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Diffusion of fluorescein-labelled molecules in suspensions of erythrocyte ghosts

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The diffusion of fluorescein isothiocyanate-labelled dextran molecules in suspensions of centrifugally, tightly packed, erythrocyte ghosts was measured by fluorescence recovery after photobleaching. In comparison with diffusion in aqueous solution, the diffusion coefficients for probe molecules of varying size were about two orders of magnitude smaller. It was established that the dextran molecules remained in the space between the ghosts. Since crosslinking membrane surface carbohydrates with antibodies further inhibits diffusion, it is assumed that interactions between surface carbohydrates and the probe molecules are the cause of slow diffusion. Two alternative models are discussed.

Intercellular diffusion; FRAP; FITC-dextran; Cell membrane carbohydrate; Carbohydrate interaction; (Erythrocyte ghost)

1. INTRODUCTION

Cells in animal tissues are set apart by an intercellular space of varying thickness. Carbohydrates are an important class of structural components in this interstitium. Glycoproteins and glycolipids as well as molecules of the proteoglycan type are anchored in the plasma membrane and form a layer covering the membrane, separating cells from one another. Various aspects of this intercellular matrix have been studied intensively, but its properties are still poorly understood [1].

In recent years the composition and the structure of the carbohydrate layer covering the erythrocyte membrane have become known [2]. The erythrocytes do not form a tissue in the usual sense, and thus do not have an interstitium of the type described above. On the other hand, the carbohydrate layer of the erythrocyte membrane is quite massive, extending about 100–200 Å outwards and having a carbohydrate concentration equivalent to 0.1 mol of monosaccharide per liter, or approx. 2% [2]. This carbohydrate is covalently attached to integral membrane proteins and lipids and should therefore exhibit diffusional properties similar to integral membrane proteins [3,4]. The high concentration of the carbohydrate will also contribute to its slow diffusion [5].

Most studies on the properties of the interstitium have been performed with model systems consisting of hyaluronan or proteoglycans, or of model carbohydrates such as dextran [5,6]. The properties of the membrane bound glycoprotein or glycolipid carbohydrate have not received much attention. In the present study we have used erythrocyte ghosts to prepare an artificial 'tissue' with closely packed cells separated only by tightly bound membrane carbohydrates. In this system we have studied the diffusion of fluorescent probes of varying molecular size.

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Abbreviations: FITC, fluorescein isothiocyanate; FRAP, fluorescence recovery after photobleaching

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2. MATERIALS AND METHODS

2.1. *Fluorescent probes*

Carboxyfluorescein was from Sigma (St. Louis, MO) and the fluorescein isothiocyanate-labelled dextrans (FITC-dextrans) were kind gifts from Dr K. Granath, Pharmacia AB (Uppsala). Carboxyfluorescein was used as a solution of approx. 0.2 mg/ml and FITC-dextrans as solutions of approx. 1 mg/ml. FITC-dextran 3 (Lot no.3565), average molecular mass 3 kDa, and FITC-dextran 20 (Lot no.0916), average molecular mass 19.4 kDa, were used. The FITC-dextrans were passed through a Sephadex G-25 column before use in order to remove possible low-molecular degradation products.

2.2. *Other reagents*

Latex particles of diameter 6.76 ± 1.06 μm from Duke Scientific Corp., Palo Alto, CA, were a kind gift of Dr H. Pertoft (Uppsala) and monoclonal anti A-antibody a gift from Dr A. Lundblad (Lund). All other reagents were of analytical grade. The solutions were made in deionized water.

