffusion coefficient of fluorescein isothiocyanate-labelled compounds in the erythrocyte membrane at 20°–23 °C.

INTRODUCTION

Recently a number of investigations has demonstrated rotational and translational movement of proteins in cellular membranes [2–9]. These observations support the idea [1] that cellular membranes basically consist of a fluid lipid bilayer within which membrane proteins are able to diffuse at comparatively high rates. Nevertheless

* A part of this study has been presented at the 43. Tagung der Deutschen Physiologischen Gesellschaft [10].

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only a few types of membranes have been studied with respect to fluidity so far and quantitative data on the diffusion of membrane proteins are available in some cases only [3, 6, 7]. The present study is concerned with translational movement in human erythrocyte membranes, which were labelled covalently with the fluorescent marker fluorescein isothiocyanate. This dye is known to fade quite rapidly [11] under conditions of visual fluorescence microscopy. By simple technical means it was therefore possible to bleach single fluorescein isothiocyanate-labelled ghosts on one half only. The gradient of fluorescent material thus established in the membrane was followed kinetically over a period of 20 min after bleaching by microscopic measurements. The results obtained in this manner indicate that lateral diffusion is much slower in the erythrocyte membrane than for instance in photoreceptor membranes. This might be due to differences in lipid composition of these two membrane types, but more likely is related to the complex protein organization of the erythrocyte membrane, in which "spectrin" apparently plays an important role.

METHODS

1. *Preparation of fluorescein isothiocyanate-labelled ghosts*

Hemoglobin-free erythrocyte ghosts were prepared according to the method of Dodge et al. [12], whereas fluorescein isothiocyanate-labelled ghosts were prepared in the following manner. 1 ml of fresh human citrate blood was washed once with 40 ml buffered saline, pH 8.0. The packed erythrocytes were then incubated with 5 mg/ml of a 10 % adsorbate of fluorescein isothiocyanate on celite (Serva, Heidelberg, G.F.R.) for 30 min at 20 °C. No appreciable hemolysis was induced by the staining procedure. The red cells were then hemolyzed in 40 ml of 7 mM sodium phosphate buffer, pH 8.0. Celite was sedimented by low-speed centrifugation and removed. The ghosts subsequently were sedimented by high-speed centrifugation (Sorvall RC-2B, Rotor SS 34, 15 000 rev./min, 10 min, 4 °C) and washed three times with 40 ml of 7 mM buffer. Finally the ghosts were resuspended to give a total volume of 1 ml. The procedure yielded ghosts of normal shape, exhibiting a strong and homogeneous fluorescence.

2. *Sodium dodecylsulfate-gel electrophoresis*

Sodium dodecylsulfate–polyacrylamide gel electrophoresis was performed following the method of Shapiro et al. [13]. Sodium dodecylsulfate and 2-mercaptoethanol were added to a small volume of a suspension of either unstained or fluorescein isothiocyanate-labelled ghosts to give a final concentration of 1 % w/v and 1 % v/v respectively. 30 µl of this solution were applied to 5 % sodium dodecylsulfate–polyacrylamide gels of 6 mm diameter and run for about 2.5 h using bromophenolblue as tracking dye. The gels were then stained with Coomassie blue and scanned for transmittance at 620 nm with a commercial apparatus (Zeiss). For fluorescence scanning, gels of fluorescein isothiocyanate-labelled ghosts were run in the same manner applying, however, approximately a three-fold amount of membrane material. Immediately after electrophoresis the gels were scanned for fluorescein isothiocyanate-fluorescence in a fluorescence microscope according to a procedure developed by Bähr and Jovin (in preparation).

3. *Preparation of specimen for microscopic study*

1 μl of a diluted suspension of fluorescein isothiocyanate-labelled ghosts was deposited on a clean glass slide and was covered with a 12 mm × 38 mm cover slip. In the absence of dust particles on slide and cover slip, the ghost suspension spread spontaneously between slide and cover slip. As follows from the volume applied and the area occupied, the average thickness of the layer of the ghost suspension obtained in this manner was about 2 μm. In the layer the ghosts therefore were flattened and acquired a discoidal shape. This fact reduced defocussing effects in bleaching and fluorescence measurements which were due to the very small depth of focus of the high power objective. The preparation of the specimen was completed by sealing the layer of the ghost suspension at the edges of the cover slip with a laboratory grease. This prevented evaporation of buffer and drying of membranes.

