ANALYSIS OF INTRACELLULAR RECEPTOR/LIGAND SORTING

Calculation of Mean Surface and Bulk Diffusion Times within a Sphere

JENNIFER J. LINDERMAN AND DOUGLAS A. LAUFFENBURGER
Department of Chemical Engineering, University of Pennsylvania, Philadelphia, Pennsylvania 19104

ABSTRACT Cell surface receptors bind extracellular ligand molecules and transport those ligands into the cell by a process termed receptor-mediated endocytosis. Receptor and ligand molecules are sorted from one another after endocytosis, apparently within a structure consisting of intracellular vesicles and connected thin tubules. The experimental observation is that most free (unbound) ligand molecules are found in the lumen of the vesicles and receptors are located primarily within the tubules. Because equilibrium and geometric considerations do not explain this segregation, a kinetic scheme involving the passive diffusion of molecules from a vesicle into a tubule is investigated. Two possible sorting mechanisms are considered: first, that receptors are able to move into tubules more rapidly than ligand molecules due to an advantage in dimensionality and, second, that receptors diffusing into tubules are trapped there while ligands are not. Mean diffusion times for receptor and ligand movement into a tubule are calculated by solving Poisson's equation in two and three dimensions, respectively, on the surface of and within a sphere. Using estimated parameter values, we found that only the second scheme is able to account for the experimentally observed sorting. An estimate is obtained for the length of time a tubule and vesicle must be connected in order to remove a significant number of receptors into a tubule. The fraction of free ligand that is "mis-sorted" with the recycling receptor population and thus exocytosed is also determined.

INTRODUCTION

Specialized cell surface molecules, receptors, are able to bind extracellular ligand molecules and, among other functions (e.g. signal transduction, adhesion), mediate their transport into the cell itself. A wide variety of molecules are internalized by eucaryotic cells through this highly specific process known as receptor-mediated endocytosis (RME). In this way, hormones and growth factors (e.g., insulin, epidermal growth factor), carrier molecules (e.g., low density lipoprotein, transferrin), and substances to be removed from the body (e.g., asialoglycoproteins) are taken up.

The basic features of this internalization process have been described in a number of reviews (1–4) and are summarized in Fig. 1. Cell surface receptors, generally transmembrane glycoproteins with a high affinity for a particular macromolecule, bind ligand present in the cell's local environment. In many cases, the newly formed receptor-ligand complexes cluster in specialized membrane regions, called coated pits, which are characterized by the presence of the protein clathrin. The plasma membrane then invaginates, pinching off vesicles containing the complexes. Within these intracellular vesicles, or endosomes, a sorting process occurs. Typically, ligands are delivered to lysosomes whereas receptors recycle to the cell surface to bind more ligand molecules. For example, Schwartz et al. (5) have found that asialoglycoprotein receptors are recycled after endocytosis and their ligand, asialoorosomucoid (ASOR), is degraded in lysosomes. In other cells, low density lipoprotein (LDL), the cholesterol transporting protein in human plasma, is degraded in the lysosomes to liberate cholesterol; its receptor apparently cycles continuously (6).

The mechanism of this sorting process is not known. Investigators suspect that prior to actual physical segregation the receptor and ligand must dissociate. Because the rate constants for dissociation of the receptor-ligand complex are frequently pH-dependent (7, 8, 9), the measurement of a low endosome pH (10, 11) suggests that a pH shift accomplishes the dissociation. For example, very little epidermal growth factor (EGF) dissociates from its receptor after 15 min at pH 7.5, but about 90% dissociates after the same time at pH 5.5 (9). Thus the ligand in an endosome is likely to have dissociated from its receptor.

Although the acidic environment of the endosome can hasten the dissociation of the receptor-ligand complex, this pH shift cannot explain the segregation of the two species that must occur if they are to follow different intracellular pathways from this point on. Electron micrographs are currently the best direct evidence for the segregation of receptors and ligands within the cell. Geuze et al. (12) have

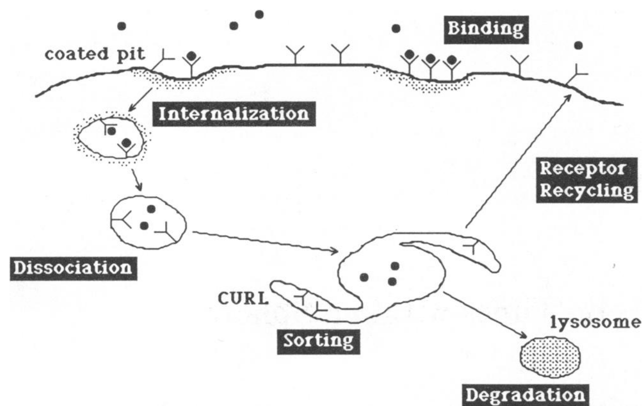

FIGURE 1 The endocytic cycle. Receptors mediate the transport of ligand from the extracellular environment to the interior of the cell. Receptor and ligand are sorted intracellularly, allowing receptor recycling to the cell surface and ligand delivery to lysosomes.

shown that endosomes are associated with tubules and that while most free ligand molecules are found in the lumen of the vesicles, receptors are located primarily in the tubules or at the tubule entrance. Other investigators have seen similar structures (11, 13, 14, 15). Sorting apparently occurs at this location, in a structure termed the compartment of uncoupling of receptor and ligand, or CURL (12). A reasonable interpretation is that after the receptor-ligand complexes dissociate, ligand molecules remain free in the vesicle lumen and receptors move into the tubules. These tubules may either become, or empty into, recycling vehicles able to transport their contents back to the cell surface.

The sorting process is a critical step in the endocytic pathway because at this point the paths of receptor and ligand molecules are determined. Inefficient sorting, whether intentional for purposes of down-regulation (a decrease in the number of available cell surface receptors upon exposure to ligand) or unavoidable due to the sorting mechanism, may result in receptor degradation or ligand exocytosis (the return of ligand to the cell surface). Both of these phenomena have been observed in amounts that vary with experimental conditions (6, 7, 16, 17, 18). Thus an understanding of the intracellular sorting of the two populations is critical to an overall understanding of the endocytic cycle, the kinetics of receptor recycling and down-regulation, and ligand exocytosis.

To explain the segregation of the receptor and ligand populations quantitatively, we first considered a simple mechanism: that CURL tubules, with a higher surface area to volume ratio than the vesicles, accomplish the segregation by an equilibration of receptor and ligand molecules between the two compartments. Marsh et al. (15) have estimated that 60–70% of the volume and 30–40% of the surface area of the CURL are contained in its vesicular portions, implying that diffusion and equilibration of receptors and ligands throughout the structure would prevent a third of the receptors from recycling. This degree of receptor loss contradicts experimental measurements on many systems (9, 16). Thus an equilibrium mechanism does not explain the sorting process, and a kinetic explanation for sorting is necessary. In this paper we consider two possible kinetic processes for intracellular receptor/ligand sorting, based on diffusion of the ligand and receptor molecules in the endosome.