2.3. *Resealed erythrocyte ghosts*

Erythrocytes (about 120 ml) were obtained from the local blood bank (Akademiska Sjukhuset, Uppsala), washed once with 3 vols of 0.15 M NaCl and centrifuged for 10 min at 3000 × *g*. The washed cells were lysed in 8 vols of 10 mM Tris-HCl, pH 7.5, containing 0.2 mM EDTA, and centrifuged 15 min at 10000 × *g*. The ghosts were washed once more in the same volume of the above buffer and once in the same volume of 10 mM Tris-HCl, pH 8.25, centrifuging both times for 15 min at 10000 × *g*. The packed ghosts were then suspended in 10 mM Tris-HCl, pH 8.25, in a final volume of 280 ml, to which 50 ml of 1 M NaCl and 3.3 ml of 10 mM $MgSO_4$ were added. The suspension was incubated 40 min at 37°C, cooled to 0°C in an ice bath and centrifuged for 15 min at 16000 × *g*. The packed colorless ghosts were stored no more than 2 weeks at 4°C until used. 120 ml blood bank erythrocytes yielded approx. 40 ml ghosts. This procedure is essentially the one described by Steck [7] and produces resealed ghosts.

2.4. *Diffusion experiments*

To 2–3 ml of packed ghosts a solution of 0.15 M NaCl, and 10 mM in Tris-HCl, pH 8.25, was added to make a total of 5 ml, and 0.25 ml of the appropriate fluorescent probe in a 10 ml centrifuge tube. The mixture was kept for 40 min at 4°C to permit equilibration and then centrifuged for 60 min at 250000 × *g*. The supernatant was removed and the sediment, the packed ghosts, was used as such for diffusion measurements. The determinations were made by fluorescence recovery after photobleaching (FRAP) using an apparatus described previously [8]. In short, the sample was placed in a small cuvette of 100 μm light path, and a beam from an argon ion laser, wavelength 496.5 nm, was focused on a small area of this cuvette. The arrangement was such that bleaching could be performed with a short flash (usually 2–10 ms) of a strong beam, and excitation of the fluorescent probe with a continuous, strongly attenuated beam. Fluorescence recovery was measured by photon counting in a time resolved manner. The data collection apparatus permitted signal averaging, and thus the results are obtained from a large number of measurements. Both the bleaching and the measuring beam have a cross section with Gaussian intensity distribution, and thus diffusion coefficients could be calculated as described previously [8]. The apparatus was calibrated by measuring the fluorescence recovery of carboxyfluorescein in water, assuming a diffusion coefficient of 3×10^{-6} $cm^2 \cdot s^{-1}$. The fluorescence recovery curves were plotted on a recorder and the recovery half times estimated from these plots. Some diffusion experiments were performed on uncentrifuged or diluted ghosts. Diffusion of the probes in suspensions of latex particles was measured in an analogous manner.

2.5. *Analytical procedures*

Protein [9], sialic acid [10] and the content of various monosaccharides [11] were estimated using standard procedures.

3. RESULTS

The diffusion of all the probes used, FITC-dextran 20, FITC-dextran 3 and carboxyfluorescein, is markedly retarded in a suspension of packed erythrocyte ghosts. In fig.1 the fluorescence recovery tracings of FITC-dextran 20 in an isotonic buffer and in a ghost suspension are shown. On the short time scale shown in fig.1, the recovery of the fluorescence is incomplete in the packed ghosts. In prolonged recordings, however, the fluorescence recovery is approximately complete. Diffusion coefficients derived from such long term scans are almost two orders of magnitude smaller than the coefficients in buffer solution. The retardation of the diffusion for the three different probe molecules is shown in the two first columns of table 1.

The diffusion coefficients obtained for the dextrans in buffer agree remarkably well with previous results obtained with other methods [5]. Moreover, the diffusion coefficients of the different probes are approximately inversely proportional to their molecular radii, assuming globular molecules, both in buffer and in packed ghost suspensions. The only notable exception to this is the diffusion of FITC-dextran 20 in antibody-linked ghosts, which will be discussed later.