4. *Diffusion studies*

The Zeiss microfluorimeter as previously described in a different context [14] was used and equipped with a 100 ×, n.a. 1.30, oil-immersion objective. In this apparatus the object is aligned and focussed by phase contrast microscopy in transmitted light, whereas bleaching and fluorescence measurements are performed with incident light. Thus the light of the excitation source (Osram, HBO 100) is focussed on the object by the objective itself, which permits to obtain a particularly high excitation energy. For a diffusion experiment a ghost of a diameter of 9 μm (as determined by an ocular micrometer) was chosen from the membranes in the specimen. The subsequent procedure is indicated schematically in Fig. 1. For bleaching, a 434 nm interference filter, a heat absorbing filter (KGl of Schott, Mainz, Germany), and a diaphragm, which cut off the light in one half of the field of vision, were placed into the excitation light path. The diaphragm was carefully aligned in order to obtain a sharp boundary between light and shadow in the object. The ghost to be bleached was placed exactly on the boundary and in this position (Fig. 1a) was irradiated for 30 s. After bleaching the diaphragm was removed and instead a neutral grey filter was inserted, which diminished the excitation intensity by approximately 98 %. Then a measuring field of 4.5 μm × 9.0 μm was adjusted by means of the variable aperture in the photometer head and the specimen was aligned in such a manner, that one half of the partially bleached ghost fitted exactly into the measuring field. In this position (Position II in Fig. 1b) a fluorescence measurement lasting 8 s was performed. Subsequently the specimen was shifted a little to bring the other half of the membrane into the measuring field (Position III in Fig. 1b). After a second measurement the ghost was shifted completely out of the measuring field (Position IV in Fig. 1b) in order to measure background intensity. The described series of measurements was repeated after 10 min and again after 20 min. The temperature was 20–23 °C.

In order to show that the membranes were not bleached by the measuring procedure, the fluorescence of single, whole fluorescein isothiocyanate-labelled ghosts was measured repeatedly. Bleaching was found to be negligible. During these experiments, however, it unexpectedly was observed, that membrane fluorescence was not stable over longer periods of time in all of the ghosts: in some membranes fluorescence decreased spontaneously with time. A conclusive explanation for this behaviour has not yet been found and several modifications of the experimental conditions (variation of pH and ionic strength of the buffer, addition of either Ca^{2+} or EDTA,

extensive cleaning of slides and cover slips) had no appreciable effect. Consequently the fluorescence of each ghost chosen for a diffusion experiment was first measured two times at a 10 min interval. Only if there was no loss of fluorescence intensity the experiment was continued.

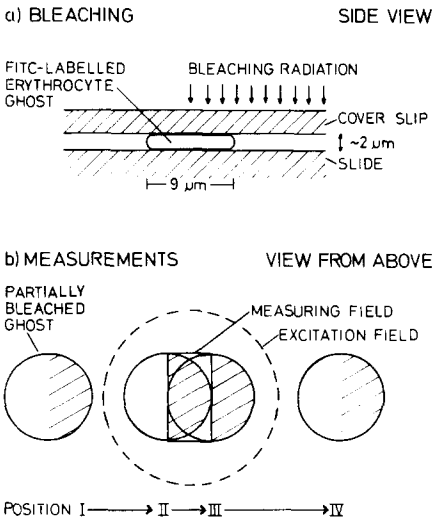

Fig. 1. Schematic representation of the procedure applied to study lateral diffusion in erythrocyte membranes. (a) A thin layer of a suspension of fluorescein isothiocyanate (FITC)-labelled erythrocyte ghosts was deposited between a slide and a cover slip. In a fluorescence microscope a ghost was chosen from the membranes in the specimen and bleached on one half only by an intensive, sharply bounded light beam. (b) The gradient of fluorescent material thus established in the membrane was followed by separately measuring the fluorescence intensities of the membrane halves. For this purpose a measuring field of the size of the diameter times the radius of the ghost was used. The specimen was shifted stepwise as indicated in the figure. In Position II the fluorescence intensity of the right half and in Position III of the left half of the ghost was measured, whereas background intensity was registered in Position IV.

