Quantitation of Surface Affinities of Red Blood Cells in Dextran Solutions and Plasma†

K. Buxbaum, E. Evans,* and D. E. Brooks

ABSTRACT: Mutual affinities of red blood cell surfaces in dextran solutions and plasma have been determined experimentally. The approach was to use dual micropipet techniques to manipulate cells and spherical cell "fragments" into position for contact. After contact, the red cells encapsulated the fragments to an extent that depended on the affinity. Surface affinity is defined as the reduction in free energy per unit area of interface that is associated with formation of adhesive contact. The surface affinity was calculated with the use of a minimum free energy analysis and knowledge of the red cell membrane elastic properties. With this approach, surface affinities were measured for normal and neuraminidase-treated red cells in plasma and various solutions of dextran. The data presented are the first direct measurements of the affinity of a biological membrane for another surface in a well-defined system. The peak surface affinities of normal red blood cells were found to be 4.9×10^{-3} erg/cm^2 in D70, 2.2×10^{-2} erg/cm^2 in D-150, and 2.0×10^{-3} erg/cm^2 in plasma. Neuraminidase-treated cells had higher affinities than normal cells: at least 2.8×10^{-2} erg/cm^2 in D70 and 1.8×10^{-3} erg/cm^2 in D28, which does not aggregate normal red cells.

The ability of plasma proteins and dextran to promote adhesion and aggregation of red blood cells is a significant factor in blood rheology. The tendency of cells to aggregate ranges from very weak, as observed in rouleaux formation, to very strong, as seen for cells in the presence of 2 or 3 g % of high molecular weight dextran. The details of the cell–cell surface interactions are of considerable interest but are not well-known. Red cells alone in salt solutions do not adhere to each other even if ionic strength is increased or if charged groups on the cell surfaces are modified.

Adhesion of red blood cells in the presence of the glucose polymer dextran has been well described by Brooks (1973b), Chien and associates (Jan & Chien, 1973), and others. Disruption of red cell aggregates has been evaluated by viscometric methods, which produce fluid shear stresses. In addition, dextran adsorption to red cell surfaces has been extensively studied by Brooks (1973a, 1980) and Chien (1980). However, the actual free energy reduction (i.e., affinity between membrane surfaces) associated with formation of surface contact has not been quantitated for the specific conditions of dextran molecular weight, concentration, and cell surface charge that promote and inhibit red cell aggregation.

Recently, methods have been developed to measure cell surface affinities in the range of those caused by modifiers such as dextran.[1] Evans (1980) introduced a minimum free energy method of analysis that provides a way to quantitate the affinity of red cells for other surfaces. The approach takes advantage of the native elastic properties exhibited by normal red blood cell membranes and the liquid interior. Subsequently, a modified experimental technique and a simpler analysis were derived to extend the measurable range of affinities (Evans & Buxbaum, 1981). The modified technique involves observation of the equilibrium encapsulation of small spherical red cell fragments by normal red cells. The simpler analytical model gives a good approximation for surface affinity as a function of fractional extent of particle encapsulation by the intact red cell. Because the range of surface affinities for which the results are applicable has been increased over previous techniques, it has been possible to quantitate surface affinities of red cells in dextran solutions and plasma. This paper details our application of the experimental method to measurement of red cell surface affinities in these solution environments.

Experimental Procedures

Experiments were performed with red blood cells (RBC) from healthy donors. Unwashed cells were always obtained from the same donor (type O, Rh$^+$) by finger prick and dilution at very low hematocrit into phosphate-buffered saline (PBS). These cells were drawn just prior to their use in experiments. Cells to be washed were drawn directly into heparinized containers on the day of the experiment and were washed 2 or 3 times in isotonic PBS.

RBC fragments were produced by exposure of cells to high temperature. Fresh cells were suspended in 250 mosM PBS and then immersed in a 58–60 °C water bath for 3 min. High temperature caused vesiculation of the cells. The result was a concentrated suspension of RBC fragments, most of which were about 2–5 μm in diameter and appeared to have little excess surface area (i.e., were essentially spherical).