MODEL DESCRIPTION

In an attempt to elucidate the mechanisms by which intracellular receptor/ligand sorting is accomplished, we begin with the assumption that dissociated receptor and ligand molecules move by passive diffusion within CURL, the sorting chamber of the cell. Our model for sorting will require knowledge of the rates at which receptor and ligand molecules enter tubules for endocytic vesicles.

We first consider the possibility that receptors are able to segregate from ligand molecules by moving into CURL tubules at a greater rate. The rate constants for the movement of receptor and ligand molecules into tubules are likely to be very different. Receptors, which diffuse on the surface of the vesicle, need only search in two dimensions for a tubule entrance. The ligand, on the other hand, must search in three dimensions. Adam and Delbrück (19) found that the mean time for a molecule to diffuse to a target is strongly dependent on the dimensionality of the system. While the ligand has the advantage of a diffusion coefficient that is likely two to three orders of magnitude greater than that of the receptor, the receptor experiences the advantage of lower dimensionality in its search. The time it takes each to find a tubule entrance will depend on the vesicle radius, the appropriate diffusion coefficient, the number of tubules connecting with the vesicle, and the size of a tubule entrance.

Using our calculated transport rate constants and appropriate parameter values, we can determine whether the receptors are able to reach the tubule entrance more rapidly than the ligand molecules. The receptor's advantage in dimensionality may result in a greater rate of transport of receptors to the tubule entrance, and we are interested in the circumstances under which this possibility could be realized. Our analysis allows this possible sorting mechanism to be evaluated.

Second, we consider another possible sorting mechanism. Clathrin, a protein thought to play a role in trapping receptors in coated pits on the cell surface, has been noted to be present around CURL tubules (12, Geuze, H.J., and J.E.A.M. Zijderhand-Bleekemolen. Personal communication). Thus receptors moving into the tubules may be unable to diffuse back out. The ligand molecules, on the other hand, may diffuse into and out of the tubules and eventually equilibrate between the vesicular and tubular volumes of CURL. For this mechanism, then, the time required for a significant fraction of the receptors to move into a tubule must be calculated, as well as the amount of ligand that would also be found in the tubules.

FORMULATION OF THE MATHEMATICAL MODEL

We want to investigate the movement of receptor and ligand molecules toward a tubule entrance on the surface of the cell's sorting chamber, CURL. More specifically, the mean time or its inverse, a rate constant, for a molecule of each species to reach a tubule is to be calculated. As shown in Fig. 2, the CURL is modeled as a sphere of radius R to which is attached a single thin tubule of radius b. The critical angle θ_c is defined as the ratio b/R. The assumption that at any given time only one tubule entrance is available to receptors and ligands is reasonable in view of the electron micrographs of Geuze et al. (12) and is made for simplicity. Calculations might also be done for the case of multiple tubules, as suggested by the measurements of Marsh et al. (15), but the general character of the rate constants is likely to be found from the single tubule model.

Initially, receptors are located randomly on the spherical surface and ligand molecules randomly within the sphere. We temporarily assume that the tubule entrance is perfectly absorbing for receptor and ligand molecules, in order to calculate and compare the rate constants for the transport step from the vesicle to the tubule.

In related problems, other investigators have calculated rate constants for the binding of extracellular ligand to receptors on the surface of a cell (20, 21, 22). The most common approach is to postulate a constant ligand concentration far from the cell and solve the steady state diffusion equation for the region exterior to a sphere with mixed boundary conditions at the sphere surface. In our problem, because the ligand molecules in a CURL diffuse within the interior and not on the exterior of a sphere, finding the mean time to reach the tubule entrance would require the solution of an unsteady state diffusion equation. We choose instead a simpler technique.

Berg and Purcell (20) and Szabo et al. (23) derive an equation for the mean time required by a molecule moving in n dimensions to reach a target when there is no interaction potential between the molecule and the sink

$$D_n \nabla_n^2 W + 1 = 0, \tag{1}$$

where D_n is the appropriate diffusion coefficient and ∇_n^2 is the Laplacian operator in the relevant dimension and coordinate system. The mean time to capture, W, is a function of the initial or starting position of the molecule and is approximately the inverse of the rate constant for a molecule finding the tubule entrance (23). Because a finite mean capture time is generated by the initial placement of a molecule in the vesicle and all initial positions are equally likely, there is a constant source term in the equation.

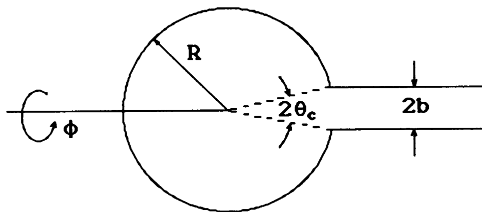

FIGURE 2 Model geometry. CURL is modeled as a sphere of radius R to which is attached a single thin tubule of radius b. The angle θ_c is equal to b/R and defines the size of the tubule opening.

The mean capture time equation, Eq. 1, can be used for both species. For the receptor, the equation becomes

$$D_R\left[\frac{1}{R^2 \sin\theta}\frac{\partial}{\partial\theta}\left(\sin\theta\frac{\partial W_R}{\partial\theta}\right) + \frac{1}{R^2\sin^2\theta}\frac{\partial^2 W_R}{\partial^2\varphi}\right] + 1 = 0 \qquad \hat{r} = R, \theta \geq \theta_c, \tag{2}$$

where D_R is the diffusion coefficient for the receptor in the membrane. The boundary conditions are

$$W_R = 0 \qquad \theta = \theta_c \tag{3a}$$

$$\frac{\partial W_R}{\partial\theta} = 0 \qquad \theta = \pi. \tag{3b}$$

For the ligand, analogously, the mean time equation for W_L is

$$D_L\left[\frac{1}{\hat{r}^2}\frac{\partial}{\partial\hat{r}}\left(\hat{r}^2\frac{\partial W_L}{\partial r}\right) + \frac{1}{\hat{r}^2\sin\theta}\frac{\partial}{\partial\theta}\left(\sin\theta\frac{\partial W_L}{\partial\theta}\right) + \frac{1}{\hat{r}^2\sin^2\theta}\frac{\partial^2 W_L}{\partial^2\varphi}\right] + 1 = 0, \tag{4}$$

subject to the following boundary conditions:

$$W_L = 0 \qquad \hat{r} = R, \theta \leq \theta_c \tag{5a}$$

$$\frac{\partial W_L}{\partial\hat{r}} = 0 \qquad \hat{r} = R, \theta > \theta_c \tag{5b}$$

$$W_L(\varphi = 0) = W_L(\varphi = 2\pi) \tag{5c}$$

$$\frac{\partial W_L}{\partial\varphi}(\varphi = 0) = \frac{\partial W_L}{\partial\varphi}(\varphi = 2\pi) \tag{5d}$$

$$\frac{\partial W_L}{\partial\theta} = 0 \qquad \theta = \pi. \tag{5e}$$

D_L is the diffusion coefficient for the ligand in the CURL lumen. Eqs. 5c and 5d are periodic boundary conditions and Eq. 5e states the condition of boundedness on θ. We assume that the tubule entrance is equivalent to a spherical cap and not the disk formed by removing that cap. For small values of the angle θ_c, the two assumptions are equivalent.