In order to study whether the fluorescence of fluorescein isothiocyanate would be restored spontaneously after bleaching, whole ghosts were bleached and their fluorescence measured. Usually a slight increase of membrane fluorescence, e.g. by a few percent was observed during the first minute after bleaching. Then fluorescence did not change any further. Therefore an equilibration time of three minutes between bleaching and the first measurement was chosen in all diffusion experiments.

In diffusion experiments the fluorescence of the membrane part directly exposed to the bleaching light beam was found to be reduced by approximately 65 %. However, the fluorescence of the "unbleached" part of the membrane (the half which remained in the shadow during bleaching) was also found to be reduced by a certain degree (approximately 30 %, e.g. see Fig. 4). This seems to be due mainly to stray light: the fluorescence of ghosts, which were placed entirely on the shadow side of the field of vision during bleaching, also decreased and this fluorescence reduction was of a comparable degree (20 %–25 %).

5. *Microphotographs*

Microphotographs of partially bleached fluorescein isothiocyanate-labelled ghosts were taken using a high-speed film (Kodak 2457 Recording Film). The exposure time was 32 s applying the full excitation energy.

6. *Calculation of diffusion coefficients*

In order to evaluate the experimental data, the diffusion on the surface of a sphere was analyzed. The diffusion equation

$$\partial_t c = D\Delta c \tag{1}$$

in polar coordinates (r, θ, ϕ) is the following:

$$\partial_t c = D\left\{\frac{1}{r^2}\partial_r r^2 \partial_r + \frac{1}{r^2 \sin\theta}\partial_\theta \sin\theta \partial_\theta + \frac{1}{r^2 \sin^2\theta}\partial_\phi^2\right\} c \tag{2}$$

D is the diffusion constant, $c = c(\vec{r}, t)$ is the concentration at point $\vec{r}$ and time t, Δ is the Laplacian operator and δ_ε stands for the partial derivative with respect to ε. If the concentration is dependent neither on r nor on ϕ, then Eqn 2 reduces to

$$\partial_t c = D\frac{1}{r^2 \sin\theta}\partial_\theta \sin\theta \partial_\theta c \tag{3}$$

With the abbreviation $x = \cos\theta$ (and consequently with $\delta_\theta = -\sin\theta\delta_x$)

$$\partial_t c = \frac{D}{r^2}\partial_x(1-x^2)\partial_x c \tag{4}$$

follows. Eqn 4 describes for example the diffusion on an isotropic spherical surface, if the initial concentration does not depend upon the angle ϕ of rotation about the polar axis. The similarity of Eqn 4 with the differential equation of Legendre (see for example, ref. 15) suggests to expand c into a series of Legendre's polynominals $P_n(x)$. As a general solution of Eqn 4 in accordance with Eqn 8 in ref. 16

$$c(x, t) = \sum_{n=0}^{\infty} A_n P_n(x) e^{-n(n+1)(D/r^2)t} \tag{5}$$

is obtained, the coefficients A_n depending neither on x nor on t but instead are only determined by the initial conditions $c(x, 0)$.

If at $t = 0$ the diffusing particles are uniformly distributed on each of the hemispheres, i.e.

$$c(x, 0) = \begin{cases} \alpha & \text{for} \quad 0 < x \leqslant 1 \\ \beta & \text{for} \quad -1 \leqslant x < 0 \end{cases}$$

then, because of the orthogonality of Legendre's polynomials, in accordance with Eqn 10 in ref. 16,

$$c(x, t) = \frac{\alpha+\beta}{2} + \frac{\alpha-\beta}{2}\sum_{k=0}^{\infty}\frac{(-)^k(4k+3)(2k)!}{2^{2k+1}k!(k+1)!}P_{2k+1}(x)e^{-(2k+1)(2k+2)(D/r^2)t} \tag{6}$$

results.