Cells to be treated with neuraminidase to reduce cell surface charge (Haydon & Seaman, 1967) were first washed as above in PBS. They were then incubated 60–90 min at 37 °C, hematocrit 10%, in 10^{-4} international units (IU) of neuraminidase (Calbiochem, protease free) per 0.75 cm^3 of packed cells. Next, cells were washed 3 times in ice-cold PBS. All washed cells were kept near 4 °C (except during incubation) until they were used. All data were collected at room temperature.

PBS used in all experiments contained 25–30 mM phosphate, which maintained solution pH at a physiological level

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[1] The affinity is defined by the reduction in free energy per unit area that is associated with the initial formation of adhesive contact. As such, the natural tendency for cells to aggregate is represented by the surface-to-surface affinity. In general, the surface affinity may be much smaller than the work per unit area that is required to separate the surfaces. The work of separation may involve subsequent chemical reactions between surface molecules; also, the work to separate the surfaces may include strong time-dependent, dissipative contributions. Consequently, for measurement of surface affinity, the equilibrium state of an adhesive contact must be observed.

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Table I: Characteristics of Dextran Fractions

dextran fraction	Pharmacia designation	$\bar{M}_w$	$\bar{M}_n$	$\bar{M}_w/\bar{M}_n$
dextran 28	RMI 726	27 900	23 000	1.21
dextran 70	FDR 1165	70 100	58 000	1.21
dextran 150	II A1	147 500	94 300	1.56

of 7.4. Human serum albumin (0.5 g %, Sigma Chemical Co.) was added to PBS suspending solutions in order to maintain cell shape and to minimize adhesion of cells to the micropipets. Plasma for suspension of cells was obtained as the supernate from whole blood centrifuged at 3600 rpm to remove red cells. Plasma was then centrifuged at 19 000 rpm for 15 min to remove all remaining cells prior to resuspension of red cells.

Specifications of the sharply cut dextran fractions (kindly supplied by Dr. Kirsti Granath) are listed in Table I. Approximate weight-average molecular weights (M_w) of the fractions are given by 10^3 times the fraction number. The ratio of M_w and the number-average molecular weight, M_n, may be taken as an indication of the heterogeneity of the sample.

Concentrated stock solutions of dextran were made in distilled water with 0.2 g % sodium azide added to prevent bacterial growth. Concentrations of stock solutions were accurately measured by polarimetry. Subsequent dilutions of dextran stock solutions into concentrated PBS were made prior to experiments to obtain solutions of desired concentration and ionic strength.

Cell surface affinity was measured for cells suspended in plasma and in solutions of each dextran fraction over a wide concentration range. Lower and upper threshold concentrations for dextran-mediated aggregation were defined to be those concentrations below or beyond which, respectively, no adhesion would occur between cells held in contact together.

Suspending solutions were slightly hypotonic (250 mosM) so the cells used for affinity measurements were swollen (by about 10%) and slightly cup shaped. The flaccid red cell and the red cell fragment were manipulated with a dual micropipet aspiration system. Each was deformed minimally by the manipulations that preceded adhesion. The red cell fragment was brought into very close proximity to the unrestrained, convex side of the red cell. The two surfaces were not pushed together in order to make contact. The cell–fragment contact was made in a central area of the convex side of the cell, and the deformations were essentially axisymmetric. The equilibrium configuration was reached within 1 s and persisted without shape change for the observation period (e.g., minutes). This was reproducible at all conditions. [Photographs of the equilibrium red cell–fragment conformation in different adhesion environments are presented in the paper by Evans & Buxbaum (1981).]

As soon as contact was made between cell and fragment, aspiration pressure was reduced and pipets were withdrawn. Measurements of cell deformation due to adhesion were taken from freely floating (unrestrained) fragment–cell pairs. [Slightly cupped red cells were used so that the extent of encapsulation could be observed more accurately. Numerical analysis has shown (Evans & Buxbaum, 1981) that the shape of the RBC (cup or disk) does not significantly affect the result.]