SOLUTION OF MODEL EQUATIONS

The mean times for receptor and ligand molecules to diffuse randomly from within the vesicular portion of the CURL to the tubule entrance are found from the solution of the appropriate mean capture time equations.

Receptor Diffusion

The straightforward solution to Eq. 2, which describes receptor diffusion, is

$$W_R(\theta) = \frac{R^2}{D_R} \ln\left[\frac{1-\cos\theta}{1-\cos\theta_c}\right] \qquad \hat{r} = R, \theta \geq \theta_c, \tag{6}$$

where θ is the initial angular position of the receptor. After averaging over all possible initial positions, the average mean capture time $\overline{W}_R$ is obtained

$$\overline{W}_R = \frac{R^2}{D_R} f_R(\theta_c), \tag{7a}$$

where

$$f_R(\theta_c) = \frac{2\ln\left[\dfrac{2}{1-\cos\theta_c}\right]}{1+\cos\theta_c} - 1. \tag{7b}$$

This result was previously reported by Bloomfield and Prager (24) in their calculation of the attachment rate of tail fibers to bacteriophages.

Ligand Diffusion

The mean capture time equation for the ligand is more difficult to solve because the boundary conditions on the surface of the sphere are mixed; over part of the surface the boundary condition is that of no flux while over the remainder of the surface an absorbing boundary is specified. This equation is solved using an approximate Green's function technique suggested by the work of Brunn (22).

To solve Poisson's equation inside a sphere with mixed boundary conditions on the surface, Eq. 4, the Green's function for a simpler problem, Laplace's equation with a constant flux boundary condition over the entire surface of the sphere, is found. This Green's function is then used to treat both the inhomogeneity in the equation itself and the inhomogeneity in the boundary condition. The boundary condition inhomogeneity can be more easily seen by rewriting boundary conditions in Eqs. 5a and 5b as

$$\frac{\partial W_L}{\partial \hat{r}} = 0 \qquad \hat{r} = R, \theta > \theta_c \tag{8a}$$

$$\frac{\partial W_L}{\partial \hat{r}} \neq 0 \qquad \hat{r} = R, \theta \leq \theta_c. \tag{8b}$$

The flux of ligand into the sink ($\theta \leq \theta_c$) is an unknown function of the angular position θ.

The Green's function for Laplace's equation is

$$G(\hat{r}, \theta\, \varphi; \hat{r}', \theta', \varphi') = \frac{1}{4\pi}\left[\frac{R}{(R^4 + \hat{r}^2\hat{r}'^2 - 2\hat{r}\mathbf{r}'R^2\cos\gamma)^{1/2}} + \frac{1}{R}\ln\left(\frac{2R^2}{R^2 - \hat{r}\hat{r}'\cos\gamma + (R^4 + \hat{r}^2\hat{r}'^2 - 2\hat{r}'R^2\cos\gamma)^{1/2}}\right) + (\hat{r}^2 + \hat{r}'^2 - 2\hat{r}\hat{r}'\cos\gamma)^{-1/2}\right], \tag{9}$$

where $(\hat{r}, \theta, \psi)$ are the coordinates of the observation point $\hat{r}$ and $(\hat{r}', \theta', \psi')$ are the coordinates of the source point $\hat{r}'$. The angle γ between (θ, ψ) and (θ, ψ') is given by

$$\cos\gamma = \cos\theta\cos\theta' + \sin\theta\sin\theta'\cos(\varphi - \varphi'). \tag{10}$$

The Green's function satisfies boundary conditions 5c, 5d, 5e, and the constant flux boundary condition

$$\frac{\partial G}{\partial \hat{r}} = \frac{-1}{4\pi R^2} \qquad \hat{r} = R. \tag{11}$$

By defining dimensionless variables for radial position,

$$\xi = \frac{\hat{r}}{R} \tag{12a}$$

$$\xi' = \frac{\hat{r}'}{R} \tag{12b}$$

the Green's function can be rewritten as

$$G(\xi, \theta, \varphi; \xi', \theta', \varphi') = \frac{1}{4\pi R}\left[(1 + \xi^2\xi'^2 - 2\xi\xi'\cos\gamma)^{-1/2} + (\xi^2 + \xi'^2 - 2\xi\xi'\cos\gamma)^{-1/2} + \ln\left(\frac{2}{(1 - \xi\xi'\cos\gamma) + (1 + \xi^2\xi'^2 - 2\xi\xi'\cos\gamma)^{1/2}}\right)\right], \tag{13}$$

which has dimensions of length^{-1}.

The inhomogeneity in Eq. 4 due to the source term $-1/D_L$ is now accounted for by a volume integral, and the inhomogeneous boundary condition (Eq. 8b) by a surface integral. The general solution to Eq. 4 is

$$W_L(\xi, \theta, \varphi) = \frac{1}{D_L}\iiint_V G(\xi, \theta, \varphi; \xi', \theta', \varphi')\mathrm{d}V' + \iint_{S_{\text{sink}}} \left.\frac{\partial W_L}{\partial \xi}\right|_{\xi=1} G(\xi, \theta, \varphi; 1, \theta', \varphi')\mathrm{d}S' + C. \tag{14}$$

The surface integration here and in later integrals is performed only over the surface of the sink because $\partial W_L/\partial\xi|_{\xi=1}$ is equal to zero elsewhere. The specification of Neumann boundary conditions in finding the Green's function means that the solution for the mean time W_L can only be determined, at this point in the development of our solution, to within a constant C.

Two unknowns in Eq. 14 prevent us from solving for the mean time directly. The first is the radial derivative of W_L appearing inside the surface integral, and the second is the constant C. We now introduce the approximation that $\partial W_L/\partial\xi$ does not vary significantly over the sink and replace the derivative by its average,

$$j = \text{avg}\left[\frac{\partial W_L}{\partial \xi} \text{ at } \xi = 1, \theta \leq \theta_c\right]. \tag{15}$$

This approximation should work well for small values of θ_c and allows us to remove the derivative from within the

surface integral. Applying Fredholm's Alternative (25), the requirement of solvability, to the approximate equation

$$W_L(\xi, \theta, \varphi) \approx \frac{1}{D_L} \iiint_V G(\xi, \theta, \varphi; \xi', \theta', \varphi')dV' + j \iint_{S\,\mathrm{sink}} G(\xi, \theta, \varphi; 1, \theta', \varphi')dS' + C \quad (16)$$

we find that

$$j = \frac{-2R}{3D_L(1 - \cos\theta_c)}. \quad (17)$$

To find the remaining unknown, C, in Eq. 14, we apply the boundary condition in Eq. 5a to require that the mean capture time is zero at the sink, or

$$0 = \frac{1}{D_L} \iiint_V G(1, \theta^*, \varphi^*; \xi', \theta', \varphi')dV' + j \iint_{S\,\mathrm{sink}} G(1, \theta^*, \varphi^*; 1, \theta', \varphi')dS' + C, \quad (18)$$

where $(1, \theta^*, \varphi^*)$ is a point on the sink. The two integrals are approximated by their average value over the sink, and we define the dimensionless integrals

$$\beta_1 = \frac{1}{R^2} \mathrm{avg}\left[\iiint_V G(1, \theta^*, \varphi^*; \xi', \theta', \varphi')dV'\right] \quad (19a)$$

$$\beta_2 = \frac{1}{R} \mathrm{avg}\left[\iint_{S\,\mathrm{sink}} G(1, \theta^*, \varphi^*; 1, \theta', \varphi')dS'\right]. \quad (19b)$$