In the experiments the fluorescence emitted by fluorescein isothiocyanate on one half of the membrane was measured. In the mathematical model this corresponds to the determination of an average concentration on the upper and lower hemisphere, which is:

$$c_+(t) \equiv \frac{1}{2\pi}\int_0^{2\pi} d\phi \int_0^1 d\cos\theta c(x,t) = \int_0^1 dx\, c(x,t) \tag{7}$$

and

$$c_-(t) \equiv \frac{1}{2\pi}\int_0^{2\pi} d\phi \int_{-1}^0 d\cos\theta c(x,t) = \int_{-1}^0 dx\, c(x,t) \tag{8}$$

respectively. The solution for this is:

$$c_+(t) = \frac{\alpha+\beta}{2} \pm \frac{\alpha-\beta}{2}\sum_{k=0}^{\infty} P_k \exp\{-(2k+1)(2k+2)(D/r^2)t\} \tag{9}$$

with

$$P_k \equiv (4k+3)\left|\frac{(2k)!}{2^{2k+1}k!(k+1)!}\right|^2 \tag{10}$$

Let

$$P(t) \equiv 1 - \sum_{k=0}^{\infty} P_k \exp\{-(2k+1)(2k+2)(D/r^2)t\} \tag{11}$$

then Eqn. 9 becomes

$$c_+(t) = \alpha - \frac{\alpha-\beta}{2}\cdot P(t) \tag{12}$$

$$c_-(t) = \beta + \frac{\alpha-\beta}{2}\cdot P(t) \tag{13}$$

P_k and $P(t)$ can easily be determined by an iterative procedure. If we take

$$P(n,t) \equiv 1 - \sum_{k=0}^{n} P_k \exp\{-(2k+1)(2k+2)(D/r^2)t\} \tag{14}$$

and choose

$$P(-1,t) = 1 \tag{15}$$

$$P_{-1} = -1 \tag{16}$$

as "intial conditions", then successively for $k = 0, 1, \ldots, n$

$$P_k = P_{k-1}\cdot\frac{4k+3}{4k-1}\cdot\left(\frac{4k-2}{4k+4}\right)^2 \tag{17}$$

$$P(k,t) = P(k-1,t) - P_k\cdot\exp\{-(2k+1)(2k+2)(D/r^2)t\} \tag{18}$$

is obtained and according to Eqns 11 and 14,

$$P(t) = \lim_{n \to \infty} P(n, t) \tag{19}$$

$P(t)$ was calculated in this manner on the UNIVAC 1108 of the "Gesellschaft für wissenschaftliche Datenverarbeitung", Göttingen and the UNIVAC 1108 des "Hochschulrechenzentrums" of the University at Frankfurt. The average concentrations c_+ and c_- are depicted in Fig. 2 in a general form as functions of the dimensionless quantity tau $= (D/r^2)t$ for $\alpha = 1$ and $\beta = 0$ (this is no restriction of generality). They were then drawn by a CALCOMP-plotter.