The surface affinity is predicted to be simply related to the fractional encapsulation of the RBC bead (Evans & Buxbaum, 1981). Consequently, the only experimental variables required in the analysis of deformation were the polar cap height of the contact region, Z_c, and the sphere (particle) diameter, $2R_s$, as shown in Figure 1. For fractional encapsulation $[Z_c/(2R_s)]$ less than 0.6, R_s and Z_c were measured directly from side views. For greater fractional encapsulation, Z_c was calculated on the basis of top-view measurement of the portion of the bead that remained exposed after cell deformation.

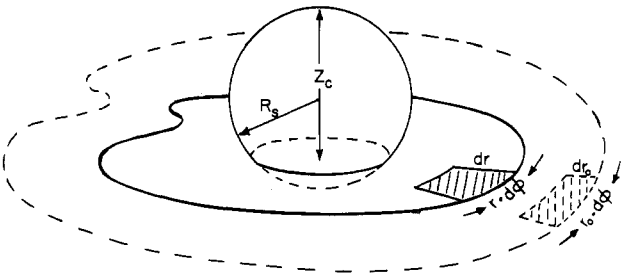

FIGURE 1: Schematic illustration of an elastic membrane sheet that has deformed to adhere to the surface of a spherical particle. Dashed lines represent the undeformed geometry of the flat sheet, and solid lines represent the corresponding regions of the sheet in the deformed state. Cylindrical coordinates (z, r, and ϕ) correspond to the symmetry axis for the deformation, the radial distance from the symmetry axis, and the azimuthal angle around the symmetry axis. [Reprinted with permission from Evans & Buxbaum (1981); copyright 1981 Rockefeller University Press.]

Analytical Concepts

Static deformation of the elastic red cell membrane stores energy conservatively. This elastic energy opposes the reduction of free energy associated with formation of adhesive contact. Equilbrium is established when small (virtual) increases in elastic deformation energy of the red cell are just balanced by small (virtual) decreases in energy due to contact. Hence, the elastic deformation is only determined by the reversible work of *formation* of the contact, not the potentially irreversible work of separation of an already formed adhesive contact. For illustration of the membrane deformation, Figure 1 shows the membrane region local to the particle, approximated as a thin, flat sheet. In the initial state, the flat sheet and the small spherical bead are not in contact. Over the contact region in the final state, the sheet is deformed into a spherical cap. The remainder of the sheet local to the particle remains flat after deformation. It can be assumed that the membrane makes a sharp "bend" at the edge of the contact region and that bending elastic effects at the corner may be neglected. [These assumptions have been shown to be valid from whole cell analyses by Evans (1980) and Evans & Buxbaum (1981).] The level of membrane deformation is expressed by surface extension ratios. These measures of deformation are given by the ratios of the differential dimensions of an element in the deformed state and of the same element in the initial state as illustrated in Figure 1. Since the red cell membrane strongly resists area dilation, the extension ratios are reciprocally related. Thus, the stretch along the meridian of the sphere and in the radial direction in the sheet completely specifies the membrane deformation. This extension ratio, λ, is a function of location given by the curvilinear distance, s, measured along the meridian from the pole of the spherical cap to the contact edge and along the circular radius in the outer surface. The maximum extension ratio in the membrane, $\hat{\lambda}$, occurs at the rim of the contact region and is equal to

$$\hat{\lambda} = 1/(1 - X)^{1/2}$$

where $X = Z_c/(2R_s)$ and is the fractional extent of encapsulation. The extension then decreases to unity as the square of the radial distance away from contact region. Consequently, it is apparent that the extent of encapsulation is a measure of the elastic deformation of the cell membrane.