The interpretation of the diffusion measurements in the ghost suspensions depends on the location of the probe. In principle the probe molecule can be extracellular, intracellular, or both, and in addition it can be bound or inserted in the membrane. A priori it was assumed that the probes would remain in the extracellular space, due to their hydrophilic nature. In order to verify this the volume of distribution of the probe was

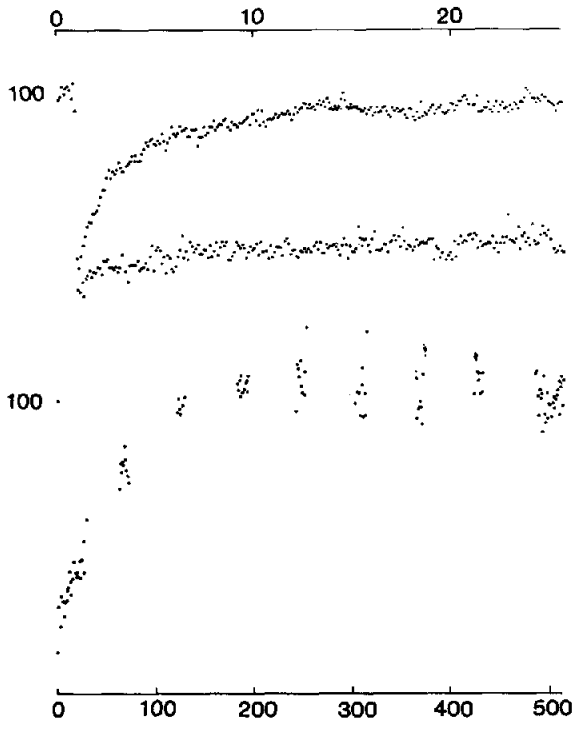

Fig.1. Recovery of FITC-dextran 20 fluorescence in buffer (upper trace) and in packed ghosts (middle and lower trace). The time scale in seconds for the upper and middle traces is shown above and for the lower trace below. The fluorescence intensity scale is linear and arbitrary. The level before bleaching is indicated by 100.

Table 1

Diffusion coefficients of fluorescent probes in buffer, ghosts and antibody-linked ghosts

Probe	D ($cm^2 \cdot s^{-1}$)		
	In buffer	In packed ghosts	In antibody crosslinked ghosts
CF	3.0×10^{-6}	4.2×10^{-8}	3.7×10^{-8}
FD3	1.79×10^{-6}	2.0×10^{-8}	1.5×10^{-8}
FD20	0.82×10^{-6}	1.2×10^{-8}	0.3×10^{-8}

measured by determining its concentration in the supernatant after centrifugation of the ghosts. Several experiments indicate that on average, 20% of the sediment volume is accessible to FITC-dextran. Measurements of the fluorescence trapped in the sediment also indicate that only a minor portion of the total volume is accessible to the fluorescent probe. If the ghosts are made permeable to FITC-dextran by hypotonic shock, the accessible volume fraction increases markedly, reaching about 100% of the total sediment volume. It was therefore concluded that the probes used were present only in the space between the packed ghosts.

If the suspension of packed ghosts is diluted with buffer, the diffusion of the probe becomes more rapid (fig.2). This confirms the above results that the probe is extracellular. In a few experiments, where the probe was introduced into the ghosts, diffusion did not become more rapid on dilution. Moreover, these dilution experiments also indicate that an equilibrium exists between the rapidly and slowly diffusing fractions of the probe. In suspensions of uncentrifuged ghosts, where the ghost concentration is about one third as large as in the packed ghosts, the diffusion rate is similar to that seen in diluted ghosts (fig.3).

In order to determine if impermeable particles as such have an effect on the rate of diffusion, suspensions of latex particles were used. The fluorescence recovery after photobleaching indicated that the diffusion of FITC-dextran 20 in a packed suspension of latex particles was somewhat slower than in the absence of the particles. A rough estimate of the accessible volume fraction in the particle suspension indicates that the diffusion coefficient in the latex particle suspension was at least 10 times higher than in a ghost suspension with a similar accessible volume fraction (table 2).