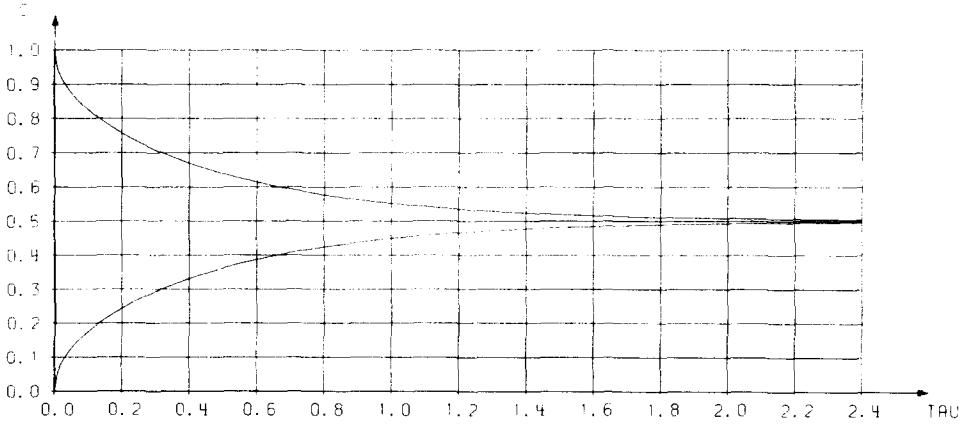

Fig. 2. The solution of the diffusion problem encountered in this study is shown in a general form. The diffusion on the surface of a sphere has been analyzed for the case, where, at $t = 0$, the particles are distributed homogeneously on each hemisphere. On the ordinate the mean concentration C on each hemisphere is plotted denoting the concentrations at $t = 0$ as 1.0 and 0.0 respectively. The abscissa is the dimensionless variable tau $= (D/r^2)t$, where D is the diffusion coefficient, r is the radius of the sphere and t the time.

It has been mentioned above that the diffusion experiments were performed on erythrocyte ghosts, which had a discoidal shape measuring 9 μm in diameter and approximately 2 μm in height. If such ghosts are thought to convert into spheres without changing their surface area, they would assume a radius of 3.6 μm (a discoidal body of 9 μm diameter and 2 μm height has a surface area of 160 μm^2, which is the same as that of a perfect sphere with a radius of 3.6 μm). Approximating the discoidal ghosts by spheres, the application of a radius of 3.6 μm therefore seems to be the best approach. Consequently we applied Eqn 9 with $r = 3.6$ μm for the evaluation of our experiments in terms of a diffusion coefficient. The diffusion coefficient, however, does not depend in a particularly sensitive manner on the radius of the sphere chosen to approximate the actual shape of the ghosts (see e.g. Fig. 2). If, for instance, the discoidal shape of the ghosts would have been completely neglected and the ghosts were approximated by spheres with a diameter of 9 μm ($r = 4.5$ μm), the diffusion coefficient derived from a given measurement would be larger by a factor of $(4.5/3.6)^2 = 1.56$.

RESULTS

Scans of sodium dodecylsulfate gels of fluorescein isothiocyanate-labelled ghosts are given in Fig. 3. The scan in Fig. 3a was obtained after staining the gel with Coomassie blue, and a pattern typical for the proteins of human erythrocyte membranes (e.g. ref. 17) was found. At low mobilities the characteristic double peak is seen, which is attributed to the protein named spectrin (ref. 26, mol. wt $>$ 200 000). A second strong peak is found approximately in the center of the gel and belongs to the 100 000 mol. wt group [18]. This pattern is very similar to those given in the literature for unstained ghosts [17]. We found no significant difference between gels of fluorescein isothiocyanate-labelled and of unstained ghosts. Fig. 3b shows the distribution of fluorescein isothiocyanate-fluorescence in the gels. A major part of the fluorescence is concentrated in the 100 000 mol. wt region, whereas in other regions fluorescence is less pronounced. In the front of the gels a fluorescein isothiocyanate-peak is observed, which does not correspond to any of the protein peaks. This might be due to fluorescein isothiocyanate which is bound to certain lipids (see discussion), or to unreacted or split-off fluorescein isothiocyanate.

In order to study the effect of the described bleaching procedure, microphotographs of bleached ghosts were taken. As a consequence of the rapid fading of fluorescein isothiocyanate at high irradiation levels it was rather difficult to obtain this kind of photographs at all, even when highly sensitive film material was used. The photographs did show, however, that it was possible to obtain a relatively sharp boundary between bleached and unbleached parts of single ghosts, and that the membranes were divided into two parts of approximately equal size.