The solution for C is then

$$C = \frac{R^2}{D_L}\left(\frac{4\beta_2}{3\theta_c^2} - \beta_1\right). \quad (20)$$

Combining Eqs. 14, 17, and 20, we obtain the mean capture time for the ligand

$$W_L(\xi, \theta, \varphi) = \frac{R^2}{D_L} f_L(\theta_c; \xi, \theta, \varphi), \quad (21a)$$

where

$$f_L(\theta_c; \xi, \theta, \varphi) = \alpha_1 - \beta_1 + \frac{2}{3}\left(\frac{2\beta_2}{\theta_c^2} - \frac{\alpha_2}{1 - \cos\theta_c}\right) \quad (21b)$$

and we define the dimensionless integrals

$$\alpha_1 = \frac{1}{R^2} \iiint_V G(\xi, \theta, \varphi; \xi', \theta', \varphi')dV' \quad (21c)$$

$$\alpha_2 = \frac{1}{R} \iint_{S\,\mathrm{sink}} G(\xi, \theta, \varphi; 1, \theta', \varphi')dS'. \quad (21d)$$

The major approximation of our technique is to treat the sink as a point on the sphere and satisfy solvability and boundary condition Eq. 5a on the average. This assumption is reasonable for small θ_c and for points far from the sink. An alternative would be to satisfy Eq. 5a only at the center of the sink by replacing β_1 and β_2 by

$$\beta_1' = \frac{1}{R^2} \iiint_V G(1, 0, 0; \xi', \theta', \varphi')dV' \quad (22a)$$

$$\beta_2' = \frac{1}{R} \iint_{S\,\mathrm{sink}} G(1, 0, 0; 1, \theta', \varphi')dS'. \quad (22b)$$

Integrals were evaluated numerically using Gauss-Legendre quadrature. For θ_c less than 1 radian, the value of β_1' is within 2.5% of β_1, and the value of β_2' is within 8.0% of β_2. β_2' was used in calculations instead of β_2 because of its convergence properties.

We assume that all ligand molecules dissociate from their receptors before a tubule forms. Therefore we average over all possible initial positions within the volume of the sphere to obtain the averaged mean capture time for the ligand, $\overline{W}_L$

$$\overline{W}_L = \frac{R^2}{D_L} f_L(\theta_c), \quad (23)$$

where $f_L(\theta_c)$ denotes the average over the sphere of $f_L(\theta_c; \xi, \theta, \varphi)$. If we instead assume that ligand molecules do not dissociate before a tubule forms, then an average of W_L over all possible initial positions on the surface of the sphere will give the average time for a ligand molecule to travel from a receptor to the tubule entrance. The results of this calculation are very similar to the volume-averaged $\overline{W}_L$ (26).

RESULTS

The average times required by receptor and ligand molecules to reach the tubule entrance are given in Eqs. 7 and

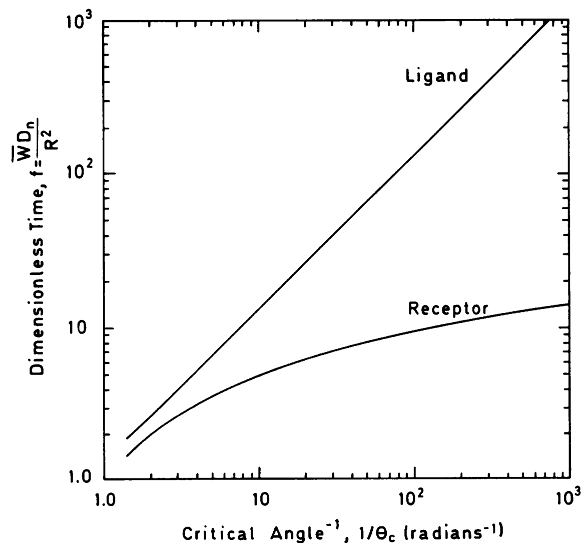

FIGURE 3 Dimensionless times for ligand and receptor to reach the tubule entrance as a function of the critical angle θ_c.

23. Both are expressed as the product of the ratio R^2/D_n and a factor f, the dimensionless time, which depends only on the angle θ_c. The variations of the dimensionless times f_R and f_L with θ_c are shown in Fig. 3. As previously discussed by Adam and Delbrück (19), this "tracking factor" f reflects the dimensionality of the system: the two dimensional f_R varies as log $(1/\theta_c)$ and the three-dimensional f_L as $1/\theta_c$ for small θ_c. Because of this difference in dimensionality, the dimensionless capture time for the ligand is always greater than that for the receptor. As the angle θ_c decreases, both dimensionless times increase and the difference between the two also increases.

Examination of the numerical results for $f_L(\theta_c)$ indicates that as θ_c decreases, the term $(4\ \beta_2'/3\ \theta_c^2)$ dominates Eq. 21b and that β_2' can be approximated by θ_c to within 9% for $\theta_c \leq 0.1$ radians and to <1% for $\theta_c \leq 5 \times 10^{-3}$ radians. Thus a good approximation for $\overline{W}_L$ is

$$\overline{W}_L \approx \frac{R^2}{D_L} \cdot \frac{4}{3\theta_c} \qquad \theta_c \leq 0.1 \text{ radians.} \tag{24}$$

To compare the average times required by the molecules to reach the sink, we calculate the ratio of the capture times

$$\frac{\overline{W}_R}{\overline{W}_L} = \frac{D_L}{D_R} \cdot \frac{f_R(\theta_c)}{f_L(\theta_c)}, \tag{25}$$

which is independent of the radius R and a function only of the angle θ_c and the ratio of diffusion coefficients. In Fig. 4, lines of constant $\overline{W}_R(\theta_c)/\overline{W}_L(\theta_c)$ are shown as a function of θ_c^{-1} and D_R/D_L. It is clear that for a constant ratio of diffusion coefficients, the receptor's advantage of searching for the tubule entrance in one less dimension than the ligand is greatest at small θ_c. In other words, for small enough θ_c, the mean time for the receptor to reach the tubule entrance can be less than that for the ligand $(\overline{W}_R/\overline{W}_L < 1)$ even though the ligand's diffusion coefficient is orders of magnitude greater than that for the receptor.