The red cell deformation provides a direct measure of the affinity through a compliance factor that involves the shear and bending elastic moduli convolved with the cell shape. The compliance relation is derived from equilibrium thermodynamics; i.e., the variation of the work of cell membrane deformation, W_D, is equal to the variation of the free energy of formation of surface contact, F_A, with respect to changes in contact area, A_c.

$$\partial W_D/\partial A_c = \partial F_A/\partial A_c$$

In the context that the contact area represents the extent of the aggregation reaction, the surface affinity, γ, is defined by (Guggenheim, 1959)

$$\gamma \equiv \partial F_A/\partial A_c \text{ (erg/cm}^2\text{)}$$

The affinity is related to the chemical potentials and mole fractions of molecular species and complexes involved in the reaction. Unfortunately, it is not presently possible to identify and quantitate all of the components involved in membrane–membrane adhesion, so we leave the definition of affinity at the thermodynamic level. Since the red cell interior is liquid, the reversible work of cell deformation depends only on the membrane material properties and the analysis of membrane deformation. It has been shown that the compliance relation for the red cell–bead encapsulation is well approximated by

$$(1/\mu)(\partial W_D/\partial A_c) = X^2/(1 - X) - X \ln (1 - X) + 2\tilde{B} \quad (1)$$

(Evans & Buxbaum, 1981) where μ is the elastic shear modulus (dyn/cm or erg/cm^2) and X is the fractional particle encapsulation $[Z_c/(2R_s)]$. $\tilde{B}$ is a dimensionless term that represents a threshold for the onset of encapsulation. $\tilde{B}$ is determined by the ratio of the bending to shear elastic rigidities $[B/(\mu R_s^2)]$. For the RBC membrane, $\tilde{B}$ is less than 10^{-2}. The opposition of $\tilde{B}$ to the affinity is, therefore, very small. In the measurable range of affinities, it is negligible.

The compliance relation above is equal to the surface affinity, γ, scaled by the shear modulus, μ, to create a "dimensionless surface affinity". the relation

$$\gamma/\mu = (1/\mu)(\partial W_D/\partial A_c)$$

illustrates the role of the red cell membrane as an elastic "transducer". In the calculations of surface affinity that follow, we have utilized the elastic shear modulus value of 6.6×10^{-3} dyn/cm published by Waugh & Evans (1979) and an estimated bending modulus, B, of 10^{-12} dyn·cm (erg) (Evans, 1980).

Results

The extent of red cell fragment encapsulation was measured for normal cells in solutions of D70 and D150 and for neuraminidase-treated cells in D28 and D70. Results are shown in Figures 2 and 3, respectively. Fractional encapsulation, $Z_c/(2R_s)$, is plotted vs. dextran concentration (gram percent). Breaks on the abscissa indicate upper and lower critical concentrations for adhesion. The extent of encapsulation in plasma is indicated by the arrow near the ordinate. Particles used in weak aggregation conditions (e.g., in D28) were in the 4–5-μm diameter range. Larger particles did not compromise the use of the simpler analytical model. For low affinities where fractional encapsulation was small, larger particles did, however, permit more accurate measurement of the contact region.

We have assumed that the red cell fragment formed by heat treatment has the same surface affinity for normal red cell membrane as would another normal red cell. This is based on our comparative observations of encapsulation by red cells

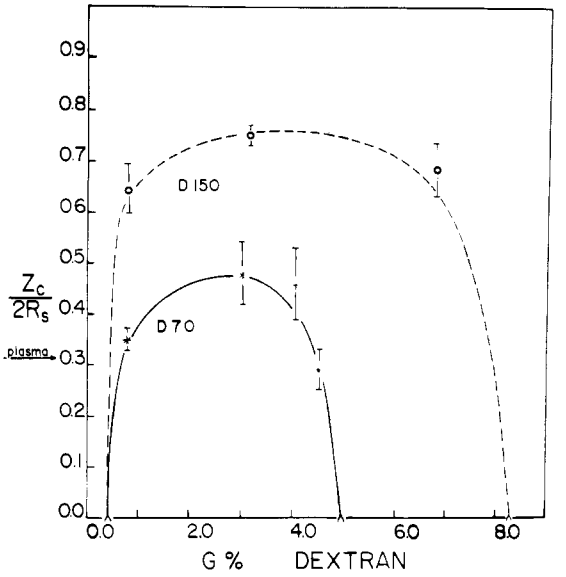

FIGURE 2: Fractional encapsulation vs. dextran concentration for normal RBCs suspended with 2–3-μm spherical red cell fragments in solutions of D70 or D150. The breaks on the abscissa indicate upper and lower limits for adhesion. Fractional encapsulation in plasma is indicated by the arrow near the ordinate. The curves are drawn to indicate the data characteristics and are not theoretical or empirical correlations.