The possible role of the carbohydrate coat of the erythrocyte membrane in causing the slower diffusion in ghost suspensions was tested by attempts to remove this coat by digestion with various glycosidases. Analytical data indicated, however,

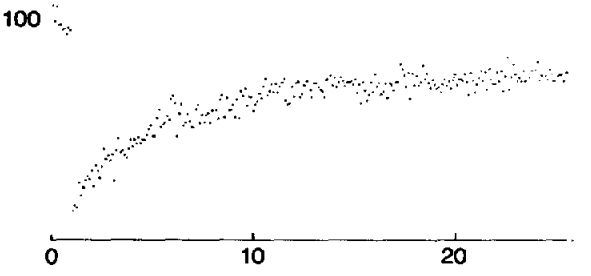

Fig.2. Fluorescence recovery in diluted ghosts. After centrifugation, the ghosts were diluted with one volume of buffer. Time scale is in seconds and the intensity is as in fig.1.

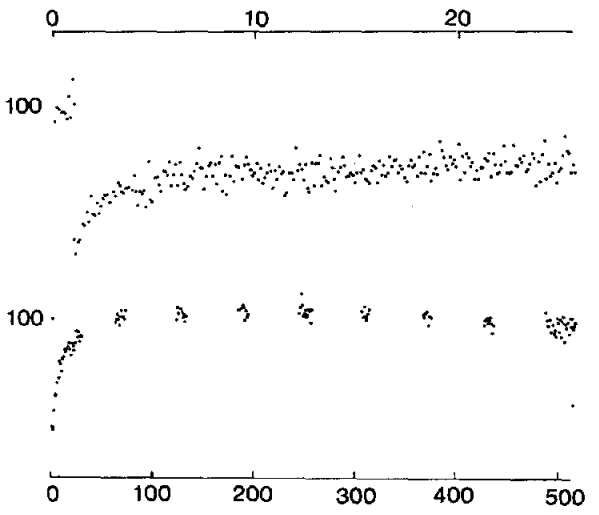

Fig.3. Fluorescence recovery in uncentrifuged ghosts. The time scale in seconds for the upper trace is shown above and for the lower trace below. The intensity is as in fig.1.

Table 2

Diffusion of FD20 in suspensions of latex particles

Accessible vol. fraction	D ($cm^2 \cdot s^{-1}$)
1.0	7.4×10^{-7}
0.65	3.5×10^{-7}
0.3	1.65×10^{-7}

that only a marginal portion of the carbohydrates could be removed under conditions in which the ghosts would still remain intact.

Crosslinking the ghosts with antibodies against blood group substances, which are carbohydrates expressed on polysaccharides linked to membrane proteins (band 3 and band 4.5) [12,13], affected the diffusion of the large molecular probe FITC-dextran 20. The last column in table 1 indicates a considerable reduction in the diffusion coefficient of this probe in a system consisting of blood group A ghosts treated with monoclonal antiA antibody, while the diffusion of the two smaller probes was not greatly affected. Control experiments with blood group O ghosts and antiA antibody showed no reduction in diffusion coefficients.

4. DISCUSSION

The findings of this study indicate that the diffusion of FITC-labelled molecules in suspensions of packed erythrocyte ghosts is considerably inhibited, both in comparison with diffusion in buffer alone, in less concentrated ghosts suspensions or in suspensions of latex particles.

Several experiments indicate that the fluorescein-labelled dextran is extracellular and is easily released from interaction with the membrane. It is therefore unlikely that the dextran probe molecules are anchored in the membrane through hydrophobic interactions. Moreover, the complete recovery of fluorescence after bleaching shows that the measured volume unit is in equilibrium with the remaining sample. Even the lowest diffusion coefficients, of the order 10^{-8}–10^{-9} $cm^2 \cdot s^{-1}$, are considerably higher than, for instance, lateral diffusion coefficients of integral membrane proteins, which are of the order 10^{-10}–10^{-11} $cm^2 \cdot s^{-1}$ [3,4]. The conclusion that we are dealing with the diffusion of molecules in the intercellular space is therefore warranted.