DISCUSSION

Mathematical Analysis

In this paper, we have solved mean capture time equations in two and three dimensions with spherical geometry. The two-dimensional problem is straightforward, but the three-dimensional problem requires some care because it involves a mixed boundary condition. Our approach to this latter problem is guided by the work of other investigators on a related problem, the calculation of the rate constant for diffusion of ligand to cell surface receptors (20, 21, 22). This problem requires the solution of the diffusion equation with mixed boundary conditions. These boundary conditions result from the situation where part of the surface is covered with absorbers (receptors) while the remainder of the surface is reflecting. Fortunately, one can postulate a constant ligand concentration infinitely far from the cell and thus solve the steady state diffusion equation. To solve the equation describing this problem, Laplace's equation on the exterior of a sphere, several methods have been used. We note particularly the method of Brunn (22), who treats the absorbing boundary condition as an unknown inhomogeneity in flux and solves the problem approximately using Green's functions. Shoup et al. (27) also solve for an unknown flux at a sink and use this in obtaining rate constants for different geometries.

To find the rate constant for ligand entry into a tubule,

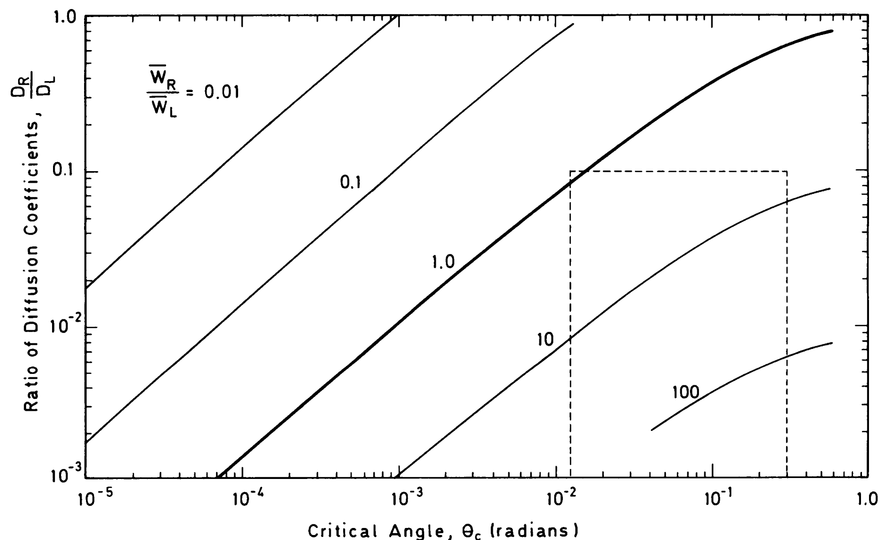

FIGURE 4 Ratio of receptor capture time, $\overline{W}_R$, to ligand capture time, $\overline{W}_L$, as a function of the ratio of diffusion coefficients and the critical angle. The curve $\overline{W}_R/\overline{W}_L = 1$ separates two regimes: when $\overline{W}_R/\overline{W}_L < 1$, receptors are able to find the tubule entrance more rapidly than ligand molecules and when $\overline{W}_R/\overline{W}_L > 1$, ligand molecules are able to find the tubule entrance more rapidly than receptors. Estimated parameter values lie within the boxed region.

our problem, the solution to the diffusion equation within a sphere with mixed boundary conditions on the surface of that sphere is needed. Because there is no infinite source of ligand for this interior problem, the equation cannot be solved at steady state. This complication is avoided by instead using the mean capture time equation, derived by Berg and Purcell (20) and Szabo et al. (23), to calculate the time required for a ligand molecule to reach the tubule entrance. If the tubule entrance can be considered perfectly absorbing, then this mean time—averaged over the volume or surface area of the sphere to account for all possible initial positions of ligand or receptor molecules—is simply the inverse of the rate constant we seek. If, however, the entrance is only partially absorbing, this first rate constant for finding the entrance can be combined with a second rate constant for adsorption or reaction, analogous to the "encounter complex" model of receptor-ligand binding (21, 28).

Thus we solve the mean time equation, Poisson's equation inside a sphere with mixed boundary conditions on the surface. To do this, an approximate Green's function technique is used. Following Brunn (22) we treat the absorbing boundary condition as an unknown inhomogeneity in flux. A volume integral is used to account for the inhomogeneity in the equation itself. Our result can be expressed as the product of a ratio, R^2/D_L, and a factor $f_L(\theta_c)$, which is plotted in Fig. 3. For small θ_c, we obtain the approximate result given in Eq. 24.

This result can be compared with the results of similar calculations in the literature, as shown in Fig. 5, in order to discern the influence of geometry on the ability of a ligand to find a generalized surface reaction site. The diffusive current of ligand to a perfectly absorbing circular cell surface receptor is equivalent to the diffusion of molecules to a circular sink on an infinite plane in the limit of an infinitely small receptor. As found by Hill (29) and by Berg and Purcell (20), the rate constant k is equal to $4Db$ where D is the diffusion coefficient and b the radius of the sink. Shoup and Szabo (30) show that the first passage time, the quantity we refer to as $\overline{W}$, is equal to a volume divided by this rate constant. The appropriate volume is that of our vesicle, $4\pi R^3/3$, and for the same size sink we set b equal to $R\theta_c$. Thus the mean time $\overline{W}^{FP}$ for diffusion to a sink on a flat plate is equal to

$$\overline{W}_L^{FP} = \frac{R^2}{D_L} \cdot \frac{\pi}{3\theta_c}. \tag{26}$$

$\overline{W}^{FP}$ is smaller than our result because of geometrical differences between the problems. In our problem, shown in Fig. 5 *a*, ligand moves within a sphere to a hole on the surface of that sphere. As shown in Fig. 5 *b*, the volume used for diffusion to a flat plate should be placed so that

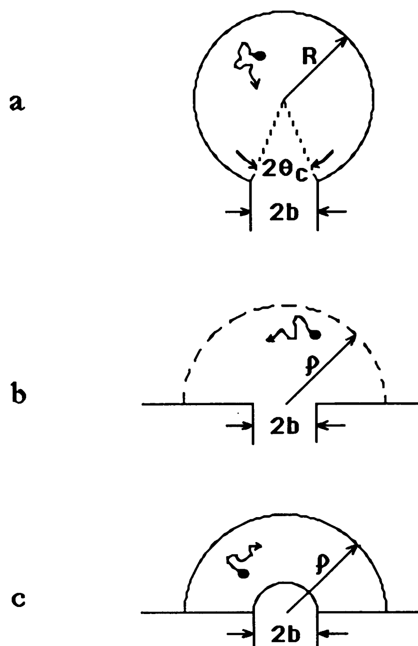

FIGURE 5 Geometries for comparison of ligand capture times. (*a*) Diffusion within a sphere to a hole on the surface. (*b*) Diffusion to a hole on an infinite plane. (*c*) Diffusion within a hemisphere to a smaller concentric hemisphere (19).