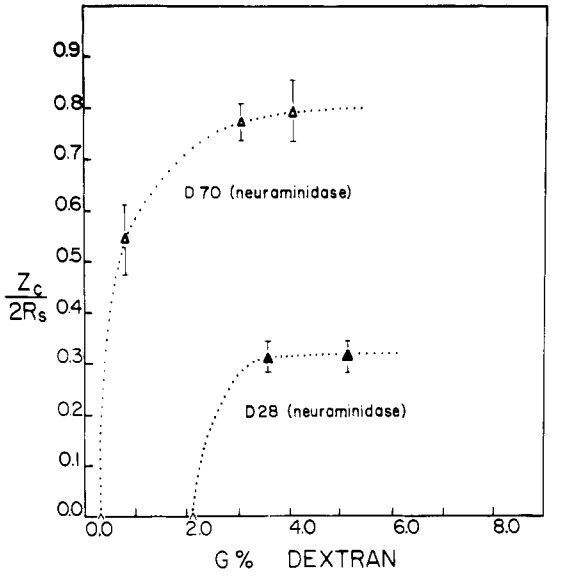

FIGURE 3: Fractional encapsulation vs. dextran concentration for neuraminidase-treated RBCs and cell fragments in solutions of D28 or D70. The breaks on the abscissa indicate lower limits for adhesion. No upper limit for adhesion was observed for cells treated with neuraminidase. Again, the curves are drawn only to indicate the data characteristics.

of the small fragments and normal (*non*-heat-treated) red cells that were forced to sphere by micropipet aspiration (Buxbaum, 1980). No difference was detectable; however, these comparative measurements could only be performed at low affinities because of the geometric constraints presented by the larger, sphered red cells (Evans & Buxbaum, 1981). Values for surface affinity were calculated from data characteristics sketched in Figures 3 and 4 with eq 1 and the previously given values for elastic properties. The calculated surface affinity, γ, is shown in Figure 4 for the experiments on cells suspended in D28, D70, and D150 as a function of dextran concentration. Again, for cells suspended in plasma the affinity is indicated by the arrow near the ordinate. Curves for surface affinity

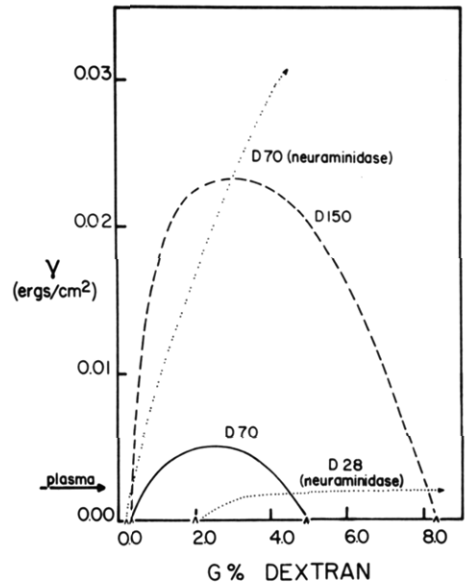

FIGURE 4: Surface affinity, γ, as a function of dextran concentration for cells suspended in solutions of D28, D70, D150, and plasma (indicated by the arrow near the ordinate). The surface affinity was calculated with the data characteristics drawn in Figures 2 and 3 plus the analytical relation for the membrane elastic compliance, eq 1.

of neuraminidase-treated cells are incomplete above 4 g % dextran because there was no obtainable dextran concentration where the encapsulation decreased (even at concentrations as high as 15 g %).

In the experimental determination of surface affinity reported above, only the formation of adhesive contact was involved. The theoretical development and the experiment were designed to study only the forward, thermodynamically reversible aspects of the problem. However, it is important to note that *separation* of an adhesive contact mediated by dextran is often not thermodynamically reversible. After contact and adhesion occurred, separation of cells appeared to require greater work than that which had gone into the formation process. Figure 5 illustrates this irreversibility. This sort of strong point attachment was often seen when cells in dextran solution were separated from each other by micromanipulation. This irreversible aspect of adhesion will be discussed further below since it may have had a significant influence on the disaggregation results reported by other laboratories.