The slow diffusion of the fluorescent molecules in suspensions of packed ghosts is thus apparently due to interactions with the external surface of the ghosts. We have envisaged two possible explanations of these results. One consists of a simple binding model. In this model, free and bound probes are in rapid equilibrium and the fraction x of free probe is given by the expression

$$D_0 = x \cdot D_F + (1 - x) D_B,$$

where D_0, D_F and D_B are observed, free and bound diffusion coefficients, respectively [8,14]. D_B, being equal to the diffusion of the ghosts, is negligible, and x emerges as the ratio D_0/D_F. On the basis of this reasoning, binding constants of the order 10^2–10^3 M^{-1} are obtained, suggesting nonspecific binding. Dilution experiments (fig.2) also indicate easy reversibility, and therefore support not very strong binding. The nature of this binding might be some form of entanglement of the dextran molecules with surface structures of the membranes.

The other explanation is given in fig.4. The ghost suspension may be regarded as a network of ghosts in between which there are crevices either open towards the surroundings or closed. The more packed the ghosts are the larger the proportion of closed crevices. The only way for a molecule to move into or out of the closed crevices

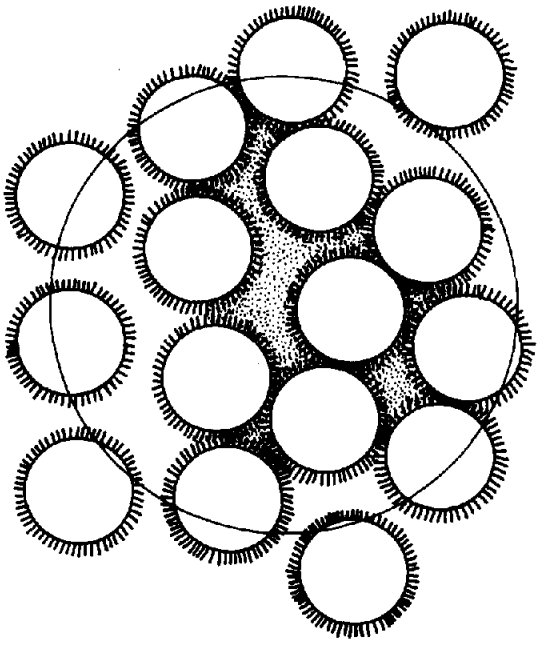

Fig.4. Sequestration of fluorescent probes in ghost suspensions. Part of the probe is present in open crevices and can therefore exchange freely with the surroundings. Another part is contained in closed crevices (shaded area) and can only exchange with the free pool by diffusion through the carbohydrate coats on the plasma membranes of the erythrocyte ghosts.

is through the pericellular coat of the erythrocytes. The latter consists of poly- and oligosaccharides, firmly anchored to the membranes via lipids or proteins [2]. We postulate that the diffusion of the dextran probes is inhibited through entanglement with this structure. Some support for this conclusion is given by experiments where the cellular carbohydrate structures were crosslinked by antibodies. The diffusion of the dextran probes, especially the higher molecular probe, was slowed down by this cross-linking.

Although our experiments do not give quantitative values of the diffusion coefficients of the probes in the pericellular coat, there is evidence that the coat affords a considerable resistance towards transport of external macromolecules.

In the present experimental setup the fluorescence of an area with a radius of about 20 μm is monitored. Therefore our experiments measure the diffusion of the probe in and out of this area. Due to the relative size of this area and of the ghosts (7 μm), bound or sequestered probes would show apparently the same behaviour, and it is therefore impossible to distinguish between the two explanations given. Measurement of diffusion in a considerably smaller area, e.g. within a sequestered pool, should show free diffusion, and thus give a possibility to distinguish between the alternatives. The apparatus is presently being reconstructed to permit such measurements.

In general, the principle used in this work seems to be applicable to the measurement of binding of fluorescent molecules to membranes or membrane fragments, and thus it would provide a fairly simple way to monitor binding of molecules to membranes. We intend to explore this possibility further.

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