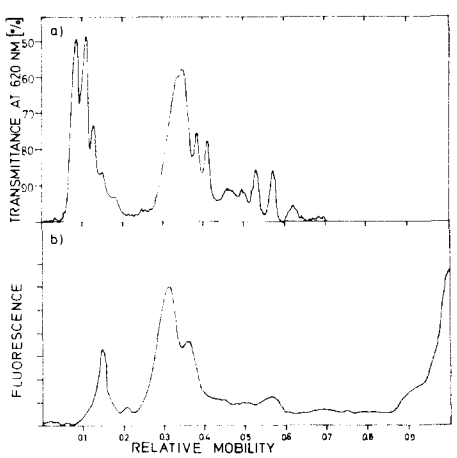

Fig. 3. Sodium dodecylsulfate–gel electrophoresis of fluorescein isothiocyanate-labelled ghosts. (a). Scanning pattern of a gel which was stained with Coomassie blue. (b). Scanning pattern of fluorescein isothiocyanate-fluorescence in unstained gels.

The recording curve in Fig. 4 shows the course of a diffusion experiment. The steps, which above have been described in detail, may be found: first measurement, bleaching, equilibration time, and measurements at $t = 0$ min, $t = 10$ min, and $t = 20$ min.

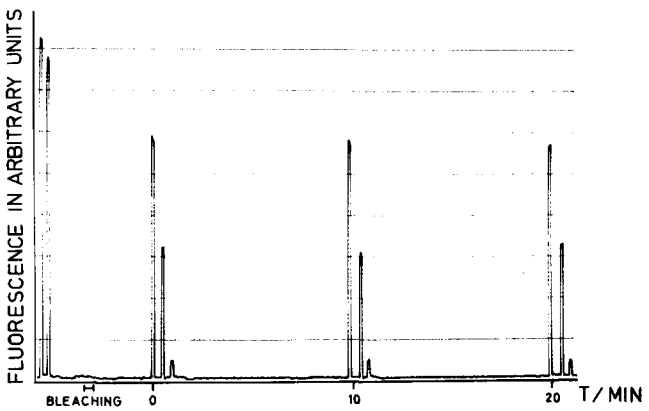

Fig. 4. A recording curve showing the course of a diffusion experiment. Ordinate: fluorescence signal; abscissa: time. In each group, measurements representing the fluorescence intensity of each membrane half and the intensity of the background are shown.

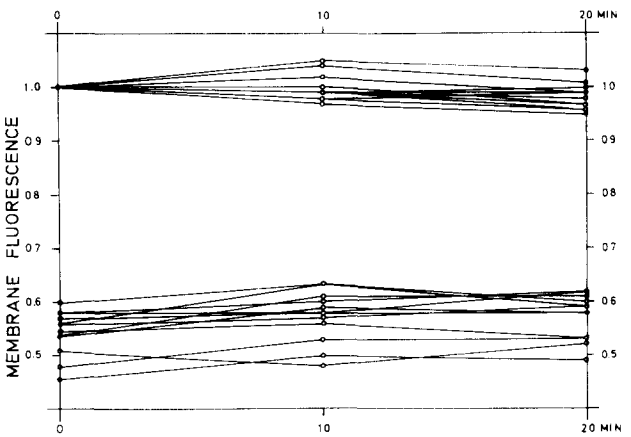

Fig. 5. The results of diffusion studies on 12 individual ghosts are shown. Ordinate: Fluorescence intensity of the unbleached and of the bleached membrane halves denoting the fluorescence intensity of the unbleached half at $t = 0$ min as 1.0; abscissa: time.

Fig. 5 gives the results of measurements on 12 ghosts, in each case denoting the fluorescence intensity of the unbleached half at $t = 0$ min as 1.0. At $t = 0$ min the ratio between the fluorescence intensities of bleached and unbleached halves was 0.55 ± 0.05 (S.D.). In the course of 20 min this gradient changed little: after 10 min the intensity of the unbleached halves was 1.00 ± 0.03, whereas that of the bleached halves was 0.57 ± 0.05. The corresponding values after 20 min were 0.98 ± 0.02, and 0.57 ± 0.04, respectively.