Discussion

The results of these experiments were qualitatively consistent with previous observations of RBC aggregation caused by macromolecules. Aggregation commenced at a critical concentration of dextran that was a function of molecular weight and neuraminidase treatment of cells. For cells *not* treated with neuraminidase, surface affinity initially increased strongly as the concentration of dextran increased and then leveled off, perhaps due to electrostatic repulsion between the cell surfaces (Brooks, 1973b; Jan & Chien, 1973). Affinity then decreased to zero as the dextran concentration approached an upper critical concentration, which was also a function of dextran molecular weight.

Cells treated with neuraminidase, a procedure reported to reduce the cell surface charge density to about 30% of its normal value (Haydon & Seaman, 1967), always had higher affinity for each other than did untreated cells. The effect was most dramatic for small macromolecules, which do not normally cause red cell aggregation. Furthermore, there was

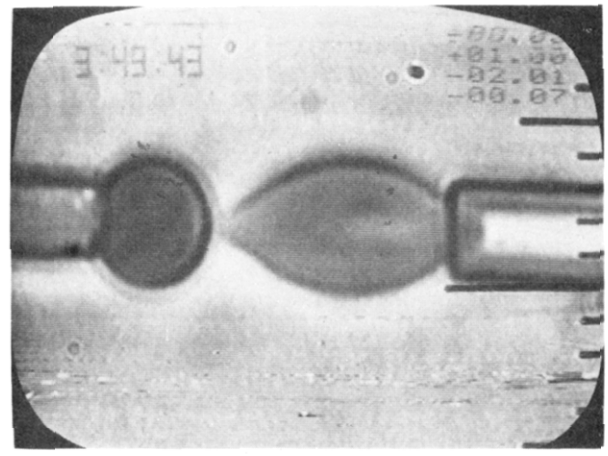

FIGURE 5: Example of a strong point attachment subsequent to dextran-mediated adhesion of normal or neuraminidase-treated RBC surfaces. This shows an irreversible aspect of the adhesion in contrast to the reversible process represented by the surface affinity.

no apparent decrease in adhesion at any obtainable dextran concentration above the lower critical concentration.

Results with D28 supported the idea that there may be a minimum molecular length of dextran, which depends on electrostatic factors, required to bridge the intercellular gap at equilibrium (Brooks, 1973b). The D28 used in these experiments was unable to mediate stable adhesive contacts between normal cells but did promote adhesion of neuraminidase-treated cells. It appears that even if cell surfaces are brought close enough together for the short-chain dextran molecules physically to bridge the gap, the intercellular repulsion that acts between the native surfaces may be too high for stable molecular bridges to form between cells.

It is interesting to note the apparent absence of an affinity decrease at high concentrations of dextran for *neuraminidase-treated* cells. Although accurate affinity measurements could not be made in solutions of very high dextran concentration, it was obvious that even in solutions of 15 g % D70 and D28, cell surface affinities were very high (data not presented). For normal cells, on the other hand, electrostatic factors are thought to be involved in the reduced affinity observed at high dextran concentration as well as for the inability of short-chain dextran fractions (e.g., D28) to act as cross bridges between cells. This view is based on previous work (Brooks, 1973b; Jan & Chien, 1973) that has shown that the upper limiting dextran concentration for cell aggregation depends strongly on ionic strength. Partial removal of cell surface sialic acid residues clearly eliminates the affinity decrease for cells in high concentrations of dextran larger than $\bar{M}_w$ 28 000.

With the micropipet technique it was also observed that the aggregation process is not readily reversible. When a cell–cell aggregate is permitted to make stable contact in the presence of dextran (e.g., at 3 g %) and is then moved to a solution of dextran above the upper critical concentration (e.g., 8 g %), they remain in contact for long periods of time, and significant forces are required to pull the cells apart. In other words, the cells do not spontaneously disaggregate. However, once the cells are separated, they will not reaggregate while in the very concentrated dextran solution. Clearly, the process of aggregate formation is *not* equivalent to the process of aggregate dispersion. Expressed differently, the decrease in surface free energy density associated with formation of adhesive contact is not equivalent to the "fracture energy", i.e., the work that would be associated with cell separation. Techniques that

utilize red cells in bulk suspensions reflect the kinetic and kinematic balance of aggregation and dispersion processes. Direct manipulation of cells, as performed in these experiments, is required to separately quantitate such aggregation and dispersion processes.