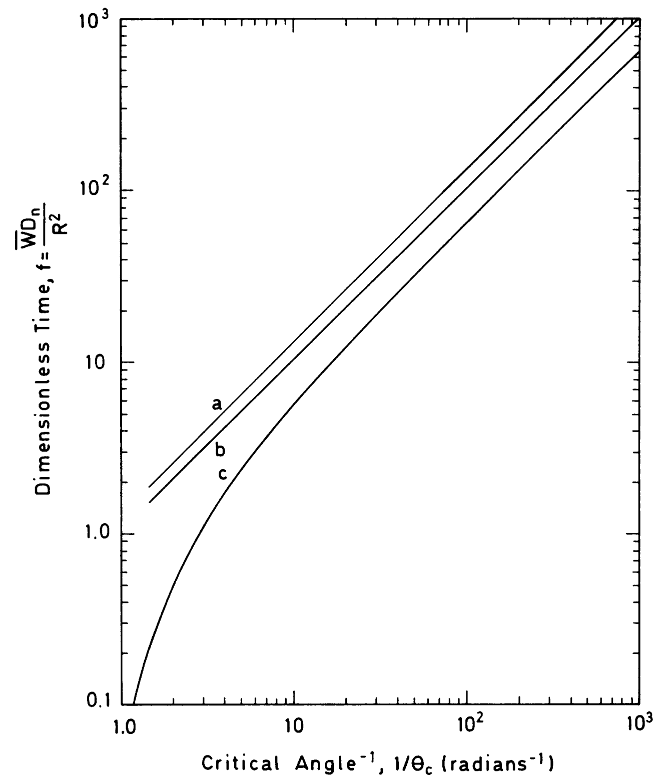

FIGURE 6 Effect of geometry on ligand capture times. Curves correspond to the geometries shown in Fig. 5. In all cases, $\theta_c = b/R$. (*a*) Diffusion within a sphere to a hole on the surface. Our result is replotted from Fig. 3. (*b*) Diffusion to a hole on an infinite plane. For $\rho = 2^{1/3}R$, $\overline{W}_L^{FP} D_L/R^2 = \pi/3\theta_c$. (*c*) Diffusion within a hemisphere to a smaller concentric hemisphere. For $\rho = 2^{1/3}R$, and $x = 2^{-1/3}\theta_c$,

$$\overline{W}_L^{H} D_L/R^2 = \frac{2^{2/3}(1-x)^2(5+6x+3x^2+x^3)}{15x(1+x+x^2)} \text{ (reference 23).}$$

the hole is most accessible to the ligand: that is, as a hemisphere that has as its center the hole. Although the radius ρ of this hemisphere is greater than R in order that the hemisphere volume is equal to the volume of a sphere with radius R, the average distance traveled to the sink is less than in Fig. 5 *a*. So it is clear that our result should be greater than the result for diffusion to a hole on a flat plate for purely geometric reasons.

Second, we compare our result with that of Adam and Delbrück (19), as simplified considerably by Szabo et al. (23), who calculated the mean time for a molecule within a hemisphere of radius ρ to diffuse to the surface of a smaller concentric hemisphere of radius b (see Fig. 5 *c*). When $\rho \gg b$, their results are well approximated by a time of $\rho^2/3D\theta$ where θ equals b/ρ. To equate the volume of this outer hemisphere with our sphere and the sink radius with our radius b, we set ρ equal to $2^{1/3}R$ and b equal to $R\theta_c$. Thus Adam and Delbrück's result becomes

$$\overline{W}_L^H \approx \frac{R^2}{D_L} \cdot \frac{2}{3\theta_c}, \quad (27)$$

which is smaller than Berg and Purcell's result because the inner hemisphere is slightly easier to find than the hole of Fig. 5 *b*. Our result is the largest of the three, illustrating the effect of geometry. In Fig. 6, we plot the three results over a range of sink sizes.

The effect of geometry is also demonstrated by the influence of dimensionality on the transport rate constants. The receptor searches for the tubule entrance in one fewer dimension than the ligand, and this dimensionality advantage can outweigh the ligand's advantage of a greater diffusion coefficient when the critical angle is small enough.

Biological Implications

Because an equilibrium mechanism does not explain the observed intracellular segregation of receptor and ligand molecules within CURL, the sorting chamber for the cell, two kinetic schemes for the separation are suggested. First, we propose that receptors may be able to move into CURL tubules from the vesicular region of CURL more rapidly than ligand molecules due to an advantage in dimensionality. Alternatively, we consider the possibility that receptors diffusing into tubules are trapped there whereas ligand molecules are free to diffuse back out of the tubule. The evaluation of these possible sorting mechanisms requires the calculation of the rates at which receptor and ligand molecules move into CURL tubules.

Beginning with a simple model of CURL, a single vesicle with an attached thin tubule, we calculate the capture time, or time to reach the tubule entrance, for molecules placed randomly within the vesicle and moving by passive diffusion. Our calculated capture times for the receptor and ligand are a function of three parameters of the system: the vesicle radius, the tubule radius, and the appropriate diffusion coefficient. To compare the receptor and ligand capture times, the ratio of times $\overline{W}_R/\overline{W}_L$ as a function of the ratio of diffusion coefficients D_R/D_L and the ratio of the tubule radius to the vesicle radius b/R (or θ_c) is plotted in Fig. 4. The curve of parameter values for which the ratio $\overline{W}_R/\overline{W}_L$ is equal to one separates two regimes in the figure. When the value of the ratio is greater than one, the transport of receptors to the tubule is slower than the transport of ligand molecules, and when the value is less than one, receptors diffuse to the tubule entrance more rapidly than ligand. Using the appropriate parameter values, the regime in which the CURL is likely to operate can be determined.

From electron micrographs, Geuze et al. (12) and Marsh et al. (15) obtain values for the vesicle radius R and tubule radius b. The parameter ranges and the calculated range of values for θ_c are given in Table I. The diffusion coefficients D_R and D_L within an endosome are not known, so we postulate that they are not significantly different from the diffusion coefficients for ligand free in solution and receptors on the cell surface. Thus we estimate that D_L is 10^{-5}–10^{-7} cm^2/s and D_R is 10^{-8}–10^{-11} cm^2/s (31, 32). Assuming these parameter values to be accurate, we can determine from the region inside the dashed lines in Fig. 4 that $\overline{W}_R(\theta_c)$ is likely 10 to 100 times greater than $\overline{W}_L(\theta_c)$. Thus the endosome operates in the regime for which $\overline{W}_R/\overline{W}_L$ is greater than one, implying that the cell cannot sort the receptors into a tubule before a significant amount of ligand reaches the tubule. In fact, these parameter values indicate that ligand will have equilibrated throughout the vesicle and tubule volumes by the time an appreciable number of receptors have diffused into the tubule. Thus our analysis reveals that our first proposed separation scheme is unlikely; the ligand's advantage of a greater diffusion coefficient predominates over the receptor's advantage in dimensionality.