In Fig. 6 the mean values of the measurements, which have been shown in Fig. 5, are given. In analogy to Fig. 2, however, this time the fluorescence intensities of the bleached halves at $t = 0$ min were denoted as 0.0 and those of the unbleached halves at $t = 0$ min as 1.0. The experimental values for the fluorescence intensities

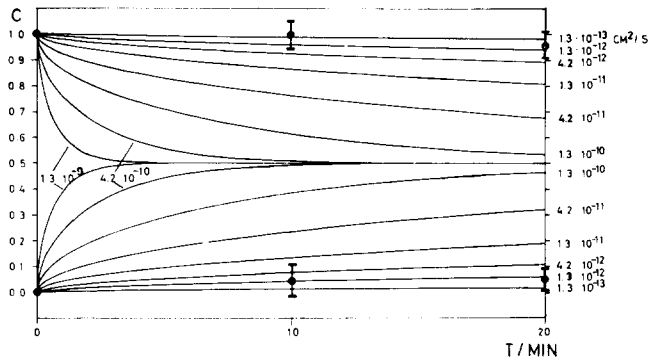

Fig. 6. The results of the diffusion studies are shown together with curves for diffusion. Coefficients as calculated from Eqn. 9 with $r = 3.6\,\mu m$. In this graph the fluorescence intensities of the bleached membrane halves at $t = 0$ min were denoted as 0.0, and those of the unbleached halves as 1.0.

of bleached and unbleached membrane halves in this graph at $t = 0$ min, $t = 10$ min, and $t = 20$ min were 0.00, 0.05 ± 0.06, 0.05 ± 0.05, and 1.00, 1.00 ± 0.06, 0.95 ± 0.05, respectively. Together with the experimental values a family of theoretical curves has been plotted, which was calculated from Eqn. 9 with a value of $r = 3.6\,\mu m$. Although there seems to be a very slight decrease of the gradient in fluorescent material between the membrane halves in 20 min, calculation of a diffusion coefficient does not seem to be justified. Instead an upper limit may be given. Taking all the experimental values, a reduction of the initial difference in fluorescence by 9 % ± 7 % in 20 min was found. According to Eqn 9 with $r = 3.6\,\mu m$ a 16 % reduction of an initial concentration difference in 20 min corresponds to a diffusion coefficient of $2.2 \cdot 10^{-12}$ cm²/s. Therefore a value of $3 \cdot 10^{-12}$ cm²/s is regarded as an upper limit for the average translational diffusion coefficient of fluorescein isothiocyanate-labelled compounds in the human erythrocyte membrane at 20–23 °C.

DISCUSSION

1. *Methods*

In the version described above, the applied technique is suited for the determination of diffusion coefficients, which are in the range of 10^{-9} cm²/s to 10^{-12} cm²/s approximately (see Fig. 6). It is obvious that this range may easily be extended. If the bleaching time and the measuring time would be reduced by a factor of 1000, diffusion coefficients as large as 10^{-6} cm²/s could be measured, which is sufficient to follow the diffusion of lipids [19] and still faster molecules in membranes. Basically the degree of bleaching is of little importance for the technique. In order to disturb the membrane as little as possible a low degree of bleaching would even be preferable but would require an increase in the resolution of the fluorescence measuring device. In order to study the diffusion in membranes at physiological temperatures, a temperature-controlled object stage should be used. With regard to the staining procedure a large number of variations seems possible. It might be particularly attractive to label cells with fluorescein isothiocyanate-conjugates of antibodies or plant agglutinins, because with these substances specific components of the membrane can be labelled and intact cells in addition to isolated membranes could be studied.

Fluorescein isothiocyanate is usually employed for fluorescent protein tracing in immunological studies and has not yet been used to label erythrocyte membranes. It is therefore unknown whether organization and function of the membrane are influenced by the conjugation process. It can be said, however, that hemolysis is not induced by staining and that sodium dodecylsulfate–gel electrophoresis does not reveal an alteration of the protein pattern. The application of fluorescein isothiocyanate–celite avoids the use of organic solvents and is a simple, rapid and mild method of labelling membranes. Fluorescein isothiocyanate reacts primarily with amino- and sulfhydryl groups [20]. In the erythrocyte membrane it may combine with membrane proteins and lipids such as phosphatidylethanolamine. The unidentified fluorescence peak in the front of the gels (Fig. 3b) therefore might be due to fluorescein isothiocyanate, which is bound to lipid. The fluorescence scan in Fig. 3b seems to exhibit a certain selectivity of labelling as documented by a concentration of the fluorescence in the 100 000 mol. wt region. A similar selectivity has been reported recently [17] upon labelling of erythrocytes with dimethylaminonaphthalene chloride which was dispersed in phospholipid micelles. Since fluorescein isothiocyanate and dimethylaminonaphthalene chloride have a number of properties in common (e.g. site of combination and hydrophobicity) this similarity is not surprising. The selective labelling of membrane proteins by hydrophobic dyes such as dimethylaminonaphthalene chloride and fluorescein isothiocyanate, which can penetrate the erythrocyte membrane is of some interest for the interpretation of surface labelling studies [28–30]. This aspect, however, is not of direct concern for the present study, a discussion may be found in the literature [17, 21].