Micromanipulation techniques were also useful in the separation of time-dependent, i.e., dissipative, processes from equilibrium processes. In general, solution viscosities and cell surface viscosities affect the rate at which cell contact and adhesion take place, but the data reported here were taken from pairs of cells that had reached mechanical equilibrium.

In conclusion, we have used the elastic deformation of the red blood cell at mechanical equilibrium to calculate the cell surface affinity in dextran solutions and plasma. The affinities are the reduction in free energy per unit of membrane area associated with formation of adhesive contact and are independent of cell geometry. The peak surface affinities of normal red blood cells were found to be 4.9×10^{-3} erg/cm^2 in D70, 2.2×10^{-2} erg/cm^2 in D150, and 2.0×10^{-3} erg/cm^2 in plasma. Neuraminidase-treated cells had higher affinities than normal cells: at least 2.8×10^{-2} erg/cm^2 in D70 and 1.8×10^{-3} erg/cm^2 in D28.

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A Method for Rapid, Continuous Monitoring of Solute Uptake and Binding†

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ABSTRACT: A method has been developed for dynamically monitoring the free concentration of diffusible, tritiated solutes. The technique utilizes particles of a solid scintillator microencapsulated in gel beads that are permeable to diffusible label. Since tritium β radioactivity has an effective range in water of only a few micrometers, only label that is free to diffuse through the gel can excite the scintillator, while sequestered label is effectively excluded. Thus, the scintillation light output monitors the freely diffusible concentration of label exclusively. A simple, preliminary encapsulation technique is described and tested, and the theory behind the method is discussed with regard to the time resolution attainable for a given label concentration and type of encapsulation. The feasibility of the method is demonstrated by measuring the uptake of [^{3}H]tetraphenylphosphonium by *Escherichia coli* membrane vesicles in response to the generation of a membrane potential (interior negative).

In biological systems, it is frequently important to quantify uptake or binding of soluble substances by colloidal or macroscopic phases such as cells, subcellular particles, or macromolecules. The time course of the phenomenon is often an important parameter. Generally, uptake or binding is measured either directly by assaying the macroscopic phase after separation from the bathing medium or indirectly by determining the the free concentration of the solute. Although a wide variety of analytical techniques have been utilized for these measurements, no single method is applicable in every instance, and most of the methods either are relatively insensitive, exhibit poor time resolution, or are applicable to a limited group of solutes.

This paper describes the use of encapsulated scintillation beads for in situ monitoring of the free solution concentration of tritiated solutes. Advantage is taken of the fact that the β particle released when a tritium atom decays has a maximum range of about 7 μm in water. Consequently, a scintillator may be totally shielded from a tritiated source that is not freely diffusible. In the procedure, a finely divided scintillator is encapsulated in microbeads of a permeable gel. The gel pore size is selected to be large enough to allow for free diffusion of the tritiated solute in solution. As a result, the light output of the microbead suspension continuously monitors the free concentration of the tritiated species with no contribution from solute that is not freely diffusible.

Experimental Procedures

Materials

D-[^{3}H]Glucose (25 Ci/mmol) was obtained from New England Nuclear. [^{3}H]Tetraphenylphosphonium (bromide salt; 2.5 Ci/mmol) was prepared by the Isotope Synthesis Group of Hoffmann-La Roche Inc. under the direction of Arnold Liebman. All other materials were of reagent grade and purchased from commercial sources.

Methods

Scintillator Preparation. Poly(vinyltoluene)-based NE102 plastic scintillator microspheres of 1–10-μm diameter (Nuclear Enterprises, Inc., San Carlos, CA) were chosen because of both their size and their relative immunity to most aqueous saline

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