It is possible, however, that the parameter values used are inaccurate and that receptors do reach the tubule more rapidly than ligand molecules. The tubule radius may be much smaller at the entrance than where it is measured, further down the tubule. Such a constriction would decrease θ_c considerably and greatly increase the receptor's dimensionality advantage over the ligand. For example, if

TABLE I

Model parameter	Range
Vesicle radius, R	10^{-5}–4×10^{-5} cm
Tubule radius, b	5×10^{-7}–3×10^{-6} cm
Critical angle, θ_c	0.0125–0.30 radians
Receptor diffusion coefficient, D_R	10^{-8}–10^{-11} cm^2/s
Ligand diffusion coefficient, D_L	10^{-5}–10^{-7} cm^2/s

Parameter values. Values for R and b are taken from references 12 and 15 and used to calculate θ_c. Diffusion coefficients are estimated as described in the text.

the tubule radius at the entrance is constricted to 25 Å, the vesicle radius is 4×10^{-5}cm, and D_R/D_L is equal to 0.1, then the receptor would move into the tubule about twice as fast as the ligand. At such small tubule diameters, the finite size of the ligand becomes important. The ligand effectively sees a smaller θ_c than the ratio b/R and this will further decrease the ratio $\overline{W}_R/\overline{W}_L$. In addition, the diffusion coefficients may have been inaccurately estimated. To our knowledge, no one has measured diffusion coefficients within an endosome, and D_R may be enhanced over its plasma membrane value or D_L may be less than its value in free solution. These changes in the values of the diffusion coefficients would decrease the ratio $\overline{W}_R/\overline{W}_L$.

Another possible mechanism could move receptors into the tubule at a greater rate. If tubules bud from vesicles, drawing membrane from the vesicle itself, then it is not difficult to envision a convective membrane current pulling the receptors toward the tubule entrance (33, 34). This is diagrammed in Fig. 7. Depending upon the magnitude of the convective current, this could be the primary mechanism for moving the receptor population.

At present, however, we believe that our model assumptions are reasonable and our parameter values are representative. The cell apparently cannot sort receptor and ligand molecules by taking advantage of a difference in the dimensionality of their diffusion processes.

As a second possible sorting mechanism, we suggested that receptors diffusing into tubules are trapped there. From the calculated rate constant for movement of free receptors into a tubule, the length of time a vesicle and tubule must remain connected in the CURL in order to allow the movement of a substantial fraction of receptors into the tubule can be estimated. Receptors not diffusing into a tubule within this sorting time might then be degraded in lysosomes with the ligand molecules. For a reasonable choice of parameter values, a vesicle radius of 4×10^{-5} cm, a tubule radius of 100 Å, and a diffusion coefficient of 10^{-10} cm^2/s, one can calculate that the mean time $\overline{W}_R$ is equal to 2.1 min. Assuming a first-order process

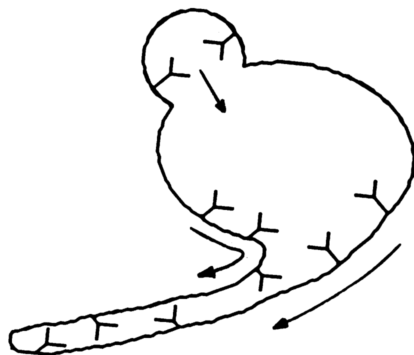

FIGURE 7 Convective membrane current. Lengthening tubule draws membrane from vesicle, pulling receptors into the tubule. Membrane might be donated from new endosomes fusing with the vesicle.

(23), 90% of the receptors will have moved into the tubule by 5 min. This is a significant portion of the time necessary to complete the endocytic cycle and also falls near experimental estimates of the time a receptor spends inside the cell (5, 16, 35). Thus, we have a means of estimating the time required to sort the two populations after the acidic environment of the endosome has released the ligand from the receptor.

Because ligand molecules apparently equilibrate throughout the vesicle and tubule volumes within this sorting time, we suggest that a fraction of the ligand is "mis-sorted" into the tubule with the recycling receptors and will be exocytosed. Such exocytosis has been detected by a number of researchers: for monovalent Fab fragments (7), and asialoglycoproteins (17, 36). The fraction of ligand in the tubule at equilibrium can be calculated from the volumes of each compartment and knowledge of the partition coefficient. The partition coefficient K is equal to the ratio of the ligand concentration in the tubule to the concentration in the vesicle and is found from

$$K = (1 - \lambda)^2, \tag{28}$$

where λ is equal to $d/2b$ and d is the diameter of the ligand molecule (37). For example, ASOR has an approximate diameter of 40 Å[1] and, for a tubule diameter of 100 Å, the partition coefficient is equal to 0.36. If the tubule volume is 25% of the total CURL volume, then 10% of the ligand would be found in tubules at equilibrium. Assuming that the tubule becomes the recycling vehicle without losing this ligand, this is the fraction of ligand that would be exocytosed. We suggest then, that the cell allows this small fraction of ligand to be returned to the cell surface in enabling the receptors to recycle. Although there are other cellular mechanisms, such as reversible pinocytosis (39), which can result in exocytosis of ligand, we believe that this "mis-sorting" also may provide a mechanism for returning ligand to the cell surface.

CONCLUSION

Two possible sorting mechanisms, based on molecular movement by pure diffusion, have been examined. For what we believe to be reasonable model assumptions and representative parameter values, only the second mechanism can account for the observed sorting of receptor and ligand molecules. On the basis of our analysis, then, we suggest that receptors diffuse into CURL tubules and are trapped there, while ligand molecules equilibrate between the vesicular and tubular volumes of CURL. The time necessary for a significant fraction of receptors to diffuse into a tubule and the fraction of ligand found with the

[1]Molecular weight of ASOR is ~40 kD. Molecular diameter is estimated from the molecular weights and diffusivities of macromolecules of similar size and the Stokes-Einstein relation (38).

receptors in those tubules, and thus exocytosed, can be calculated.

Although one might expect cells to sort the receptor and ligand populations very efficiently, our analysis indicates that this is not the case. We have shown that a kinetic scheme based on molecular movement by diffusion does allow a separation but that the efficiency of the separation is $<100\%$. Quantitative experimental confirmation of the "mis-sorting" of ligands (ligand exocytosis) and of receptors (receptor degradation) and more detailed modeling will further elucidate the mechanism of the sorting process.

Finally, additional modeling work needs to be pursued. Even at the low endosome pH, all ligands may not dissociate from their receptors. We are currently examining the consequences of this failure to dissociate, particularly as it may explain the effects of ligand valency and affinity on sorting (7, 17, 18).