2. *Results*

Frye and Edidin [2] are among the investigators who first reported on the lateral motion of proteins in a biological membrane. From their observations pertaining to the movement of surface antigens in the membranes of mouse–human cell hybrids a diffusion coefficient of $0.2 \cdot 10^{-9}$ cm^2/s has been estimated [3]. An even higher value ($D = 1\text{–}3 \cdot 10^{-9}$ cm^2/s) was found by Edidin and Fambrough [3] for the diffusion coefficient of surface antigens in the membranes of cultured muscle fibers. Recently Poo and Cone [7] have studied the lateral diffusion of rhodopsin in the photoreceptor membranes of frog and mudpuppy and have reported a value of $D = 4 \cdot 10^{-9}$ cm^2/s at 20 °C.

In contrast [33] to photoreceptor membranes, erythrocyte membranes are characterized by a high content of cholesterol and of saturated fatty acid chains. Both properties suggest a comparatively low fluidity of the lipid membrane matrix. For the interpretation of our results, it furthermore might be relevant that a great deal of fluorescein isothiocyanate was bound to the 100 000 mol. wt proteins of the erythrocyte membrane. Hence, the results of the present diffusion experiments are largely determined by the mobility of these proteins. The 100 000 mol. wt proteins are tightly bound to the membrane [18] and cannot be released by variation of ionic strength or by denaturants, but are solubilized only by detergents. These proteins therefore can be characterized as "intrinsic" and apparently are deeply embedded in the membrane matrix, probably spanning it [27]. The 100 000 mol. wt proteins consequently may interact with lipids and intrinsic membrane proteins and in addition with other membrane components such as extrinsic proteins on both surfaces of the

membrane. This possibility seems to be particularly important with respect to the protein called spectrin. It recently has been observed [32], that agglutination of spectrin is accompanied by a clustering of anionic sites on the membrane surface opposite to the location of spectrin. This suggests [31], that spectrin interacts with other membrane proteins such as the 100 000 mol. wt proteins to form large structures, which span the membrane. Depending on the size of such hypothetical aggregates their lateral diffusion could be more or less restricted.

That the erythrocyte membrane is a rather rigid structure has been indicated by previous investigations as well. By ESR studies [25] it has been shown, that erythrocyte membranes are much more rigid than nerve fibers and phospholipid dispersions. On the basis of their mechanical properties [23, 24] erythrocyte membranes have been characterized as "tough viscoelastic solids" [24]. For these and other reasons a recent review on the proteins of the erythrocyte membrane [18] questioned, that the fluid mosaic model [1] is applicable to the erythrocyte membrane. Our results indicate, that a comparatively rapid lateral diffusion as observed in photoreceptor membranes [7] is not typical for the erythrocyte membrane. However, a lower rate of diffusion, which nevertheless could be quite effective in the sub-light microscopic range is not ruled out by the results of the present study.

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Recently, the role of spectrin has been further analyzed by freeze fracture electron microscopy and sodium dodecylsulfate-polyacrylamide gel elcrophoresis (refs 34, 35 and Elgsaeter, A. and Branton, D. (1974) to be published). These studies provide further evidence for the hypothesis that spectrin forms a dense molecular meshwork at the cytoplasmic surface of the membrane which limits translational diffusion in the membrane matrix.

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