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REFERENCES

1. Brown, M. S., R. G. W. Anderson, and J. L. Goldstein. 1983. Recycling receptors: the round-trip itinerary of migrant membrane proteins. *Cell.* 32:663–667.
2. Pastan, I., and M. C. Willingham. 1983. Receptor-mediated endocytosis: coated pits, receptosomes, and the Golgi. *Trends Biochem. Sci.* 8:250–254.
3. Steinman, R. M., I. S. Mellman, W. A. Muller, and Z. A. Cohn. 1983. Endocytosis and the recycling of plasma membrane. *J. Cell Biol.* 96:1–27.
4. Helenius, A., I. Mellman, D. Wall, and A. Hubbard. 1983. Endosomes. *Trends Biochem. Sci.* 8:245–250.
5. Schwartz, A. L., S. E. Fridovich, and H. F. Lodish. 1982. Kinetics of internalization and recycling of the asialoglycoprotein receptor in a hepatoma cell line. *J. Biol. Chem.* 257:4230–4237.
6. Anderson, R. G. W., M. S. Brown, U. Beisiegel, and J. L. Goldstein. 1982. Surface distribution and recycling of the low density lipoprotein receptor as visualized with antireceptor antibodies. *J. Cell Biol.* 93:523–531.
7. Mellman, I., H. Plutner, and P. Ukkonen. 1984. Internalization and rapid recycling or macrophage Fc receptors tagged with monovalent antireceptor antibody: possible role of a prelysosomal compartment. *J. Cell Biol.* 98:1163–1169.
8. Tietze, C., P. Schlesinger, and P. Stahl. 1982. Mannose-specific endocytosis receptor of alveolar macrophages: demonstration of two functionally distinct intracellular pools of receptor and their roles in receptor recycling. *J. Cell Biol.* 92:417–424.
9. Dunn, W. A., and A. L. Hubbard. 1984. Receptor-mediated endocytosis of epidermal growth factor by hepatocytes in the perfused rat liver: ligand and receptor dynamics. *J. Cell Biol.* 98:2148–2159.
10. Tycko, B., C. H. Keith, and F. R. Maxfield. 1983. Rapid acidification of endocytic vesicles containing asialoglycoprotein in cells of human hepatoma line. *J. Cell Biol.* 97:1762–1776.
11. Yamashiro, D. J., and F. R. Maxfield. 1984. Acidification of endocytic compartments and the intracellular pathways of ligands and receptors. *J. Cell. Biochem.* 26:231–246.
12. Geuze, H. J., J. W. Slot, G. J. A. M. Strous, H. F. Lodish, and A. L. Schwartz. 1983. Intracellular site of asialoglycoprotein receptor-ligand uncoupling: Double-label immunoelectron microscopy during receptor-mediated endocytosis. *Cell.* 32:277–287.
13. Wall, D. A., G. Wilson, and A. L. Hubbard. 1980. The galactose-specific recognition system of mammalian liver: The route of ligand internalization in rat hepatocytes. *Cell.* 21:79–93.
14. Harding, C., M. A. Levy, and P. Stahl. 1985. Morphological analysis of ligand uptake and processing: The role of multivesicular endosomes and CURL in receptor-ligand processing. *Eur. J. Cell Biol.* 36:230–238.
15. Marsh, M., G. Griffiths, G. E. Dean, I. Mellman, and A. Helenius. 1986. Three-dimensional structure of endosomes in BHK-21 cells. *Proc. Natl. Acad. Sci. USA.* 83:2899–2903.
16. Marshall, S. 1985. Kinetics of insulin receptor internalization and recycling in adipocytes: Shunting of receptors to a degradative pathway by inhibitors of recycling. *J. Biol. Chem.* 260:4136–4144.
17. Townsend, R. R., D. A. Wall, A. L. Hubbard, and Y. C. Lee. 1984. Rapid release of galactose-terminated ligands after endocytosis by hepatic parenchymal cells: evidence for a role of carbohydrate structure in the release of internalized ligand from receptor. *Proc. Natl. Acad. Sci. USA.* 81:466–470.
18. Mellman, I., and H. Plutner. 1984. Internalization and degradation of macrophage Fc receptors bound to polyvalent immune complexes. *J. Cell Biol.* 98:1170–1177.
19. Adam, G., and M. Delbrück. 1968. Reduction of dimensionality in biological diffusion processes. *In* Structural Chemistry and Molecular Biology. A. Rich and N. Davidson, editors. W.H. Freeman and Co., San Francisco. 198–215.
20. Berg, H. C., and E. M. Purcell. 1977. Physics of chemoreception. *Biophys. J.* 20:193–219.
21. DeLisi, C., and F. W. Wiegel. 1981. Effect of nonspecific forces and finite receptor number on rate constants of ligand-cell bound-receptor interactions. *Proc. Natl. Acad. Sci. USA.* 78:5569–5572.
22. Brunn, P. O. 1981. Absorption by bacterial cells: Interaction between receptor sites and the effect of fluid motion. *J. Biomech. Eng.* 103:32–37.
23. Szabo, A., K. Schulten, and Z. Schulten. 1980. First passage time approach to diffusion controlled reactions. *J. Chem. Phys.* 72:4350–4357.
24. Bloomfield, V. A., and S. Prager. 1979. Diffusion-controlled reactions on spherical surfaces: application to bacteriophage tail fiber attachment. *Biophys. J.* 27:447–453.
25. Ramkrishna, D., and N. R. Amundson. 1985. Linear Operator Methods in Chemical Engineering with Applications to Transport and Chemical Reaction Systems. Prentice-Hall, Inc., Englewood Cliffs, New Jersey. 209 pp.
26. Linderman, J. J. 1987. Analysis of Intracellular Receptor/Ligand Sorting. PhD thesis, University of Pennsylvania.
27. Shoup, D., G. Lipari, A. Szabo. 1981. Diffusion-controlled biomolecular reaction rates: The effect of rotational diffusion and orientation constraints. *Biophys. J.* 36:697–714.
28. DeLisi, C. 1980. The biophysics of ligand-receptor interactions, *Q. Rev. Biophys.* 13:201–230.
29. Hill, T. L. 1975. Effect of rotation on the diffusion-controlled rate of ligand-protein association. *Proc. Natl. Acad. Sci. USA.* 72:4918–4922.
30. Shoup, D., and A. Szabo. 1982. Role of diffusion in ligand binding to macromolecules and cell-bound receptors. *Biophys. J.* 40:33–39.
31. Wiegel, F. W. 1984. Diffusion of proteins in membranes. *In* Cell Surface Dynamics: Concepts and Models. A. S. Perelson, C. DeLisi, and F. W. Wiegel, editors. Marcel Dekker, Inc., New York. 135–150.
32. Schlessinger, J., Y. Schecter, P. Cuatrecasas, M. C. Willingham, and I. Pastan. 1978. Quantitative determination of the lateral diffusion coefficients of the hormone-receptor complexes of insulin and

epidermal growth factor on the plasma membrane of cultured fibroblasts. *Proc. Natl. Acad. Sci. USA.* 75:5343–5357.
33. Bretscher, M. S. 1976. Directed lipid flow in cell membranes. *Nature (Lond.).* 260:21–23.
34. Wiegel, F. W. 1980. Fluid Flow Through Porous Macromolecular Systems. I. Lecture Notes in Physics. Vol. 121. Springer-Verlag, New York. 56–60.
35. Oka, J. A., and P. H. Weigel. 1983. Recycling of the asialoglycoprotein receptor in isolated rat hepatocytes: dissociation of internalized ligand from receptor occurs in two kinetically and thermally distinguishable compartments. *J. Biol. Chem.* 258:10253–10262.
36. Simmons, C. F., Jr., and A. L. Schwartz. 1984. Cellular pathways of galactose-terminal ligand movement in a cloned human hepatoma cell line. *Mol. Pharmacol.* 26:509–519.
37. Pappenheimer, J. R. 1953. Passage of molecules through capillary walls. *Physiol. Rev.* 33:387–423.
38. Tanford, C. 1961. Physical Chemistry of Macromolecules. John Wiley & Sons., Inc. New York.
39. Daukas, G., D. Lauffenburger, and S. Zigmond. 1983. Reversible pinocytosis in polymorphonuclear leukocytes. *J. Cell Biol.* 96:1642–1650.